GENE REARRANGEMENTS IN HUMAN T-CELLS

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Breit, Timo Markus

Gene rearrangements in human T-cells / Timo Markus Breit ;
[ill.: Tar van Os]. - Rotterdam : Afdeling Immunologie,
Erasmus Universiteit. - ill.
Thesis Rotterdam. - With ref. - With summary in Dutch.
ISBN 90-73436-18-4
NUGI 743
Subject headings: T-cell receptors / T-cell
differentiation / gene rearrangement.

No part of this thesis may be reproduced or transmitted in any form by any means, electronic or mechanical, including photocopying, recording or any information storage and retrieval system, without permission in writing from the author (T.M. Breit) and the publisher (Department of Immunology, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands).

GENE REARRANGEMENTS IN HUMAN T-CELLS

Genherschikkingen in humane T cellen

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
prof. dr. P.W.C. Akkermans M.A.
en volgens besluit van het college voor promoties.
De openbare verdediging zal plaatsvinden op
woensdag 23 november 1994 om 13.45 uur

door

Timo Markus Breit

geboren te Rotterdam

PROMOTIECOMMISSIE

Promotor : Prof. dr. J.J.M. van Dongen

Overige leden : Prof. dr. R. Benner
Prof. dr. H.C. Clevers
Prof. dr. J.H.J. Hoeijmakers

The studies described in this thesis were performed at the Department of Immunology, Erasmus University Rotterdam.

The printing of this Ph.D. thesis was financially supported by the Journal LEUKEMIA (and the Killmann Leukemia Foundation) and the DAKO Corporation (Carpinteria, CA USA). Additional support was obtained from Perkin-Elmer Nederland B.V. (Gouda, the Netherlands), Biozym Nederland B.V. (Landgraaf, the Netherlands), Bio-Rad Laboratories B.V. (Veenendaal, the Netherlands), and Schleicher & Schuell Nederland B.V. ('s-Hertogenbosch, the Netherlands).

Front cover : Cedar Hill shunting yard, USA.
Back cover : Detail Cedar Hill shunting yard, USA.
Illustrations : Tar van Os.
Printing : Ridderprint, Ridderkerk.

Jongens waren we - maar aardige jongens. Al zeg ik 't zelf.
We zijn nu veel wijzer, stakkerig wijs zijn we, behalve Bavink,
die mal geworden is.

Titaantjes, Nescio

GENE REARRANGEMENTS IN HUMAN T-CELLS

CONTENTS

Chapter 1 General introduction 9

Chapter 2 Repertoire of human TcR-$\gamma\delta^+$ T-cells 13

2.1 Receptor diversity of human TcR-$\gamma\delta$ expressing cells 15
Parts published in: Prog Histochem Cytochem 1992;26:182-193.

2.2 Limited combinatorial repertoire of $\gamma\delta$ T-cell receptors expressed by T-cell acute lymphoblastic leukemias 27
Published in: Leukemia 1991;5:116-124.

2.3 Unravelling human T-cell receptor junctional region sequences 41
Published in: Thymus 1994;22:177-199.

2.4 Extensive junctional diversity of $\gamma\delta$ T-cell receptors expressed by T-cell acute lymphoblastic leukemias: implications for the detection of minimal residual disease 59
Published in: Leukemia 1991;5:1076-1086.

2.5 Unique selection determinant in polyclonal Vδ2-Jδ1 junctional regions of human peripheral $\gamma\delta$ T-lymphocytes 75
Published in: J Immunol 1994;152:2860-2864.

Chapter 3 Rearrangements and deletions of the human TcR-δ gene 83

3.1 Regulation of TcR-δ gene rearrangements in human T-cell differentiation 85

3.2 Southern blot patterns, frequencies and junctional diversity of T-cell receptor δ gene rearrangements in acute lymphoblastic leukemia 95
Published in: Blood 1993;82:3063-3074.

3.3 Two pathways of sequential TcR-δ gene rearrangements in human thymocytes 113
Submitted for publication.

3.4 Rearrangements of the human TcR-δ deleting elements, δREC and ψJα 129
Published in: Immunogenetics 1994;40:70-75.

3.5 Human T-cells with an active V(D)J recombinase complex for TcR-δ gene deletion 137
Submitted for publication.

Chapter 4 Gene rearrangement of the *tal*-1 gene 153

4.1 TAL1, a bHLH protein implicated in T-ALL 155

4.2 Site-specific deletions involving the *tal*-1 and *sil* genes are restricted to cells of the T-cell receptor $\alpha\beta$ lineage: T-cell receptor δ gene deletion mechanism affects multiple genes 169
Published in: J Exp Med 1993;177:965-977.

4.3 Lineage specific demethylation of *tal*-1 gene breakpoint region determines the frequency of *tal*-1 deletions in $\alpha\beta$ lineage T-cells 187
Published in: Oncogene 1994;9:1847-1853.

Chapter 5 Concluding remarks 199

Summary 209

Dutch summary (samenvatting) 214

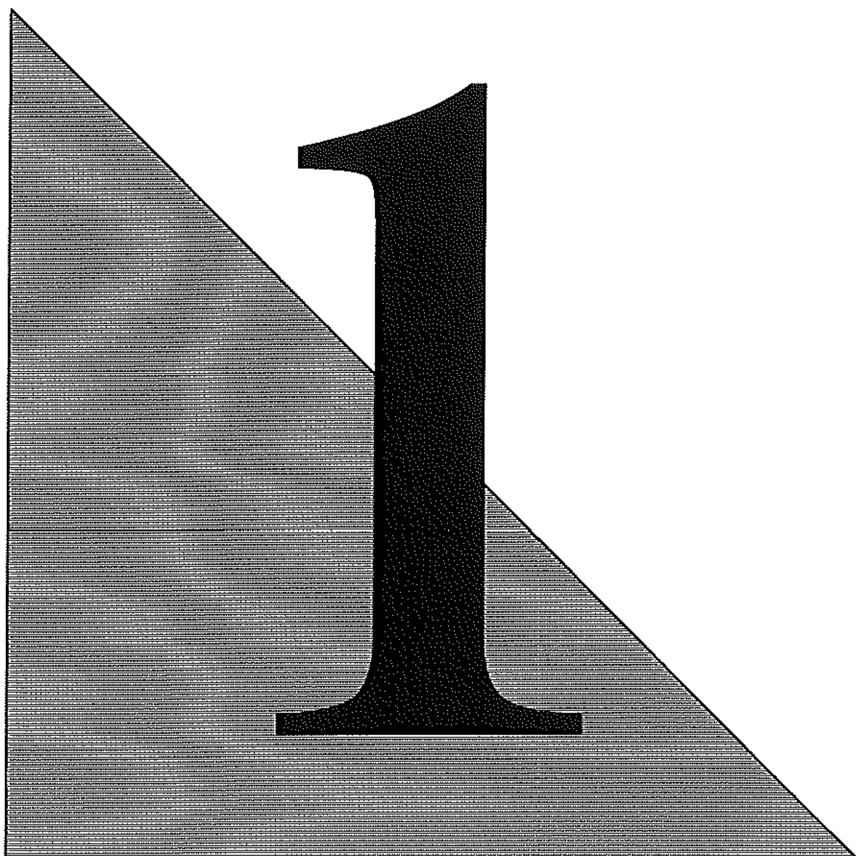
List of abbreviations 217

Acknowledgements (dankwoord) 219

Curriculum vitae 221

List of publications 222

GENERAL INTRODUCTION



CHAPTER 1

GENERAL INTRODUCTION

One of the most basal requirements of the immune system is that it must be capable of specifically recognizing and responding to foreign antigens, while remaining indifferent to self-components. For T-cells the antigen recognizing function is fulfilled by the TcR (1). Because of the stochastic nature of antigen receptor formation, selection for appropriate TcR is needed (2). These selection processes take place in the thymus, presumably in complex interactions with the thymic stromal cells (3). Negative selection (clonal deletion) prevents self-reactive T-cells from becoming auto-aggressive (4). Positive selection (clonal selection) prevents the accumulation of useless T-cells with either no antigen receptor at all, or with for the organism useless receptors (5).

In general TcR are designed to fit with a short linear peptide anchored within the peptide-binding groove of a MHC molecule present at the surface of an antigen presenting cell (6). The TcR of $CD4^+$ T-helper cells recognize peptide anchored within the cleft of MHC class II molecules, whereas the TcR of $CD8^+$ T-killer cells recognize peptides anchored within the cleft of MHC class I (7). Coengagement of the TcR and coreceptor (CD4 or CD8) by the same MHC molecules generates cell-activating signals, part of which are transmitted through the cell membrane by the TcR associated CD3 complex (8).

TcR are disulphide-linked cell surface heterodimers and occur in two configurations: TcR-$\alpha\beta$ and TcR-$\gamma\delta$ (1,9,10). While most characterized cell-mediated immune responses are carried out by TcR-$\alpha\beta^+$ T-cells, T-cells expressing a TcR-$\gamma\delta$ have yet to be assigned any specific functional role (11). However, the topology of TcR-$\gamma\delta$ interaction with MHC molecules is distinct from that of TcR-$\alpha\beta$, which suggests that the molecular nature of TcR-$\gamma\delta$ recognition is fundamentally different from that of TcR-$\alpha\beta$ (12).

Functional TcR genes are assembled by joining the V, (D), and J gene segments which are reiteratively present in the four TcR gene complexes (1,13). By this means, an enormous number of combinations of gene segments, and therefore different TcR can be generated in T-cells from a limited amount of germline sequences (1). The gene segments are imperfectly joined during the rearrangement processes, with random insertion and deletion of nucleotides at the sites of the junctions (13). The thus created junctional diversity enlarges the total antigen recognizing repertoire of the TcR (1,13). The V(D)J recombination processes that rearrange TcR gene segments into functional genes are present in all vertebrates and probably use highly conserved mechanisms (14). But, although V(D)J recombination is intensively studied worldwide, and some of the involved proteins are recently characterized, the overall process still remains a "black box" (13).

Thus, early T-cell differentiation in the thymus is characterized by rearrangement of the TcR genes, expression of the TcR on the membrane, and selection of the antigen-specific receptor. This thesis focusses on the gene rearrangements in human T-cells, especially those involved in the TcR-$\alpha\beta$/TcR-$\gamma\delta$ lineage commitment. Experimental data are

derived from studies on T-cell leukemias, normal thymocytes, or normal peripheral T-lymphocytes. In the T-ALL model each leukemia is considered to consist of clonal cells which represent a "frozen" stage in T-cell differentiation, as the leukemic cells of T-ALL are assumed to originate from malignantly transformed cortical thymocytes (15).

The following chapters in this thesis deal with experimental work on the repertoire of the human TcR (Chapter 2), the rearrangements of the TcR-δ gene in T-cell differentiation (Chapter 3), and the relation between aberrations in the *tal*-1 gene and TcR gene rearrangement processes (Chapter 4).

Chapter 2 describes the guidelines for determination of the receptor diversity of TcR gene rearrangements present in human T-cells. In TcR-$\gamma\delta^+$ T-ALL a restricted recombinatorial repertoire, but an extensive junctional diversity is reported. These data are compared with the repertoire of normal thymocytes. The majority of human peripheral T-lymphocytes are characterized by an invariant Vγ9/Vδ2 TcR, in which we discovered an invariant selection determinant in the Vδ2-Jδ1 junctional region. Implications for this particular TcR, and antigen receptor specificity in general are discussed.

Chapter 3 deals with the ordered TcR-δ gene rearrangements in early human T-cell differentiation. Preferential pathways of TcR-δ gene rearrangement are proposed after studies on polyclonal thymocytes. The characteristics and the role of the TcR-δ deleting rearrangement, (δREC-ψJα), are discussed, and new experimental models are proposed to further investigate this special rearrangement.

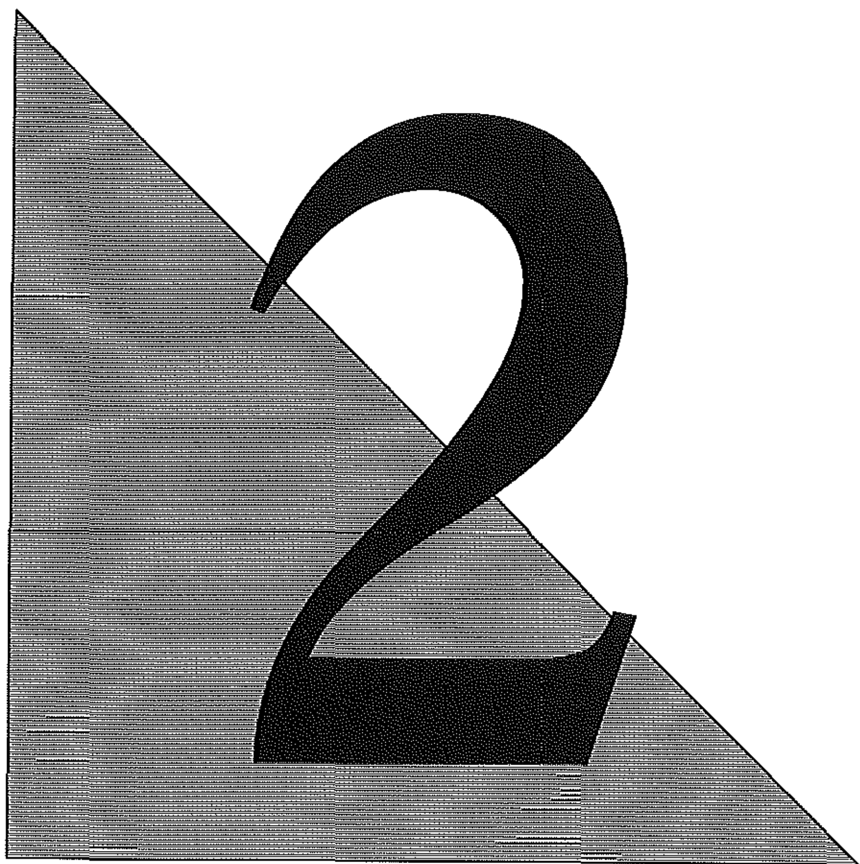
Chapter 4 summarizes the characteristics of the *tal*-1 gene aberrations in T-ALL. Also is reported that the so-called *tal*-1 deletions are restricted to T-ALL of the TcR-αβ lineage. Demethylation of the breakpoint region is proposed as a mechanism for this restricted occurrence. A possible relationship between TcR-δ gene deletion and *tal*-1 deletion is suggested.

Chapter 5 integrates several of the studied T-cell characteristics, i.e. TcR-δ gene configuration, TcR/CD3/CD4/CD8 expression, and (de)methylation of the *tal*-1 gene, into a linear human T-cell differentiation model. The role of the TcR-δ gene in human T-cell differentiation is discussed.

REFERENCES

1. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988;334:395-402.
2. Von Boehmer H. Thymic selection: a matter of life and death. Immunol Today 1992;13:454-458.
3. Van Ewijk W. T-cell differentiation is influenced by thymic microenvironments. Annu Rev Immunol 1991;9:591-615.
4. Nossal GJV. Negative selection of lymphocytes. Cell 1994;76:229-239.
5. Von Boehmer H. Positive selection of lymphocytes. Cell 1994;76:219-228.
6. Germain RN. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell 1994;76:287-299.
7. Janeway CA Jr, Bottomly K. Signals and signs for lymphocyte response. Cell 1994;76:275-285.
8. Weiss K, Rambaud S, Lavau C, Jansen J, Carvalho T, Carmo-Fonseca M, Lamond A, Dejean A. Retinoic acid regulates aberrant nuclear localization of PML-RAFα in acute promyelocytic leukemia cells. Cell 1994;76:345-356.
9. Strominger JL. Development biology of T cell receptors. Science 1989;244:943-50.
10. Porcelli S, Brenner MB, Band H. Biology of the human γδ T-cell receptor. Immunol Rev 1991;120:137-183.
11. Haas W. Gamma/delta cells. Annu Rev Immunol 1993;11:637-85.
12. Schild H, Mavaddat N, Litzenberger C, Ehrich EW, Davis MM, Bluestone JA, Matis L, Draper RK, Chein Y-H. The nature of major histocompatibility complex recognition by γδ T cells. Cell 1994;76:29-37.
13. Lewis SM. The mechanism of V(D)J joining: lessons from molecular, immunological, and comparative analyses. Adv Immunol 1994;56:27-150.
14. Litman GW, Rast JP, Shamblott MJ, Haire RN, Hulst M, Roess W, Litman RT, Hinds-Frey KR, Zilch A, Amemiya CT. Phylogenetic diversification of immunoglobulin genes and the antibody repertoire. Mol Biol Evol 1993;10:60-72.
15. Van Dongen JJM. Human T cell differentiation: basic aspects and their clinical applications. Thesis, Department of Immunology, Erasmus University Rotterdam, 1990.

REPERTOIRE OF HUMAN TcR-$\gamma\delta^+$ T-CELLS



REPERTOIRE OF HUMAN TcR-$\gamma\delta^+$ T-CELLS

2.1	Receptor diversity of human TcR-$\gamma\delta$ expressing cells Parts published in: Prog Histochem Cytochem 1992;26:182-193.	15
2.2	Limited combinatorial repertoire of $\gamma\delta$ T-cell receptors expressed by T-cell acute lymphoblastic leukemias Published in: Leukemia 1991;5:116-124.	27
2.3	Unravelling human T-cell receptor junctional region sequences Published in: Thymus 1994;22:177-199.	41
2.4	Extensive junctional diversity of $\gamma\delta$ T-cell receptors expressed by T-cell acute lymphoblastic leukemias: implications for the detection of minimal residual disease Published in: Leukemia 1991;5:1076-1086.	59
2.5	Unique selection determinant in polyclonal Vδ2-Jδ1 junctional regions of human peripheral $\gamma\delta$ T-lymphocytes Published in: J Immunol 1994;152:2860-2864.	75

CHAPTER 2.1

RECEPTOR DIVERSITY OF HUMAN TcR-$\gamma\delta$ EXPRESSING CELLS*

Timo M. Breit, Ingrid L.M. Wolvers-Tettero and Jacques J.M. van Dongen

Department of Immunology, University Hospital Dijkzigt/Erasmus University, Rotterdam, The Netherlands.

Introduction

The antigen specific receptor expressed on the cell surface of mature T-lymphocytes is called TcR and is non-covalently associated with the signal-transducing CD3 protein complex (1,2). The TcR molecule consists of two different glycoproteins (TcR-α and TcR-β or TcR-γ and TcR-δ), which generally are disulfide-linked (3). The TcR-$\alpha\beta$ is expressed by 85-98% of the T-lymphocytes in human PB, whereas the TcR-$\gamma\delta$ is expressed by the remaining 2-15% of the PB T-lymphocytes (4-8). On CD3$^+$ thymocytes this distribution is >95% and ~1%, respectively (7-10).

Each chain of the TcR heterodimer contains a variable antigen-recognizing domain and a C domain. The variable domain is encoded by a V gene segment, a J gene segment and a junctional region, which links the V and J gene segments (1,2,11). This junctional region includes D gene segments in case of TcR-β and TcR-δ genes (8,9).

The total diversity of the TcR molecules is obtained via gene rearrangement processes during T-cell differentiation by combining various V, (D) and J gene segments (combinatorial repertoire) as well as by generating diverse junctional regions (junctional diversity) (1-3,11). The combinatorial repertoire is theoretically determined by the possible combinations of functional V, (D) and J gene segments of the various TcR genes, but is actually limited by preferential usage of particular V-(D)-J rearrangements. The junctional diversity is determined by the junctional region, which is made up by D-gene-derived nucleotides in the case of TcR-β and TcR-δ genes (12,13), by N-region nucleotides (14), so called P-region nucleotides (15,16), and by deletion of nucleotides by trimming of the ends of the involved gene segments. Guidelines to determine these components of junctional regions of TcR rearrangements are described in Chapter 2.3 (17). The actual junctional diversity is limited by the fact that only "in frame" rearrangements without a stop-codon (less than one in three rearrangements) can be expressed.

Here we present a short review of the combinatorial repertoire and the junctional diversity of human TcR-γ genes and TcR-δ genes of normal mature TcR-$\gamma\delta^+$ PB T-lymphocytes, TcR-$\gamma\delta^+$ thymocytes, and TcR-$\gamma\delta^+$ T-ALL cells (18).

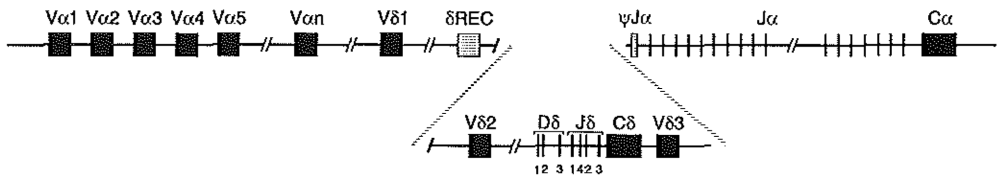
Potential repertoire of human T-cell receptors

As described above, the potential repertoire of TcR molecules is determined by the

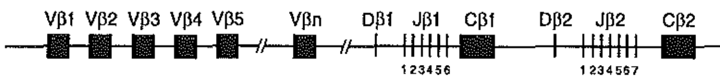
* Parts of this chapter are published in: Prog Histochem Cytochem 1992;26:182-193.

different possible combinations of available functional V, (D) and J gene segments of the various TcR genes as well as by the junctional diversity (1-3,11). The human TcR-α and TcR-β genes contain large series of functional V gene segments (>50 and ~70, respectively) and J gene segments (55 and 13, respectively), whereas the TcR-γ and TcR-δ contain only a few functional V gene segments (8 and ~4, respectively) and J gene segments (5 and 4, respectively) (Figure 1 and Table 1) (Chapter 2.3). Hence, it is obvious that TcR-αβ has a far greater potential combinatorial repertoire than TcR-γδ (Table 1). This is in contrast to the junctional diversity of the TcR-α and TcR-β rearrangements, which is relatively small compared to that of TcR-γ and TcR-δ rearrangements (Table 1) (Chapter 2.3). TcR-δ genes have the most extensive junctional diversity of all TcR genes, due to the fact that either none, one, two, or three Dδ gene segments can be used, leading to rearrangements as V-J, V-D-J, V-D-D-J, or V-D-D-D-J. This results in one, two, three, or four N-regions per junctional region, respectively (13). In the TcR-β genes only one or two Dβ gene segments can be used per rearrangement (12). After combining the combinatorial repertoire and the junctional diversity of TcR-α and TcR-β as well as TcR-γ and TcR-δ, it appears that the potential antigen-recognizing repertoire of TcR-γδ more or less equals that of TcR-αβ (Table 1). However, the actual combinatorial diversity as well as the junctional diversity and thus the total repertoire of TcR-γδ, is limited by the fact that the V and J gene segments are used in preferential patterns and there is selection for invariant amino acid residues in some junctional regions (see below and Chapters 2.2, 2.5 and 3.3).

TcR-α and TcR-δ gene complex



TcR-β gene complex



TcR-γ gene complex

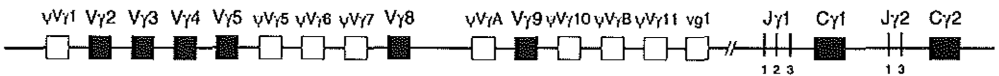


Figure 1. Schematic diagram of the germline configuration of the four human TcR genes. The TcR-α gene consists of many Vα gene segments (>50), a long stretch of J gene segments (61) and one Cα gene segment. The TcR-δ locus is located within the TcR-α gene complex between the Vα and the Jα gene segments and consists of a few Vδ (~6), four Dδ, four Jδ and one Cδ gene segments. The TcR-δ deleting elements, δREC and ψJα flank the majority of TcR-δ gene segments. The TcR-β gene contains many Vβ (~70) and two Cβ gene segments, which are preceded by one Dβ and six or seven Jβ gene segments. The TcR-γ gene consists of two Cγ gene segments, preceded by a few Jγ gene segments (2 or 3) and a restricted number of Vγ gene segments: six functional gene segments (solid boxes) and nine pseudo-gene segments (ψ; open boxes). The Vγ gene segments are grouped in four families (VγI, VγII, VγIII and VγIV) based on sequence homology.

TABLE 1. Estimation of the potential antigen-recognizing repertoire of human TcR molecules.

	TcR-αβ molecule		TcR-γδ molecule	
	TcR-α	TcR-β	TcR-γ	TcR-δ
Germline diversity number of functional gene segments[a]				
- V gene segments	>50	~70	6[b]	~4
- D gene segments	0	2	0	3
- J gene segments	55	13	5	4
Combinatorial diversity				
- per chain	>2750	~1800	30	~128[c]
- per dimer	$>5 \times 10^6$		$\sim 4 \times 10^3$	
Junctional diversity				
- number of joining sites	1	2	1	1-4
- average N-region insertion	5 bp	7 bp	5 bp	11 bp
- estimated degree of increase in diversity	$\times 10^3$	$\times 10^4$	$\times 10^3$	$\times 10^7$
Estimation of the total potential receptor diversity	$>10^{13}$		$>10^{13}$	

a. TcR-α and TcR-β genes were described in references 12,55-59. TcR-γ and TcR-δ genes were described in references 13,60-65.
b. In addition to the six functional Vγ genes, nine pseudogenes have been described (61-66).
c. The variable domain of the TcR-δ chain cannot only be encoded by a V-D-J rearrangement, but also by V-J, V-D-D-J or V-D-D-D-J rearrangements (13).

Actual repertoire of T-cell receptor γδ

The repertoire of TcR-γδ⁺ T-cells in different compartments of the human body has been studied analyzing freshly obtained PB lymphocytes and thymocytes (13,19-24) as well as TcR-γδ⁺ T-cell clones derived from PB and thymus (25-27). In addition, malignant counterparts of cortical thymocytes, i.e. T-ALL have been investigated (28-31). In early studies, the V gene usage of TcR-γδ⁺ T-cells was determined by use of a few V-gene-specific monoclonal antibodies (McAb): i.e. anti-TcR-Vγ9 (Ti-γA) (32), anti-TcR-Vδ1 (δTCS1) (33) and anti-TcR-Vδ2 (BB3) (34). These McAb only give information about V gene expression and not about J or C gene expression. Recently, a new monoclonal antibody (23D12) has been described that recognizes several VγI segments, i.e. Vγ2, Vγ3, and Vγ4 (35,36). The combined results of these studies are summarized in Table 2. In some studies Southern blot analysis, PCR analysis, and sequencing of the rearrangements were used to evaluate J and C gene usage (Cγ1 versus Cγ2) of TcR-γδ⁺ T-cell clones and TcR-γδ⁺ T-ALL (26,30,31,37).

Nucleotide insertion (N-region, Dδ gene derived, and P-region) and nucleotide deletion form the junctional region of rearranged TcR-γ or TcR-δ genes, and determine their junctional diversity. In Table 3 we have summarized information about a large number of published TcR-γ and TcR-δ junctional regions from human TcR-γδ⁺ PB T-lymphocytes, thymocytes and T-ALL cells.

The observed combinatorial repertoire as well as the junctional diversity will be discussed in more detail for each different cell type.

TABLE 2. V gene expression in human TcR-$\gamma\delta^+$ PB T-lymphocytes, thymocytes, and T-ALL cells.

	TcR-γ expression		TcR-δ expression	
	$V\gamma I$	$V\gamma II$ ($V\gamma 9$)	$V\delta 1$	$V\delta 2$
PB T-lymphocytes[a]	11–30%	66–87%	11–30%	66–87%
Thymocytes[b]	47–55%	20–25%	60–75%	20–25%
T-ALL[c] (n = 45)[d]	67%	33%	80%	4%

a. Data concerning TcR expression on PB T-lymphocytes are from references 49,25,26, and 38. Differences in V gene expression as found by different research groups may be due to the fact that in early childhood higher frequencies of Vδ1 expressing cells occur, whereas in adults most TcR-$\gamma\delta^+$ PB T-lymphocytes express Vγ9/Vδ2 (10).
b. Data concerning TcR expression on thymocytes are shown in references 9 and 26.
c. Data concerning TcR expression on T-ALL cells are shown in references 28-31,37,65, and unpublished results.
d. In addition to 43 TcR-$\gamma\delta^+$ T-ALL patients, the TcR-$\gamma\delta^+$ cell lines PEER and MOLT-13 are included (65).

TcR-$\gamma\delta^+$ T-lymphocytes

Human TcR-$\gamma\delta^+$ T-lymphocytes are characterized by the fact that the far majority express a receptor existing of a Vγ9-Jγ1.2-Cγ1 chain combined with a Vδ2-Dδ3-Jδ1-Cδ chain (5-7,10,26,38,39). Although the frequency may vary between individuals, the average percentage of Vγ9/Vδ2 T-lymphocytes is >70% of the total TcR-$\gamma\delta^+$ PB T-lymphocytes (10). The majority of the remaining T-lymphocytes express a VγI-Jγ2.3-Cγ2 chain combined with a Vδ1-Dδ2-Dδ3-Jδ1-Cδ chain (19,26,40). Thus, although the potential combinatorial repertoire of the human TcR-$\gamma\delta^+$ is about 4,000 different receptors, only two different TcR-γδ are expressed on the far majority of human PB T-lymphocytes.

It might be that this limited combinatorial repertoire is also reflected in a limited junctional diversity. The initial studies performed, showed an extensive and random junctional diversity in the Vγ9/Vδ2 T-lymphocytes (13,19-24)). However, we and others did observe an invariant element in the junctional region of the Vδ2-Dδ3-Jδ1 rearrangements (Chapter 2.5) (41,42). This selection determinant was identified by the presence of an invariant T-nucleotide at the relative second position in 90% of these junctional region sequences (Figure 2). Translation of the relative first codon of the Vδ2-Jδ1

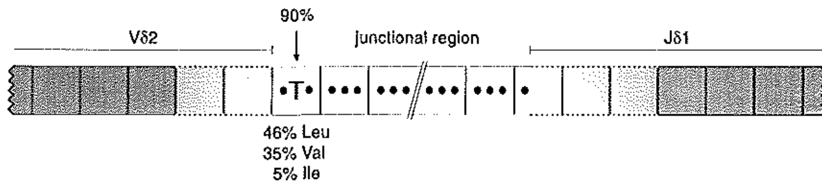


Figure 2. Invariant T-nucleotide coding for Leu, Val or Ile in polyclonal Vδ2-Jδ1 junctional regions of TcR-$\gamma\delta^+$ PB T-lymphocytes. Schematic presentation of the Vδ2-Jδ1 junctional region in TcR-$\gamma\delta^+$ PB T-lymphocytes. •••• represent random or Dδ gene derived junctional region nucleotides. The shaded areas indicate the sides in which deletion of nucleotides from the germline Vδ2 and Jδ1 sequences frequently occurs. The arrow indicates the relative second position of the junctional region with the invariant T-nucleotide (90%). The percentages Leu, Val and Ile found in the relative first codon of these junctional regions are indicated.

TABLE 3. Junctional diversity of TcR-γ and TcR-δ gene rearrangements in human PB T-lymphocytes, thymocytes, and T-ALL cells[a].

	TcR-γ genes					TcR-δ genes					
	N-region[b] nucleotides	P-region[c] nucleotides	Junctional[d] nucleotides	Deleted[e] nucleotides	No. of alleles analyzed	D-gene[f] nucleotides	N-region[b] nucleotides	P-region[c] nucleotides	Junctional[d] nucleotides	Deleted[e] nucleotides	No. of alleles analyzed
PB T-lymphocytes[g]	4.4 (0–18)	0.3 (0–2)	4.7 (0-18)	6.3 (0–20)	295	10.1 (0–24)	10.6 (0–34)	1.2 (0–6)	22.0 (7-55)	3.9 (0–17)	516
Thymocytes:											
- fetal[h]	1.9 (0–7)	0.1 (0–1)	2.1 (0-7)	5.3 (0–11)	28	9.0 (3–16)	3.1 (0–24)	1.1 (0–5)	13.1 (5-39)	2.3 (0–9)	73
- post natal[i]	NR	NR	NR	NR	0	11.5 (3–21)	14.4 (2–32)	1.5 (0–4)	27.4 (11-46)	6.4 (0–30)	42
T-ALL cells[j]	8.1 (0–25)	0.2 (0–2)	8.3 (0-25)	10.3 (1–35)	47	9.7 (0–20)	14.9 (0–41)	1.1 (0–4)	25.7 (3-55)	5.5 (0–29)	54

a. The figures in the table represent mean values, while ranges are given in parentheses. NR: not reported.
b. N-region nucleotides: random inserted nucleotides of all N-regions.
c. P-region nucleotides: nucleotides recognized as fulfilling the conditions for P-regions (15 and Chapter 2.3).
d. Junctional region nucleotides: total of (Dδ gene,) N-region and P-region nucleotides.
e. Deleted nucleotides: total loss of nucleotides per junctional region as caused by trimming at 3′ and 5′ sides of the rearranging V and J gene segments, respectively.
f. D gene nucleotides: nucleotides which aligne with a Dδ gene segment. Rules for alignment are described in Chapter 2.3.
g. Junctional region sequences of PB T-lymphocytes were published in references 19,20,62, and 67.
h. Junctional region sequences of fetal thymocytes were published in references 27,47 and 67.
i. Junctional region sequences of post natal thymocytes were published in references 18,27, and 47.
j. Junctional region sequences of T-ALL were published in references 28,31,68-70, and unpublished results.

junctional regions revealed strikingly high frequencies of the homologous amino acids leucine (46%), valine (35%), and isoleucine (5%) at this position (41,42). We could prove that this determinant was selected for at the protein level, because in out-of-frame Vδ2-Jδ1 junctional regions and polyclonal thymocytes the invariant T-nucleotide was absent (Chapter 2.5) (42).

No selection determinant could be identified in Vγ9-Jγ1.2 junctional regions, although the frequently occurring invariable, so-called canonical, junctional region provided evidence for a biased recombination processes (Chapter 2.5) (42). It might be though, that the relative size of the Vγ9-Jγ1.2 junctional regions is an important selection determinant, as the majority of these junctional regions in PB T-lymphocytes contains an almost constant number ($\pm$ 1) of amino acid residues (41).

The Vγ9/Vδ2 T-lymphocyte subpopulation is characterized by polyclonal expansion after birth, whereas no parallel post-natal expansion of Vγ9/Vδ2 thymocytes takes place (10). In contrast, at birth the majority of PB T-lymphocytes as well as thymocytes express a Vδ1 TcR. The percentage of $CD3^+$ PB T-lymphocytes that express a Vδ1 chain remains relatively constant during aging (10). It is also of interest that the majority of Vγ9/Vδ2 T-lymphocytes express high levels of CD45RO (10), which suggest prior activation of these cells, whereas the Vδ1 T-lymphocytes are either negative or express only low levels of CD45RO. No link could be found between MHC haplotypes as striking differences of TcR-γδ V gene usage did occur between identical twins (10). Altogether these data suggest peripheral expansion of the Vγ9/Vδ2 T-lymphocyte subset by exposure to foreign antigens.

It is remarkable that there are so many (five or six) strict selection determinants needed for Vγ9/Vδ2 T-lymphocytes: the Vδ2 gene segment, the Jδ1 gene segment, the invariant amino acid residue at the relative first position of the Vδ2-Jδ1 junctional region, the Vγ9 gene segment, the Jγ1.2 gene segment, and perhaps the relative size of the Vγ9-Jγ1.2 junctional region. Therefore, one has to keep an open mind to the possibility that not all determinants are selected for at the same site and/or at the same time. This suggestion is supported by reports that the Vγ9 segment is able to respond to certain antigens independently from the co-expressed Jγ, Vδ, and Jδ segments. Thymic selection processes might select for Vδ2 + invariant junctional amino acid residue + Jδ1, whereas peripheral expansion might only need Vγ9 (43,44).

These are all speculations, and there are several options to study these phenomena. For instance, by introducing different TcR-γ and TcR-δ genes into T-cell clones and measurement of their reactivity (44), or secretion of the Vγ9/Vδ2 TcR-γδ heterodimer to characterize their interactions with ligand (45). Another interesting analysis could be the determination of the selection determinants within the Vδ2, Jδ1, Vγ9, Jγ1.2 segments. This should be possible with transfection studies of mutated Vδ2-Jδ1 and Vγ9-Jγ1.2 chains.

A potential selection determinant in the Vδ2 segment could be the cysteine, two residues N-terminal of the invariant junctional region residue (42). This is not the cysteine involved in the immunoglobulin domain formation, because that cysteine is located another two residues N-terminal. There are reasons to suspect this cysteine to be involved in the selection processes; firstly, the invariant Vδ2-Jδ1 junctional region residue is positioned relative to the Vδ2 segment and because it is so inflexibly located, one can assume that the Vδ2 selection determinant is close-by. Secondly, the extra cysteine is unusual because it

is not present in any other TcR V segment (17). Finally, there might be also an unusual cysteine residue present in the N-terminus of the Vγ9 segment (46), which may form a disulphide bound with the extra cysteine in the Vδ2 segment. Although preliminary studies seem to disqualify this hypothesis, no definitive proof has been published, yet (45).

TcR-$\gamma\delta^+$ thymocytes

As elegantly described by Parker et al. (10), the percentage Vδ1 as well as Vδ2 expressing thymocytes remains constant during aging. The majority of the TcR-$\gamma\delta^+$ thymocytes expresses a VγI-Jγ2-Cγ2 chain in combination with a Vδ1-Jδ1-Cδ chain (26,40). Aside from the selection processes on the protein level or the DNA level, biased recombination processes lead to preferential rearrangements. The preferential TcR-δ gene rearrangements in both fetal and post-natal thymocytes are described in Chapter 3.3. The preferential TcR-γ and TcR-δ gene rearrangements in thymocytes cause the limited combinatorial repertoire of TcR-$\gamma\delta^+$ T-cells.

However, the junctional diversity of TcR-δ gene rearrangements in thymocytes is extensive (13,27,47). It is remarkable that the TcR-γ and TcR-δ junctional regions in fetal thymocytes are much smaller than those in PB T-lymphocytes (Table 3). This is mainly caused by the lesser insertion of N-region nucleotides, which is in line with low expression or absence of TdT in fetal thymocytes (<20 weeks of gestation) (48). Fetal thymocytes therefore are the only type of T-cells in which the Dδ-gene derived nucleotides contribute more (69%) to the total TcR-δ junctional region than N-region nucleotides (24%). In all other T-cells, N-region nucleotides are the most prominent element in the TcR-δ junctional region, whereas the contribution of Dδ-gene nucleotides is lower. P-region nucleotides are of minor importance in the total junctional diversity, because they only represent a small proportion of the junctional regions.

TcR-$\gamma\delta^+$ T-ALL cells

The combinatorial repertoire and junctional diversity of TcR-$\gamma\delta^+$ T-ALL are extensively discussed in Chapters 2.2 and 2.4, respectively (30,31). Here, we like to summarize the data from our large series (n = 29) of TcR-$\gamma\delta^+$ T-ALL. As indicated in Tables 2 and 4, the combinatorial repertoire of the Vγ and Jγ genes is rather limited. A large fraction of TcR-$\gamma\delta^+$ T-ALL expresses a VγI-Jγ2.3-Cγ2 chain. The combinatorial repertoire of the TcR-δ chain is even more restricted as 80% of the TcR-$\gamma\delta^+$ T-ALL express a Vδ1-Jδ1 chain.

In contrast with this overall extremely limited combinatorial repertoire is the presence of an extensive junctional diversity (Tables 3 and 4). Although the nucleotide insertion in TcR-γ gene rearrangements is about one third of that in TcR-δ gene rearrangements, it still exceeds that of the Vγ9-Jγ1.2 junctional regions in PB T-lymphocytes.

Because Vδ2-Jδ1 rearrangements use only one D gene segment (Dδ3) in their junctional region, whereas Vδ1-Jδ1 rearrangements use two D gene segments (Dδ2 and Dδ3), the junctional regions of the latter rearrangements are significantly longer.

The restricted combinatorial repertoire and extensive junctional diversity of TcR-$\gamma\delta^+$ T-ALL are comparable with that of TcR-$\gamma\delta^+$ thymocytes, which is in line with the assumption that T-ALL originate from differentiating cortical thymocytes.

TABLE 4. Repertoire of TcR-γ and TcR-δ alleles in TcR-γδ⁺ T-ALL

	Combinatorial diversity (n=29)	(n)[b]	Junctional diversity[a]						
			N-region	Dδ	P-region	Total ins	5′ del	3′ del	Total del
TcR-γ gene									
Vγ-Jγ1.1	7%	(3)	2.3		0	2.3	3.7	10.0	13.7
Vγ-Jγ1.2	0%	(0)	–		–	–	–	–	–
Vγ-Jγ1.3	21%	(10)	7.7		0.5	8.2	2.8	5.2	8.0
Vγ-Jγ2.1	7%	(2)	6.0		0	6.0	3.5	6.0	9.5
Vγ-Jγ2.3	60%	(26)	9.9		0.1	10.0	3.9	6.7	10.7
Germline/Deletion	5%	NR	–		–	–	–	–	–
TcR-δ gene									
Vδ1-Jδ1	53%	(25)	17.8	11.5	1.2	30.6	2.1	2.1	4.2
Vδ2-Jδ1	9%	(5)	11.0	7.4	0.8	19.2	6.0	1.4	7.4
Vδ3-Jδ1	7%	(4)	21.3	11.2	0.8	33.3	2.8	3.8	6.5
Dδ2-Jδ1	14%	(8)	9.9	6.9	1.4	18.1	7.1	3.4	10.5
Vδ2-Dδ3	3%	(2)	7.0	3.5	2.5	1.0	1.5	1.5	3.0
Other/Deletion	14%	NR	–	–	–	–	–	–	–

a. Junctional diversity is composed of insertion (ins): N-region, Dδ-gene derived, and P-region nucleotides; and deletion (del) of the involved V or D (5′ del) gene segments and D or J (3′ del) gene segments (Chapter 2.3).
b. n, number of junctional regions analyzed to determine the average nucleotide insertion and deletion. NR, not relevant.

Concluding remarks

The far majority of the human TcR-γδ⁺ T-lymphocytes express a receptor with Vγ9/Vδ2 chains. This specific T-lymphocyte population is characterized by peripheral expansion after birth (10). Although an enormous amount of effort has been put into research on these Vγ9/Vδ2 T-lymphocytes and a recent study identified one of the ligands of these receptors (49), still almost nothing is known about the function of these particular T-lymphocytes in the human immune system. The identification of homologous amino acid residues at a fixed position in the Vδ2-Jδ1 junctional region of Vγ9/Vδ2 PB T-lymphocytes revealed not only the existence of (peripheral) selection processes for these T-cells, but also falsified the hypothesis of superantigen involvement, because superantigens seize by definition only at germline encoded regions (10,23,44). In a more general way, the presence of a selection determinant at a fixed position in a junctional region of such a large percentage of the human TcR-γδ⁺ T-lymphocytes that is not present in thymocytes, also provided new insight in antigen specificity of TcR. This is by far the most consistent selection determinant published yet, and also one of the few that concerned a single junctional region amino acid residue (50), which is in contrast with the observed junctional region motifs (35). Therefore, it might be useful to use this invariant selection determinant as a model to identify selection determinants in junctional regions of other TcR.

For instance, there are two occasions in which γδ TcR with a Vδ1-Dδ2-Dδ3-Jδ1 chain, show peripheral selection: in the human intestinal tract (52,53) and in humans during post-natal aging (54). After (superficial) examination of the published amino acid sequences of the Vδ1-Jδ1 junctional regions we observed an interesting increase of arginine and isoleucine residues at fixed positions relative to the Jδ1 gene segment in the most frequently occurring junctional regions of T-lymphocytes from the small intestine, or from adult donors, but not from the colon (53,54). It might be useful to further investigate these putative determinants in these junctional regions.

Because TcR-$\gamma\delta^+$ T-cells have a limited number of gene segments and therefore a limited combinatorial diversity, their function is probably confined to their enormous junctional regions. In general, one might hope that the observed restricted junctional region diversity will be a clue towards the determination of the role of TcR-$\gamma\delta^+$ T-cells. It has to be noticed though, that although a strict selection determinant was observed in the Vδ2-Jδ1 junctional regions of Vγ9/Vδ2 T-lymphocytes, this does not exclude an active role in the selection processes for the remaining part of these junctional regions.

REFERENCES

1. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988;334:395-402.
2. Raulet DH. The structure, function, and molecular genetics of the γ/δ T cell receptor. Annu Rev Immunol 1989;7:175-207.
3. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of Immunoglobulin and T cell receptor genes. Part I: Basic and technical aspects. Clin Chim Acta 1991;198:1-92.
4. Bottino C, Tambussi G, Ferrini S, Ciccone E, Varese P, Mingari MC, Moretta L, Moretta A. Two subsets of human T lymphocytes expressing γ/δ antigen receptor are identifiable by monoclonal antibodies directed to two distinct molecular forms of the receptor. J Exp Med 1988;168:491-505.
5. Triebel F, Faure F, Graziani M, Jitsukawa S, Lefranc M-P, Hercend T. A unique V-J-C-rearranged gene encodes a γ protein expressed on the majority of CD3$^+$ T cell receptor-α/β^- circulating lymphocytes. J Exp Med 1988;167:694-699.
6. Triebel F, Faure F, Mami-Chouaib F, Jitsukawa S, Griscelli A, Genevée C, Roman-Roman S, Hercend T. A novel human Vδ gene expressed predominantly in the TiγA fraction of γ/δ^+ peripheral lymphocytes. Eur J Immunol 1988;18:2021-2027.
7. Borst J, Van Dongen JJM, Bolhuis RLH, Peters PJ, Hafler DA, De Vries E, Van de Griend RJ. Distinct molecular forms of human T cell receptor γ/δ detected on viable T cells by a monoclonal antibody. J Exp Med 1988;167:1625-1644.
8. Van Dongen JJM, Comans-Bitter WM, Wolvers-Tettero ILM, Borst J. Development of human T lymphocytes and their thymus-dependency. Thymus 1990;16:207-234.
9. Falini B, Flenghi L, Pileri S, Pelicci P, Fagioli M, Martelli MF, Moretta L, Ciconne E. Distribution of T cells bearing different forms of the T cell receptor γ/δ in normal and pathological human tissues. J Immunol 1989;143:2480-2488.
10. Parker CM, Groh V, Band H, Porcelli SA, Morita C, Fabbi M, Glass D, Strominger JL, Brenner MB. Evidence for extrathymic changes in the T cell receptor γ/δ repertoire. J Exp Med 1990;171:1597-1612.
11. Lewis SM. The mechanism of V(D)J joining: lessons from molecular immunological, and comparative analyses. Adv Immunol 1994;56:27-150.
12. Toyonaga B, Yoshikai Y, Vadasz V, Chin B, Mak TW. Organization and sequences of the diversity, joining, and constant region genes of the human T-cell receptor β chain. Proc Natl Acad Sci USA 1985;82:8624-8628.
13. Loh EY, Cwirla S, Serafini AT, Phillips JH, Lanier LL. Human T-cell-receptor δ chain: genomic organization, diversity, and expression in populations of cells. Proc Natl Acad Sci USA 1988;85:9714-9718.
14. Alt FW, Baltimore D. Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-JH fusions. Proc Natl Acad Sci USA 1982;79:4118-4122.
15. Lafaille JJ, DeCloux A, Bonneville M, Takagaki Y, Tonegawa S. Junctional sequences of T cell receptor $\gamma\delta$ genes: Implications for $\gamma\delta$ T cell lineages and for a novel intermediate of V-(D)-J joining. Cell 1989;59:859-870.
16. Meier JT, Lewis SM. P nucleotides in V(D)J recombination: a fine-structure analysis. Mol Cell Biol 1993;13:1078-1092.
17. Breit TM, Van Dongen JJM. Unravelling human T-cell receptor junctional region sequences. Thymus 1994;22:177-199.
18. Breit TM, Wolvers-Tettero ILM, Van Dongen JJM. Receptor diversity of human T-cell receptor $\gamma\delta$ expressing cells. Prog Histochem Cytochem 1992;26:182-193.
19. Loh EY, Elliott JF, Cwirla S, Lanier LL, Davis MM. Polymerase chain reaction with single-sided specificity: analysis of T cell receptor δ chain. Science 1989;243:217-220.

20. Tamura N, Holroyd KJ, Banks T, Kirby M, Okayama H, Crystal RG. Diversity in junctional sequences associated with the common human Vγ9 and Vδ2 gene segments in normal blood and lung compared with the limited diversity in a granulomatous disease. J Exp Med 1990;172:169-181.
21. De Libero G, Casorati G, Giachino C, Carbonara C, Migone N, Matzinger P, Lanzavecchia A. Selection by two powerful antigens may account for the presence of the major population of human peripheral γ/δ T cells. J Exp Med 1991;173:1311-1322.
22 Ohmen JD, Barnes PF, Uyemura K, Lu S, Grisso CL, Modlin RL. The T cell receptors of human γδ T cells reactive to *Mycobacterium tuberculosis* are encoded by specific V genes but diverse V-J junctions. J Immunol 1991;147:3353-3359.
23 Panchamoorthy G, McLean J, Modlin RL, Morita CT, Ishikawa S, Brenner MB, Band H. A predominance of the T cell receptor Vγ2/Vδ2 subset in human Mycobacteria-responsive T cells suggests germline gene encoded recognition. J Immunol 1991;147:3360-3369.
24. Uyemura K, Deans RJ, Band H, Ohmen J, Panchamoorthy G, Morita CT, Rea TH, Modlin RL. Evidence for clonal selection of γ/δ T cells in response to a human pathogen. J Exp Med 1991;174:683-692.
25. Borst J, Wicherink A, Van Dongen JJM, De Vries E, Comans-Bitter WM, Wassenaar F, Van den Elsen P. Non-random expression of T cell receptor γ and δ variable gene segments in functional T lymphocyte clones from human peripheral blood. Eur J Immunol 1989;19:1559-1568.
26. Casorati G, De Libero G, Lanzavecchia A, Migone N. Molecular analysis of human γ/δ$^+$ clones from thymus and peripheral blood. J Exp Med 1989;170:1521-1535.
27. McVay LD, Carding SR, Bottomly K, Hayday AC. Regulated expression and structure of T cell receptor γ/δ transcript in human thymic ontogeny. EMBO J 1991;10:83-91.
28. Macintyre E, D'Auriol L, Amesland F, Loiseau P, Chen Z, Boumsell L, Galibert F, Sigaux F. Analysis of junctional diversity in the preferential Vδ1-Jδ1 rearrangement of fresh T-acute lymphoblastic leukemia cells by in vitro gene amplification and direct sequencing. Blood 1989;74:2053-2061.
29. Gouttefangeas C, Bensussan A, Boumsell L. Study of the CD3-associated T-cell receptors reveals further differences between T-cell acute lymphoblastic lymphoma and leukemia. Blood 1990;75:931-934.
30. Breit TM, Wolvers-Tettero ILM, Hählen K, Van Wering ER, Van Dongen JJM. Limited combinatorial repertoire of γδ T-cell receptors expressed by T-cell acute lymphoblastic leukemias. Leukemia 1991;5:116-124.
31. Breit TM, Wolvers-Tettero ILM, Hählen K, Van Wering ER, Van Dongen JJM. Extensive junctional diversity of γδ T-cell receptors expressed by T-cell acute lymphoblastic leukemias. Leukemia 1991;5:1076-1086.
32. Jitsukawa S, Faure F, Lipinski M, Triebel F, Hercend T. Novel subset of human lymphocytes with a T cell receptor-γ complex. J Exp Med 1987;166:1192-1197.
33. Wu Y-J, Tian W-T, Snider RM, Rittershaus C, Rogers P, LaManna L, Ip SH. Signal transduction of γ/δ T cell antigen receptor with a novel mitogenic anti-δ antibody. J Immunol 1988;141:1476-1479.
34. Ciconne E, Ferrini S, Bottino C, Viale O, Prigione I, Pantaleo G, Tambussi G, Moretta A, Moretta L. A monoclonal antibody specific for a common determinant of the human T cell receptor γ/δ directly activates CD3$^+$ WT31$^-$ lymphocytes to express their functional program(s). J Exp Med 1988;168:1-11.
35. Schondelmaier S, Wesch D, Pechhold K, Kabelitz D. Vγ gene usage in peripheral blood γδ T cells. Immunol Letters 1993;38:121-126.
36. Kabelitz D, Ackermann T, Hinz T, Davodeau F, Band H, Bonneville M, Janssen O, Arden B, Schondelmaier S. New monoclonal antibody (23D12) recognizing three different Vγ elements of the human γδ T cell receptor: 23D12$^+$ cells comprise a major subpopulation of γδ T cells in postnatal thymus. J Immunol 1994;152:3128-3136.
37. Loiseau P, Guglielmi P, Le Paslier D, Macintyre E, Gessain A, Borries J-C, Flandrin G, Chen Z, Sigaux F. Rearrangements of the T cell receptor δ gene in T acute lymphoblastic leukemia cells are distinct from those occurring in B lineage acute lymphoblastic leukemia and preferentially involve one Vδ gene segment. J Immunol 1989;9:3305-3311.
38. Triebel F, Hercend T. Subpopulations of human peripheral T gamma delta lymphocytes. Immunol Today 1989;10:186-188.
39. Vietor H, Koning F. γδ T-cell receptor repertoire in human peripheral blood and thymus. Immunogenetics 1990;31:340-346.
40. Mathoudakis G, Chen P-F, Li Y-D, Chernajovsky Y, Platsoucas CD. Preferential rearrangements of the VγI subgroup of the γ-chain of the T-cell antigen receptor to Jγ2Cγ2 gene segments in peripheral blood lymphocyte transcripts from normal donors. Scand J Immunol 1993;38:31-36.
41. Davodeau F, Peyrat MA, Hallet MM, Houde I, Vie H, Bonneville M. Peripheral selection of antigen receptor junctional features in a major human γδ subset. Eur J Immunol 1993;23:804-808.
42. Breit TM, Wolvers-Tettero ILM, Van Dongen JJM. Unique selection determinant in polyclonal Vδ2-Jδ1 junctional regions of human peripheral γδ T lymphocytes. J Immunol 1994;152:2860-2864.

43. Rust CJJ, Verreck F, Vietor H, Koning F. Specific recognition of staphylococcal enterotoxin A by human T cells bearing receptors with the Vγ9 region. Nature 1990;346:572-574.
44. Loh EY, Wang M, Bartkowiak J, Wiaderkiewicz R, Hyjek E, Wang Z, Kozbor D. Gene tranfer studies of T cell receptor-γδ recognition: specificity for staphylococcal enterotoxin A is conveyed by Vγ9 alone. J Immunol 1994;152:3324-3332.
45. Davodeau F, Houde I, Boulot G, Romagné F, Necker A, Canavo N, Peyrat MA, Hallet MM, Vié H, Jacques Y, Mariuzza R, Bonneville M. Secretion of disulphide-linked human T-cell receptor γδ heterodimers. J Biol Chem 1993;268:15455-15460.
46. Huck S, Dariavach P, Lefranc M-P. Variable region genes in the human T-cell rearranging gamma (TRG) locus: V-J junction and homology with the mouse genes. EMBO J 1988;7:719-726.
47. Krangel MS, IJssel H, Brocklehurst C, Spits H. A distinct wave of human T cell receptor γ/δ lymphocytes in the early fetal thymus: evidence for controlled gene rearrangement and cytokine production. J Exp Med 1990;172:847-849.
48. Campana D, Janossy G, Coustan-Smith E, Amlot PL, Tian W-T, Ip S, Wong L. The expression of T cell receptor-associated proteins during T cell ontogeny in man. J Immunol 1989;142:57-66.
49. Constant P, Davodeau F, Peyrat M-A, Poquet Y, Pozu G, Bonneville M, Fournié J-J. Stimulation of human γδ T cells by nonpeptidic mycobacterial ligands. Science 1994;264:267-270.
50. Engel I, Hedrick SM. Site-directed mutations in the VDJ junctional region of a T cell receptor β chain cause changes in antigenic peptide recognition. Cell 1988:54:473-484.
51. Oksenberg JR, Panzara MA, Begovich AB, Mitchell D, Erlich HA, Murray RS, Schimonkevitz R, Sherritt M, Rothbard J, Bernard CCA, Steinman L. Selection for T-cell receptor Vβ-Dβ-Jβ gene rearrangements with specificity for a myelin basic protein peptide in brain lesions of multiple sclerosis. Nature 1993;362:68-70.
52. Deusch K, Lüling F, Reich K, Classen M, Wagner H, Pfeffer K. A major fraction of human intraepithelial lymphocytes simultaneously express the γ/δ T cell receptor, the CD8 accessory molecule and preferentially uses the Vδ1 gene segment. Eur J Immunol 1991;21:1053-1059.
53. Chowers Y, Holtmeier W, Harwood J, Morzycka-Wroblewska E, Kagnoff MF. The Vδ1 T cell receptor repertoire in human small intestine and colon. J Exp Med 1994;180:183-190.
54. Beldjord K, Beldjord C, Macintyre E, Even P, Sigaux F. Peripheral selection of Vδ1$^+$ cells with restricted T cell receptor δ gene junctional repertoire in the peripheral blood of healthy donors. J Exp Med 1993;178:121-127.
55. Yoshikai Y, Clark SP, Taylor S, Sohn U, Wilson BI, Minden MD, Mak TW. Organization and sequences of the variable, joining and constant region genes of the human T-cell receptor α-chain. Nature 1985;316:837-840.
56. Yoshikai Y, Kimura N, Toyonaga B, Mak TW. Sequences and repertoire of human T cell receptor α chain variable region genes in mature T lymphocytes. J Exp Med 1986;164:90-103.
57. Concannon P, Pickering LA, Kung P, Hood L. Diversity and structure of human T-cell receptor β-chain variable region genes. Proc Natl Acad Sci USA 1986;83:6598-6602.
58. Kimura N, Toyonaga B, Yoshikai Y, Du R-P, Mak TW. Sequences and repertoire of the human T cell receptor α and β chain variable region genes in thymocytes. Eur J Immunol 1987;17:375-383.
59. Klein MH, Concannon P, Everett M, Kim LDH, Hunkapiller T, Hood L. Diversity and structure of human T-cell receptor α-chain variable region genes. Proc Natl Acad Sci USA 1987;84:6884-6888.
60. Quertermous T, Strauss WM, Van Dongen JJM, Seidman JG. Human T cell γ chain joining regions and T cell development. J Immunol 1987;138:2687-2690.
61. Lefranc M-P. The human T-cell rearranging gamma (TRG) genes and the gamma T-cell receptor. Biochimie 1988;70:901-908.
62. Takihara Y, Reimann J, Michalopoulos E, Ciccone E, Moretta L, Mak TW. Diversity and structure of human T cell receptor δ chain genes in peripheral blood γ/δ-bearing T lymphocytes. J Exp Med 1989;169:393-405.
63. Takihara Y, Tkachuk D, Michalopoulos E, Champagne E, Reimann J, Minden M, Mak TW. Sequence and organization of the diversity, joining, and constant region genes of the human T-cell δ-chain locus. Proc Natl Acad Sci USA 1988;85:6097-6101.
64. Baer R, Boehm T, Yssel H, Spits H, Rabbitts TH. Complex rearrangements within the human Jδ-Cδ/Jα-Cα locus and aberrant recombination between Jα segments. EMBO J 1988;7:1661-1668.
65. Hata S, Clabby M, Devlin P, Spits H, De Vries JE, Krangel MS. Diversity and organization of human T cell receptor δ variable gene segments. J Exp Med 1989;169;41-57.
66. Zhang X-M, Tonnelle C, Lefranc M-P, Huck S. T cell receptor γ cDNA in human fetal liver and thymus: variable regions of γ chains are restricted to VγI or V9, due to the absence of splicing of the V10 and V11 leader intron. Eur J Immunol 1994;24:571-578.
67. Van der Stoep N, De Krijger R, Bruining J, Koning F, Van den Elsen P. Analysis of early fetal T-cell receptor δ chain in humans. Immunogenetics 1990;32:331-336.

68. Macintyre EA, D'Auriol L, Duparc N, Leverger G, Galibert F, Sigaux F. Use of oligonucleotide probes directed against T cell antigen receptor gamma delta variable-(diversity)-joining junctional sequences as a general method for detecting minimal residual disease in acute lymphoblastic leukemias. J Clin Invest 1990;86:2125-2135.
69. Littman DR, Newton M, Crommie D, Ang S-L, Seidman JG, Gettner SN, Weiss A. Characterization of an expressed CD3-associated Ti γ-chain reveals Cγ domain polymorphism. Nature 1987;326:85-88.
70. Hata S, Satyanarayana K, Devlin P, Band H, Mclean J, Strominger JL, Brenner MB, Krangel MS. Extensive junctional diversity of rearranged human T cell receptor δ genes. Science 1988;240:1541-1544.

CHAPTER 2.2

LIMITED COMBINATORIAL REPERTOIRE OF $\gamma\delta$ T-CELL RECEPTORS EXPRESSED BY T-CELL ACUTE LYMPHOBLASTIC LEUKEMIAS*

Timo M. Breit[1], Ingrid L.M. Wolvers-Tettero[1], Karel Hählen[2], Elisabeth R. van Wering[3], and Jacques J.M. van Dongen[1]

1. Department of Immunology, Erasmus University/University Hospital Dijkzigt, Rotterdam;
2. Department of Pediatrics, Subdivision of Hematology-Oncology, Sophia Children's Hospital, Rotterdam;
3. Dutch Childhood Leukemia Study Group, The Hague, The Netherlands.

SUMMARY

Detailed analysis of the rearrangement and expression of the TcR-γ and TcR-δ genes was performed in ten TcR-$\gamma\delta^+$ T-ALL. In nine T-ALL the TcR-γ genes were rearranged on both alleles, whereas in the tenth leukemia one allele was rearranged and the other in germline configuration. Twelve out of the 19 rearranged alleles contained rearrangements of the Jγ2.3 gene segment, five of which to the Vγ8 gene segment and three to the Vγ3 gene segment. This implies that the combinatorial repertoire of the rearranged TcR-γ gene is restricted due to preferential usage of several Vγ and Jγ gene segments. The TcR-δ genes were rearranged on both alleles in nine T-ALL, whereas in the tenth leukemia one allele was rearranged and the other deleted. The combinatorial repertoire of the TcR-δ genes was homogeneous, as in all ten T-ALL at least one allele contained a Vδ1-Jδ1 rearrangement. In at least nine of the ten T-ALL the Vδ1-Jδ1 allele coded for the expressed TcR-δ chain, as was supported by reactivity with the anti-Vδ1-Jδ1 (δTCS1) antibody in all T-ALL tested. As the total repertoire of TcR molecules is not only dependent on combinations of gene segments, but also on the size and diversity of the junctional regions, we studied the Vδ1-Jδ1 junctional regions using the PCR technique. These PCR analyses showed that the size of the Vδ1-Jδ1 junctional regions differed markedly (up to approximately 30 bases or more) between the leukemias. Therefore we conclude that the combinatorial repertoire of TcR-$\gamma\delta^+$ T-ALL is limited, especially due to the homogeneous TcR-δ gene rearrangements, but that the junctional repertoire of the TcR-δ genes seems to be extensive.

INTRODUCTION

Mature T-lymphocytes express on their cell surface a CD3-associated TcR, which consists of two different glycoproteins, each containing a variable antigen-recognizing region and a C region. Two forms of TcR exist, the classical TcR-$\alpha\beta$ and the alternative TcR-$\gamma\delta$ (1,2), which are expressed on >85% and <15% of CD3$^+$ T-lymphocytes in PB, respectively (3,4). Whereas $\alpha\beta$ receptors are disulfide-linked heterodimers, the human $\gamma\delta$

* Published in: Leukemia 1991;5:116-124.

receptor can occur in a disulfide-linked form or a non-disulfide-linked form, depending on the use of Cγ1 or Cγ2 gene segments, respectively (5-8).

The variable parts of the four TcR chains are encoded by several gene segments that have to be joined via a process of gene rearrangements during T-cell differentiation to obtain diverse mature TcR chains (1,2). This concerns V and J gene segments in case of the TcR-α and TcR-γ gene loci (9-12), whereas the TcR-β and TcR-δ gene loci contain D gene segments in addition (13-19). The possible different combinations of V, (D) and J gene segments determine the so-called potential combinatorial repertoire of TcR molecules (1).

The TcR-δ gene locus is located within the TcR-α gene locus between the long stretch of Vα and the Jα gene segments (15,16,18) and is flanked by TcR-δ deleting elements. In humans these deleting elements are: ψJα, located 3′ of the TcR-δ gene locus, and δREC, located 5′ of the major part of the TcR-δ gene locus but 3′ of some Vδ gene segments (20,21). Rearrangement of the deleting elements to each other causes deletion of the intermediate TcR-δ gene locus (20-22). This process may represent an important mechanism in separating the αβ and γδ differentiation pathways (23). Although murine ontogenic data and human leukemia studies indicate that a hierarchic order of TcR gene rearrangement exists with the TcR-δ gene segments rearranging before all other TcR genes (23-28), little is known about the precise mechanisms which induce separation of the αβ and γδ lineages (23).

There is a noticeable difference in the potential combinatorial diversity between TcR-αβ and TcR-γδ (1). TcR-αβ has an extended combinatorial diversity due to the numerous V and J gene segments, whereas the combinatorial diversity of the TcR-γδ is limited, because only a few V, (D) and J gene segments are available in both TcR gene loci (11,12,17,19,29-33). The actual TcR-γδ combinatorial diversity seems to be even more limited by the fact that the V and J gene segments are used in preferential patterns. In PB of most individuals more than 85% of TcR-$\gamma\delta^+$ T-lymphocytes express receptors consisting of a Vγ9-Jγ1.2-Cγ1 chain disulfide linked to a Vδ2-Jδ1-Cδ chain (34-36). However, the total TcR-γδ repertoire is substantially increased by the extensive junctional diversity, which is due to the random insertion and/or deletion of nucleotides (N regions) at the joining sites of the V, (D) and J gene segments during rearrangements (19,33,37,38).

In this study we extensively analyzed ten TcR-$\gamma\delta^+$ T-ALL by immunologic marker analysis. In addition, the combinatorial diversity of the TcR-γ and TcR-δ genes of these T-ALL was studied by Southern blot and Northern blot analysis. The extent of the junctional diversity of the TcR-δ genes was studied by PCR-mediated amplification of the junctional regions.

MATERIALS AND METHODS

Cell samples

Cell samples were obtained from ten different TcR-$\gamma\delta^+$ T-ALL patients at initial diagnosis. MNC were isolated from PB and BM by Ficoll-Paque (density, 1.077 g/ml, Pharmacia, Uppsala, Sweden) density centrifugation. The cell samples were frozen and stored in liquid nitrogen. Some data concerning the T-ALL cells of patients MA, HZ, DD and SA have been reported previously (8,28). These four patients were described in reference 28 as patients 6, 7, 8 and 9, respectively.

Immunologic marker analysis

The MNC of the T-ALL patients were analyzed for the nuclear expression of TdT, for the cell membrane expression of the T-cell markers CD1 (66IIC7), CD2 (Leu-5b), CD3 (Leu-4), CD4 (Leu-3a), CD5 (Leu-1), CD6

(OKT17), CD7 (3A1), and CD8 (Leu-2a), for the HLA-DR antigen and for reactivity with the McAb, WT31 (TcR-αβ), BMA031 (TcR-αβ), 11F2 (TcR-γδ), TCRδ1 (TcR-δ), Ti-γA (TcR-Vγ9), δTCS1 (TcR-Vδ1-Jδ1), and BB3 (TcR-Vδ2). The rabbit anti-TdT antiserum was purchased from Supertechs (Bethesda, MD, USA); the McAb of the Leu series, anti-HLA-DR, WT31 and 11F2 were obtained from Becton Dickinson (San Jose, CA, USA); the CD1 antibody was obtained from Monosan/Sanbio (Nistelrode, The Netherlands); OKT17 from Ortho Diagnostic Systems (Raritan, NJ, USA); the 3A1 hybridoma from the American Type Culture Collection (Rockville, MD, USA); TCRδ1 and δTCS1 were obtained from T Cell Sciences (Cambridge, MA, USA). McAbs BMA031, Ti-γA and BB3 were kindly provided by Dr. R. Kurrle (Behring, Marburg, Germany), Dr. T. Hercend (Villejuif, France), and Dr. L. Moretta (Genova, Italy), respectively. Immunofluorescence stainings were performed as described and evaluated with Zeiss fluorescence microscopes (Carl Zeiss, Oberkochen, Germany) and/or FACScan (Becton Dickinson) (39).

Northern blot analysis

Total RNA was isolated from frozen MNC by the LiCl/urea method (40). Approximately 15 μg of total RNA was size-fractionated on 1% agarose gels containing formaldehyde and blotted to Biodyne nylon membranes (Pall Ultrafine Filtration Corporation, Glen Core, NY, USA). ^{32}P random oligonucleotide-labeled cDNA probes were used to detect specific RNA sequences for TcR-γ (pTγ-1) and TcR-δ (pCTcRδ2c) (28,41).

Southern blot analysis

DNA was isolated from frozen MNC as described previously (40,42). A 15 μg sample was digested with the appropriate restriction enzymes, obtained from Pharmacia, size-fractionated on 0.7% agarose gels and transferred to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) as described (40,42). TcR-β gene rearrangements were detected with ^{32}P random oligonucleotide-labeled Jβ1, Jβ2 and Cβ probes (13,43) in *Eco*RI, *Hin*dIII and *Bam*HI digests. The configuration of the TcR-γ genes was analyzed by use of the VγI, Jγ1.2, Jγ1.3, Jγ2.1 and Cγ probes (11,12) in *Eco*RI, *Kpn*I, *Bam*HI and *Bgl*II digests. The configuration of the TcR-δ genes was analyzed by use of the Vδ1, Vδ2, Vδ3, Jδ1, Jδ2, Cδ, δREC and ψJα probes (15,19-21,28,44,45) in *Eco*RI, *Hin*dIII, *Bam*HI, *Kpn*I and *Bgl*II digests.

Polymerase chain reaction amplification analysis

PCR was essentially performed as described previously (40). A 1 μg sample of DNA, 0.2 μg of the 5′ and the 3′ oligonucleotide primer (Figure 1) and 2 units of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA) were used in each reaction. The oligonucleotide primers are listed in Table 1. These oligonucleotides were synthesized according to published TcR-δ gene sequences on an Applied Biosystems 381A DNA

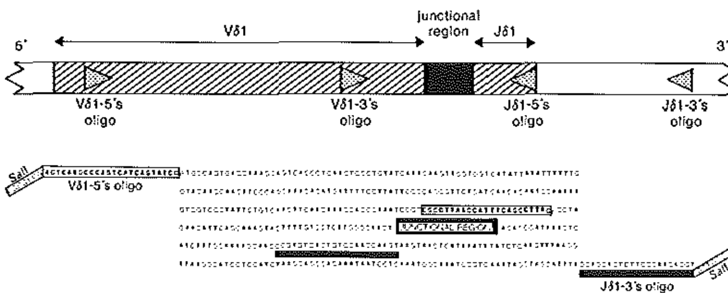


Figure 1. Schematic diagram of the Vδ1 gene segment, joined to the Jδ1 gene segment via a fictitious junctional region which may contain one or more Dδ gene segments as well as additional nucleotides at the joining sites. Four oligonucleotide primers for PCR-mediated amplification of the junctional region are indicated. *Upper panel*: The Vδ1-5′s and Vδ1-3′s oligonucleotides and the Jδ1-3′s and Jδ1-5′s oligonucleotides are indicated as dotted arrows, which point to the right and to the left, respectively. *Lower panel*: Nucleotide sequence of the rearranged Vδ1-Jδ1 gene segments (18). The Vδ1-5′s and Vδ1-3′s oligonucleotides are boxed, while the Jδ1-3′s and Jδ1-5′s oligonucleotides are indicated by bars, which are complementary to the opposite nucleotide sequences. The *Sal*I restriction site at the 5′ ends of the oligonucleotide primers can be used for cloning of the PCR product.

TABLE 1. Oligonucleotide primers used in the PCR analysis of the TcR-δ genes.

Code	Sequence[a]	Reference[b]
Vδ1-5's	CGCGTCGACTCAAGCCCAGTCATCAGTATCC	18
Vδ1-3's	CGCGTCGACGCCTTAACCATTTCAGCCTTAC	18
Vδ2-5's	CGCGTCGACCAAACAGTGCCTGTGTCAATAGG	46
Vδ2-3's	CGCGTCGACCTGGCTGTACTTAAGATACTTGC	46
Vδ3-5's	CGCGTCGACCAGACGGTGGCGAGTGGC	32
Vδ3-3's	CGCGTCGACTTGGTGATCTCTCCAGTAAGG	32
Dδ1-5's	CGCGTCGACTCCATGTTCAAATAGATATAGTATT	19
Jδ1-3's	CGCGTCGACCTCTTCCCAGGAGTCCTCC	18
Jδ1-5's	CGCGTCGACTTGGTTCCACAGTCACACGG	18
δREC-5'X	TGCTCTAGATCTTCAAGGGTCGAGACTGTC	47

a. The underlined sequences represent the aspecific 5' tail of the oligonucleotide primers, which includes an artificial *Sal*I site, or in case of δREC-5'X an artificial *Xba*I site.
b. Sequence information used to design the oligonucleotide primers was derived from the indicated literature references.

synthesizer (Forster City, CA, USA) using the solid-phase phosphotriester method (18,19,32,46,47). The reaction mixture was incubated at 94°C for 5 min, at 55°C for 90 s and at 72°C for 210 s in a thermal cycler (Perkin-Elmer cetus). Following this initial cycle, denaturing, annealing and extension steps were performed for another 34 cycli at 94°C for 90 s, at 55°C for 90 s and at 72°C for 210 s, respectively. PCR products were size-fractionated on ethidium bromide stained 1% agarose gels and transferred to a Nytran-13N nylon membrane (Schleicher and Schuell). The blotted PCR products were detected with the internal Vδ1-3's, Vδ2-3's, Vδ3-3's or Jδ1-5's oligonucleotides which were ^{32}P-labeled with polynucleotide kinase (Pharmacia).

RESULTS

Phenotype of the T-ALL

All ten T-ALL expressed the CD3 (Leu-4) antigen as well as TcR-γδ on the cell membrane, as determined by immunofluorescence staining with McAb 11F2 and TCRδ1, and/or previously reported immunoprecipitation studies (8,28). They did not express TcR-αβ as determined by use of staining with McAb WT31 and/or BMA031. All T-ALL expressed TdT and six of them were also positive for the CD1 antigen. Seven T-ALL showed the CD4^{+}/CD8^{-} phenotype, one leukemia was positive for both CD4 and CD8 and only two leukemias showed the 'γδ-associated' CD4^{-}/CD8^{-} phenotype. Two out of eight tested T-ALL expressed Vγ9 (McAb Ti-γA), eight out of eight tested expressed Vδ1-Jδ1 (McAb δTCS1) and none of six tested T-ALL expressed Vδ2 (McAb BB3). Further details concerning the marker analysis are given in Table 2 and Table 3.

Transcription of TcR-γ and TcR-δ genes

In all nine T-ALL tested, 1.6 kb TcR-γ gene transcripts were detected in Northern blot analysis. Four different types of TcR-δ gene transcripts exist: the immature 1.9 and 1.2 kb transcripts which probably lack V-region sequences, due to incomplete rearranged TcR-δ genes, and the mature 2.2 and 1.5 kb transcripts (28,44,48). All nine tested T-ALL contained both types of mature transcripts, whereas in four T-ALL both types of immature transcripts were also detected (Table 2).

TABLE 2. Immunologic marker, Southern blot, and Northern blot analysis of cell samples from ten patients with TcR-γδ⁺ T-ALL[a].

Patient (Cell Sample)		DD (PB)	IJ (PB)	HZ (PB)	AV (PB)	BP (PE)	JB (PB)	EP (PB)	MA (BM)	SA (PB)	WC (PB)
Immunologic markers[b]											
TdT		+	+	+	66%	+	+	63%	+	+	60%
HLA-DR	(L243)	18%	21%	—	—	—	—	—	—	—	NT
CD1	(66IIC7)	—	34%	40%	49%	74%	+	—	25%	—	—
CD2	(Leu-5b)	+	57%	+	66%	+	+	+	+	+	+
CD3	(Leu-4)	+	+	+	+	+	+	+	27%	+	66%
CD4	(Leu-3A)	—	26%	59%	49%	+	+	+	36%	—	22%
CD5	(Leu-1)	+	+	+	+	+	+	+	+	+	55%
CD6	(OKT17)	69%	+	+	+	44%	+	+	NT	NT	NT
CD7	(3A1)	+	73%	+	+	+	+	+	+	+	71%
CD8	(Leu-2A)	—	—	—	57%	—	—	—	—	—	—
TcR-αß	(WT31)	—	—	—	—	—	—	—	—	—	NT
TcR-αß	(BMA031)	—	—	—	—	—	—	—	—	NT	19%
TcR-γδ	(11F2)	49%[c]	+	71%[c]	59%	+	+	+	+	+[c]	61%
TcR-δ	(TCRδ1)	67%[c]	+	+[c]	61%	+	+	+	NT	NT[c]	52%
Southern blot analysis[d]											
TcR-ß1 genes		R/G	R/R	D/D	D/D	D/G	R/G	D/D	D/G	D/R	D/G
TcR-ß2 genes		G/G	G/G	R/R	R/R	R/G	G/G	R/G	R/R	R/G	R/R
TcR-γ1 genes[e]		R/R[c]	D/R	D/D[c]	D/D	D/R	D/R	D/D	D/D	D/D[c]	R/G
TcR-γ2 genes[e]		G/G[c]	R/G	R/R[c]	R/R	R/G	R/G	R/R	R/R	R/R[c]	G/G
TcR-δ genes[e]		R/R	R/R	R/R	R/R	R/R	R/R	D/R	R/R	R/R	R/R
Nothern blot analysis[f]											
TcR-γ (1.6 kb)		+	+	+	+	+	+	+	+	+	NT
TcR-δ (2.2 kb)		+	+	+	+	+	+	+	+	+	NT
TcR-δ (1.9 kb)		+	+	—	+	—	+	—	—	—	NT
TcR-δ (1.5 kb)		+	+	+	+	+	+	+	+	+	NT
TcR-δ (1.2 kb)		+	+	—	+	—	+	—	—	—	NT

a. Abbreviations used in this table: PB, peripheral blood; BM, bone marrow; PE, pleural exudate; NT, not tested.
b. Immunologic marker analysis: +, ≥75% of the cells are positive; —, ≤15% of the cells are positive; percentages positivity between 15% and 75% are indicated.
c. Immunoprecipitation studies on the T-ALL cells of patients DD, HZ, and SA have proven that the T-ALL cells of patient DD expressed disulfide-linked TcR chains (Cγ1-derived), whereas the T-ALL cells of patients HZ and SA expressed a TcR without an interchain disulfide bond (Cγ2-derived) (8). This was in line with the Southern blot data.
d. Southern blot analysis: G, allele in germline configuration; R, rearranged allele; D, deletion of involved gene segment.
e. Detailed information concerning the configuration of the TcR-γ genes and TcR-δ genes is summarized in Table 3.
f. Northern blot analysis: +, transcripts present; —, no transcripts could be detected.

TcR gene rearrangement

All T-ALL had rearranged TcR-β genes. Seven had both alleles rearranged, whereas the other three had one allele rearranged and the other allele in germline configuration. Further details concerning the TcR-β gene configurations are summarized in Table 2.

TcR-γ gene rearrangements had occurred in 19 of the 20 alleles, whereas the twentieth allele was in germline configuration. The rearrangements were analyzed using four different restriction enzyme digests and the far described TcR-γ probes in successive hybridizations (Figure 2A). Identification of the used Vγ and Jγ gene segments was accomplished by combining the information of the calculated sizes of the rearranged bands and the available data in the literature (29-31,49,50). There seems to be a preference in the rearrangement of the TcR-γ gene segments. Twelve out of the 19 rearranged alleles contained rearrangements of the Jγ2.3 gene segment, five to the Vγ8 gene segment and three to the Vγ3 gene segment. The detected Vγ9 (McAb Ti-γA) expression in patients DD and IJ was confirmed by rearrangements of the Vγ9 gene segment to the Jγ1.3 gene segment. In patient AV no Vγ9 expression was found, which indicates that the Vγ9-Jγ2.3 rearrangement in this patient is probably non-functional and that the Vγ3-Jγ2.3 rearrangement on the other allele is expressed. The configurations of the TcR-γ genes are summarized in Table 3.

All ten T-ALL had at least one Vδ1-Jδ1 gene rearrangement, which confirmed the Vδ1-Jδ1 expression in all patients tested with the McAb δTCS1. The Vδ1-Jδ1 rearrangement was proven by successive hybridization of *Eco*RI, *Hin*dIII and *Bgl*II filters with the Jδ1 probe (Figure 2B) and the Vδ1 probe, which resulted in rearranged Jδ1 and Vδ1 bands of identical size in all patients except patient IJ. The *Eco*RI digest in the latter patient showed, after hybridization with the Jδ1 probe, a 1.0 kb band (Figure 2B) and with the Vδ1 probe a 2.3 kb band, whereas in the *Hin*dIII and *Bgl*II digests the Jδ1 and Vδ1 bands had an identical size. From this and from the fact that the sizes of the 1.0 kb Jδ1 band, plus the 2.3 kb Vδ1 band, add up to a 3.3 kb band (which is the exact size of the Vδ1-Jδ1 rearranged band in a *Eco*RI digest), we concluded that an *Eco*RI restriction site had been generated in the junctional region of the Vδ1-Jδ1 rearrangement in patient IJ. This was confirmed by the fact that *Eco*RI digested the PCR product of the Vδ1-Jδ1 rearrangement in this patient, whereas a control Vδ1-Jδ1 PCR product was not digested.

Of the two T-ALL cell samples from patients MA and SA, which were not tested with the McAb δTCS1, the cells from patient MA had Vδ1-Jδ1 rearrangements on both alleles and therefore expressed a Vδ1-Jδ1 TcR-δ chain. Patient SA had a Vδ1-Jδ1 rearrangement on one allele and a Vδ3-Jδ1 rearrangement on the other, so it is not clear whether this ALL expressed a Vδ1-Jδ1 or a Vδ3-Jδ1 TcR-δ chain. In a Vδ3-Jδ1 rearrangement the 3′ to Cδ located Vδ3 gene segment rearranges to the 5′ to Cδ located Jδ1 gene segment, which results in an inversion of the DNA sequence between Jδ1 and Vδ3 (51). As a consequence of this inversion the Jδ2 and Jδ3 gene segments seem to be rearranged in *Kpn*I and/or *Bam*HI digests, whereas in *Eco*RI, *Hin*dIII and *Bgl*II digests these gene segments show germline configuration bands.

Four patients had an incomplete Dδ2-Jδ1 rearrangement on their second allele. This was in line with the presence of immature TcR-δ gene transcripts in three patients tested by Northern blot analysis (Table 2). Unexpectedly, immature transcripts were also found in patient JB who had a Vδ2-Jδ1 rearrangement in addition to the expressed Vδ1-Jδ1

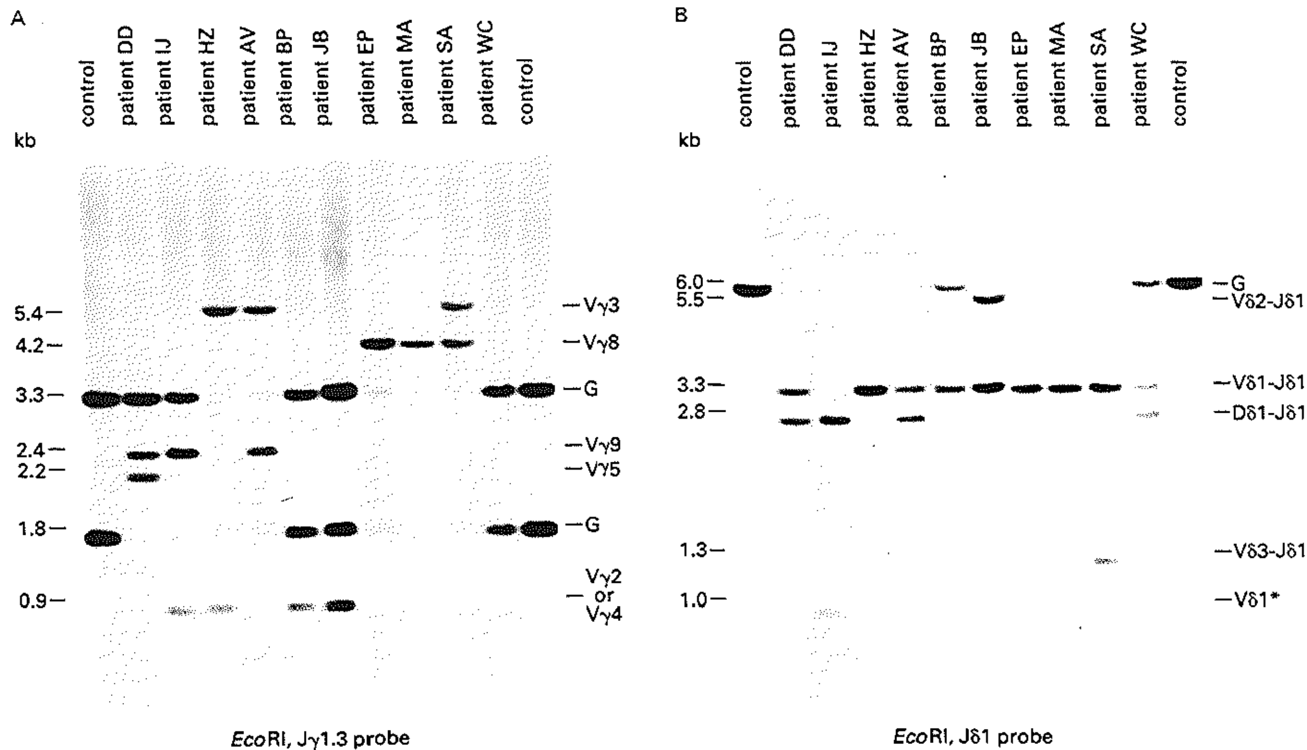


Figure 2. Southern blot analysis of the TcR-γ and TcR-δ genes. DNA samples from the ten TcR-$\gamma\delta^+$ T-ALL and from a B-cell leukemia as control were digested with *Eco*RI. The filter was successively hybridized with the ^{32}P-labeled Jγ1.3 probe (A) and Jδ1 probe (B). Vδ1*: the low band in patient IJ is caused by an *Eco*RI site in the junctional region of the Vδ1-Jδ1 rearrangement, as proven by PCR analysis (see text). In all other digests this allele showed a normal Vδ1-Jδ1 rearrangement pattern.

TABLE 3. Configuration and expression of TcR-γ and TcR-δ genes in ten TcR-γδ⁺ T-ALL.

Patient	DD	IJ	HZ	AV	BP	JB	EP	MA	SA	WC
TcR-V gene expression[a]										
Vγ9 (Ti-γA)	68%	74%	–	–	–	–	–	NT	NT	–
Vδ1-Jδ1 (δTCS1)	33%	69%	+	+	+	+	+	NT	NT	56%
Vδ2 (BB3)	NT	–	NT	–	–	–	–	NT	NT	–
TcR-γ gene configuration[b]										
Jγ1.1 gene segment	D/D	D/D	D/D	D/D	Vγ2[c]/D	Vγ2/D	D/D	D/D	D/D	VγI/G[d]
Jγ1.2 gene segment	D/D	D/D	D/D	D/D	G/D	G/D	D/D	D/D	D/D	G/G[d]
Jγ1.3 gene segment	Vγ5/Vγ9	Vγ9/D	D/D	D/D	G/D	G/Vγ2	D/D	D/D	D/D	G/G[d]
Jγ2.1 gene segment	G/G	G/D	D/D	D/D	G/D	G/G	D/D	D/D	D/D	G/G[d]
Jγ2.3 gene segment	G/G	G/Vγ2	Vγ3/Vγ4	Vγ3/Vγ9	G/Vγ4	G/G	Vγ8/Vγ8	Vγ8/Vγ8	Vγ3/Vγ8	G/G[d]
TcR-δ gene configuration[b]										
Jδ1 gene segment	Vδ1/Dδ2	Vδ1/Dδ2	Vδ1/Vδ1	Vδ1/Dδ2	Vδ1/δREC[e]	Vδ1/Vδ2	Vδ1/D[f]	Vδ1/Vδ1	Vδ1/Vδ3	Vδ1/Dδ2
Jδ2 gene segment	G/G	G/G	G/G	G/G	G/G	G/G	G/D[f]	G/G	G/G[g]	G/G
Jδ3 gene segment	G/G	G/G	G/G	G/G	G/G	G/G	G/D[f]	G/G	G/G[g]	G/G
Cδ gene segment	G/G	G/G	G/G	G/G	G/G	G/G	G/D[f]	G/G	G/G[g]	G/G
PCR analysis[h]										
Vδ1↔Jδ1 amplification	+	+	+[i]	+	+	+	+	+[i]	+	+
Vδ2↔Jδ1 amplification	–	–	–	–	–	+	–	–	–	–
Vδ3↔Jδ1 amplification	–	–	–	–	–	–	–	–	+	–
Dδ1↔Jδ1 amplification	+	+	–	+	–	–	–	–	–	+
δREC↔Jδ1 amplification	–	–	–	–	+	–	–	–	–	–

a. Immunologic marker analysis: +, ≥75% of the cells are positive; –, ≤15% of the cells are positive; percentages positivity between 15% and 75% are indicated; NT, not tested.
b. Southern blot analysis: Interpretation of the results using the five described TcR-γ probes, the seven described TcR-δ probes and the ψJα probe in five different restriction enzyme digests, except for DNA from patient WC which was digested only with *Eco*RI. G, gene segment in germline configuration; D, deletion of involved gene segment.
c. Vγ identification deduced from measured rearranged bands and available data from the literature (29-31,49,50).
d. Germline configuration, although interpretation was hampered by the presence of ~40% non-leukemic cells (cf. Table 2).
e. Four digests of DNA from patient BP hybridized consecutively with the Jδ1 and the δREC probe showed identically sized bands concerning one allele.
f. Five digests of DNA from patient EP showed on one allele a deletion for all TcR-δ gene segments and a rearranged band after hybridization with the ψJα probe.
g. GI, Germline inversion. The Jδ2 and Jδ3 gene segments appeared as rearranged bands in *Kpn*I and/or *Bam*HI digests and as germline bands in *Eco*RI, *Hin*dIII and *Bgl*II digests as consequence of the inversion caused by the Vδ3-Jδ1 rearrangement (51).
h. PCR analysis: PCR-mediated amplification between a Vδ1-5´S, Vδ2-5´S, Vδ3-5´S, Dδ1-5´S or δREC-5´X oligonucleotide primer on the one hand and the Jδ1-3´S oligonucleotide primer on the other hand: +, PCR product detected; –, no PCR product could be detected (cf. Figure 3).
i. PCR amplification of DNA from patient MA and patient HZ showed two bands after gel electrophoresis (cf. Figure 3).

rearrangement.

Patient BP showed in four digests an identically sized rearranged band after successive hybridization with the Jδ1 probe and the δREC probe. This suggested that a δREC-Jδ1 rearrangement had occurred in this patient, which was proven by PCR amplification with the δREC-5´X and Jδ1-3´s oligonucleotide primers (Table 3).

In only one patient (EP) was a deletion of a TcR-δ gene locus found on one allele. This was probably a result of rearrangement of a Vα gene segment to the ψJα gene segment, because on one chromosome in this patient all δ gene segments, including Vδ1 and Vδ3, were deleted and a rearranged band was found in digests hybridized with the ψJα probe. Further details concerning the TcR-δ gene rearrangements are summarized in Table 3.

TcR-δ junctional regions

PCR-mediated amplification of the TcR-δ gene rearrangements from the ten T-ALL with specific Vδ1-5´s and Jδ1-3´s oligonucleotide primers (Figure 1) showed bands on ethidium bromide stained agarose gel (Figure 3A) confirming the Vδ1-Jδ1 rearrangement as detected by Southern blot analysis (Table 3). The origin of the PCR products, approximately 500 bp, was confirmed by hybridization of the blotted PCR products with the ^{32}P-labeled internal Vδ1-3´s oligonucleotide (Figure 3B). Patient MA showed after amplification with the Vδ1-5´s and Jδ1-3´s primers two PCR product bands with a difference in size of approximately 30 bp; this is most probably due to the Vδ1-Jδ1 rearrangement on both alleles. Patient HZ, who also had two Vδ1-Jδ1 rearrangements, showed only one PCR band in Figure 3. This was due to co-migration of the two Vδ1-Jδ1 PCR products, which slightly differed in size as determined in a long run in a 2% agorose gel. Both patient MA and patient HZ had an additional 1.0 kb PCR product band, which probably represents doublets of PCR products (Figure 3) (52). The origin of these doublets may be related to the Vδ1-Jδ1 rearrangements on both alleles, which were only found in patients MA and HZ.

The amplification results with the Vδ1-5´s and Jδ1-3´s primers of the TcR-δ gene rearrangements indicate that the size of the PCR products, size-fractionated on an ethidium bromide stained agarose gel, vary from approximately 470 to 510 bases, implying that the size of the junctional region can vary extensively, up to approximately 30 bases or more.

PCR products of amplification with Vδ2-5´s and Jδ1-3´s, Vδ3-5´s and Jδ1-3´s, Dδ1-5´s and Jδ1-3´s and δREC-5´X and Jδ1-3´s primers also confirmed the Southern blot data after gel electrophoresis and hybridization with the ^{32}P-labeled internal probes Vδ2-3´s, Vδ3-3´s or Jδ1-5´s, respectively (Table 3). The size of the PCR products after amplification with the Dδ1-5´s and Jδ1-3´s primers was approximately 700 bp, which corresponds to a Dδ2-Jδ1 rearrangement, as a Dδ1-Jδ1 and a Dδ2-Dδ3 rearrangement are expected to give 200 bp and 1700 bp PCR products, respectively.

DISCUSSION

Ten TcR-$\gamma\delta^+$ T-ALL samples have been analyzed for their immunologic phenotype as well as TcR gene rearrangement and expression. Unlike previously published data which describe that most TcR-$\gamma\delta^+$ T-cells express the $CD4^-/CD8^-$ phenotype (3,4,53), seven out of ten TcR-$\gamma\delta^+$ T-ALL had the $CD4^+/CD8^-$ phenotype, one T-ALL was positive for

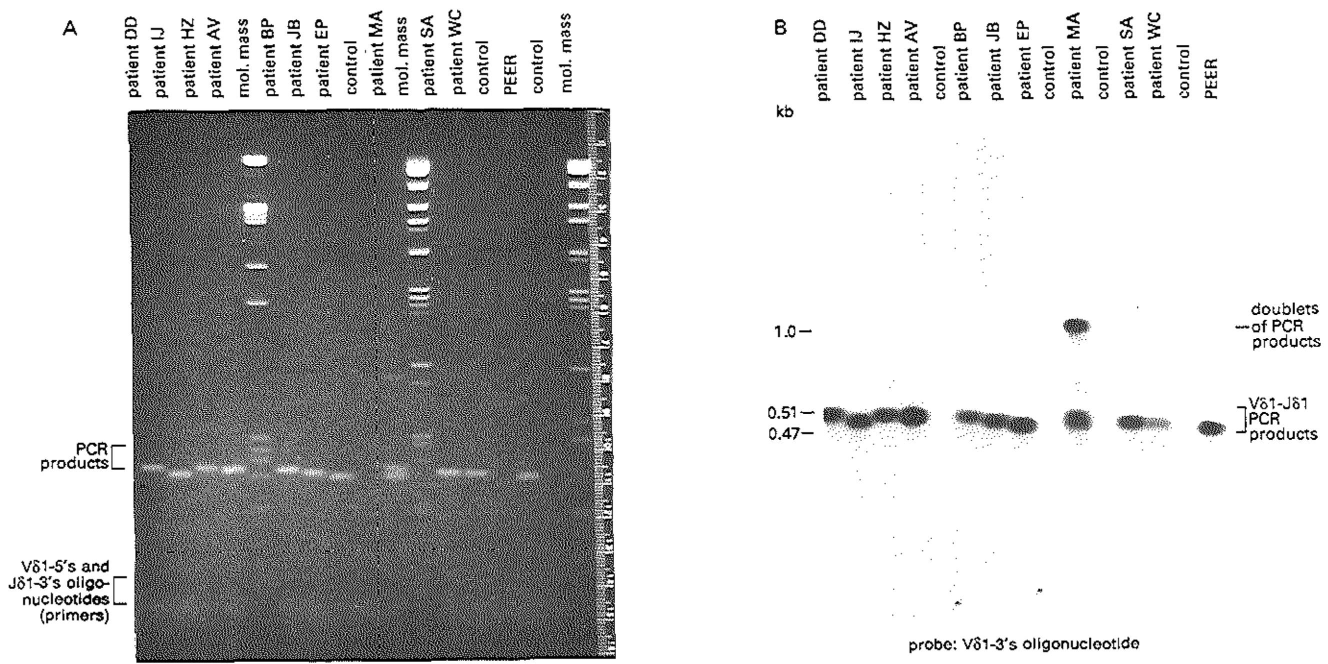


Figure 3. PCR analysis of the Vδ1-Jδ1 gene rearrangement in the ten TcR-$\gamma\delta^+$ T-ALL. The cell line PEER was used as a positive control. Water and DNA from an ANLL patient with germline TcR gene configuration were used as negative controls. PCR-mediated amplification of the junctional regions was performed by use of Vδ1-5's and Jδ1-3's oligonucleotide primers (cf Table 1). The PCR products were separated on an ethidium bromide stained 1% agarose gel (**A**) and blotted to a nylon membrane which was hybridized with the ^{32}P-labeled Vδ1-3's oligonucleotide (**B**). The weak 1.0 kb band in the lane of patient HZ and the strong 1.0 kb band in the lane of patient MA probably represent doublets of PCR products (52). The origin of these doublets may be related to the fact that the leukemia cells of these two patients had Vδ1-Jδ1 gene rearrangements on both alleles.

CD4 and CD8 and only two were negative for both assessory molecules. In addition, six T-ALL expressed the CD1 antigen. Another study on ten TcR-$\gamma\delta^+$ T-ALL also described such a heterogeneity in CD1, CD4 and CD8 expression (54). This immunophenotypic heterogeneity as well as the positivity for TdT suggests that TcR-$\gamma\delta^+$ T-ALL develop from cells in various stages of T-cell differentiation.

The TcR-γ gene rearrangements in the T-ALL appeared to be restricted due to preferential usage of Vγ and Jγ gene segments: 12 out of 19 rearranged alleles contained a rearrangement of the Jγ2.3 gene segment, of which five to Vγ8 and three to Vγ3. The twentieth TcR-γ allele was in germline configuration. Vγ9 rearrangements occurred in three alleles, two to Jγ1.3 and one to Jγ2.3, but none to Jγ1.2. This is in contrast to the TcR-$\gamma\delta^+$ T-lymphocytes in PB, most of which express Vγ9-Jγ1.2 in combination with Vδ2-Jδ1 (34-36,55). Only one T-ALL contained a Vδ2-Jδ1 rearrangement, but this allele was not expressed. All T-ALL had at least one Vδ1-Jδ1 rearrangement, and in two cases a Vδ1-Jδ1 rearrangement was found on both alleles. From our analyses it can be concluded that in at least nine T-ALL a Vδ1-Jδ1 chain was expressed and that the tenth T-ALL (patient SA) expressed a TcR with either a Vδ1-Jδ1 or a Vδ3-Jδ1 chain. In normal PB 10-20% of TcR-$\gamma\delta^+$ T-lymphocytes express Vδ1-Jδ1 chains, while in the thymus this frequency is approximately 60% (35,36,55). Therefore, the high frequency of Vδ1-Jδ1 expression in our series of T-ALL suggests that these T-ALL have developed from thymocytes. Generally, a Vδ1-Jδ1-Cδ chain is non-disulfide-linked to a Cγ2 gene derived TcR-γ chain, which can use various Vγ gene segments. At least four out of the ten T-ALL showed a similar association of TcR chains.

As expected, mature TcR-δ and TcR-γ RNA transcripts were found in all T-ALL samples, whereas immature TcR-δ transcripts were found in addition in T-ALL with a Dδ2-Jδ1 rearrangement, which is in line with this incomplete TcR-δ gene rearrangement. However, immature TcR-δ transcripts were also detected in the T-ALL with an expressed Vδ1-Jδ1 rearrangement and a non-expressed complete Vδ2-Jδ1 rearrangement. It is unclear how the immature TcR-δ transcripts were produced in this leukemia.

Interestingly, in two TcR-$\gamma\delta^+$ T-ALL, rearrangements were found of elements, which are thought to be involved in the separation process of the $\alpha\beta$ and $\gamma\delta$ differentiation lineages (20-23). One concerned a δREC-Jδ1 rearrangement which deleted the intermediate Vδ gene segments, whereas the other probably represented a Vα-ψJα rearrangement which deleted the complete TcR-δ locus. Apparently δREC and ψJα do not only rearrange to each other but can also be involved in other deleting rearrangements (47).

The potential combinatorial repertoire of the TcR-γ and TcR-δ genes is limited in comparison to the TcR-α and TcR-β genes due to the small numbers of V and J gene segments. In the 20 TcR-γ and TcR-δ alleles studied only ten different Vγ-Jγ and three different Vδ-Jδ rearrangements were found. This distinct limited combinatorial repertoire was not only caused by the restricted numbers of available V and J gene segments, but also by preferential rearrangement, which was prominent in the TcR-δ genes with Vδ1-Jδ1 rearrangements in 12 out of 20 alleles. To achieve a diverse TcR-$\gamma\delta$ repertoire the rearranged TcR-γ and TcR-δ genes contain large junctional regions, consisting of extensive N regions, each of up to nine or more random nucleotides and, in the case of the TcR-δ gene, one, two or three extra D gene segments (19,33,37,38). In the T-ALL studied the junctional regions vary extensively in size, up to 30 bases or more. Therefore the junctional

diversity in these TcR-$\gamma\delta^+$ T-ALL is probably considerable.

Further studies including cloning and sequencing of the junctional regions need to be performed to elucidate the N region and Dδ gene segment involvement. Sequencing should also answer the question which of the two rearrangements in patient SA, Vδ1-Jδ1 or Vδ3-Jδ1, is expressed.

ACKNOWLEDGMENTS. We gratefully acknowledge Prof. Dr. R. Benner, Dr. H. Hooijkaas and Dr. H.J. Adriaansen for their continuous support; the Dutch Childhood Leukemia Study Group (The Hague, The Netherlands); Dr. D. Campana, Dr. J.C. Kluin-Nelemans, Dr. R.J. van de Griend and Dr. C.E. van der Schoot for kindly providing TcR-$\gamma\delta^+$ T-ALL cell samples; Dr. R. Kurrle (Behring, Marburg, Germany), Dr. T. Hercend (Villejuif, France) and Dr. L. Moretta (Genova, Italy) for kindly providing the BMA031, Ti-γA and BB3 antibodies; Dr. T.H. Rabbitts, Dr. T. Quertermous, Dr. P. van de Elsen, Dr. S.J. Korsmeyer and Dr. M.-P. Lefranc for kindly providing the TcR-γ and TcR-δ probes; Mr. T.M. van Os for excellent assistance in the preparation of the figures; and Ms. A.D. Korpershoek for her secretarial support.

REFERENCES

1. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988;334:395-402 and corrigenda 1988;335:744.
2. Strominger JL. Developmental biology of T cell receptors. Science 1989;244:943-950.
3. Lanier LL, Weiss A. Presence of Ti (WT31) negative T lymphocytes in normal blood and thymus. Nature 1986;324:268-270.
4. Borst J, Van Dongen JJM, Bolhuis RLH, Peters PJ, Hafler DA, De Vries E, Van de Griend RJ. Distinct molecular forms of human T cell receptor γ/δ detected on viable T cells by a monoclonal antibody. J Exp Med 1988;167:1625-1644.
5. LeFranc M-P, Forster A, Rabbitts TH. Genetic polymorphism and exon changes of the constant regions of the human T-cell rearranging gene γ. Proc Natl Acad Sci USA 1986;83:9596-9600.
6. Littman DR, Newton M, Crommie D, Ang S-L, Seidman JG, Gettner SN, Weiss A. Characterization of an expressed CD3-associated Ti γ-chain reveals Cγ domain polymorphism. Nature 1987;326:85-88.
7. Krangel MS, Band H, Hata S, McLean J, Brenner MB. Structurally divergent human T cell receptor γ proteins encoded by distinct Cγ genes. Science 1987;237:64-67.
8. Van Dongen JJM, Wolvers-Tettero ILM, Seidman JG, Ang S-L, Van de Griend RJ, De Vries EFR, Borst J. Two types of gamma T cell receptors expressed by T cell acute lymphoblastic leukemias. Eur J Immunol 1987;17:1719-1728.
9. Yoshikai Y, Clark SP, Taylor S, Sohn U, Wilson BI, Minden MD, Mak TW. Organization and sequences of the variable, joining and constant region genes of the human T-cell receptor α-chain. Nature 1985;316:837-840.
10. Yoshikai Y, Kimura N, Toyonaga B, Mak TW. Sequences and repertoire of human T cell receptor α chain variable region genes in mature T lymphocytes. J Exp Med 1986;164:90-103.
11. LeFranc M-P, Forster A, Baer R, Stinson MA, Rabbitts TH. Diversity and rearrangement of the human T cell rearranging γ genes: nine germ-line variable genes belonging to two subgroups. Cell 1986;45:237-246.
12. Quertermous T, Strauss WM, Van Dongen JJM, Seidman JG. Human T cell γ chain joining regions and T cell development. J Immunol 1987;138:2687-2690.
13. Duby AD, Klein KA, Murre C, Seidman JG. A novel mechanism of somatic rearrangement predicted by a human T-cell antigen receptor β-chain complementary DNA. Science 1985;228: 1204-1206.
14. Toyonaga B, Yoshikai Y, Vadasz V, Chin B, Mak TW. Organization and sequences of the diversity, joining, and constant region genes of the human T-cell receptor β chain. Proc Natl Acad Sci USA 1985;82:8624-8628.
15. Boehm T, Baer R, Lavenir I, Forster A, Waters JJ, Nacheva E, Rabbitts TH. The mechanism of chromosomal translocation t(11;14) involving the T-cell receptor Cδ locus on human chromosome 14q11 and a transcribed region of chromosome 11p15. EMBO J 1988;7:385-394.
16. Griesser H, Champagne E, Tkachuk D, Takihara Y, Lalande M, Baillie E, Minden M, Mak TW. The human T cell receptor α-δ locus: a physical map of the variable, joining and constant region genes. Eur J Immunol 1988;18:641-644.

17. Takihara Y, Tkachuk D, Michalopoulos E, Champagne E, Reimann J, Minden M, Mak TW. Sequence and organization of the diversity, joining, and constant region genes of the human T-cell δ-chain locus. Proc Natl Acad Sci USA 1988;85:6097-6101.
18. Satyanarayana K, Hata S, Devlin P, Grazia Roncarolo M, De Vries JE, Spits H, Strominger JL, Krangel MS. Genomic organization of the human T-cell antigen-receptor α/δ locus. Proc Natl Acad Sci USA 1988;85:8166-8170.
19. Loh EY, Cwirla S, Serafini AT, Phillips JH, Lanier LL. Human T-cell receptor δ chain: genomic organization, diversity, and expression in populations of cells. Proc Natl Acad Sci USA 1988;85:9714-9718.
20. De Villartay J-P, Hockett RD, Coran D, Korsmeyer SJ, Cohen DI. Deletion of the human T-cell receptor δ-gene by a site-specific recombination. Nature 1988;335:170-174.
21. Hockett RD, De Villartay J-P, Pollock K, Poplack DG, Cohen DI, Korsmeyer SJ. Human T-cell antigen receptor (TCR) δ-chain locus and elements responsible for its deletion are within the TCR α-chain locus. Proc Natl Acad Sci USA 1988;85:9694-9698.
22. Begley CG, Aplan PD, Davey MP, De Villartay J-P, Cohen DI, Waldmann TA, Kirsch IR. Demonstration of δRec-pseudo Jα rearrangement with deletion of the δ locus in a human stem-cell leukemia. J Exp Med 1989;170:339-342.
23. Van Dongen JJM, Comans-Bitter WM, Wolvers-Tettero ILM, Borst J. Development of human T lymphocytes and their thymus-dependency. Thymus 1990;16:207-234.
24. Raulet DH, Garman RD, Saito H, Tonegawa S. Developmental regulation of T-cell receptor gene expression. Nature 1985;314:103-107.
25. Born W, Rathbun G, Tucker P, Marrack P, Kappler J. Synchronized rearrangement of T-cell γ and β chain genes in fetal thymocyte development. Science 1986;234:479-482.
26. Chien Y, Iwashima M, Wettstein DA, Kaplan KB, Elliott JF, Born W, Davis MM. T-cell receptor δ gene rearrangements in early thymocytes. Nature 1987;330:722-727.
27. Van Dongen JJM, Quertermous T, Bartram CR, Gold DP, Wolvers-Tettero ILM, Comans-Bitter WM, Hooijkaas H, Adriaansen HJ, De Klein A, Raghavachar A, Ganser A, Duby AD, Seidman JG, Van den Elsen P, Terhorst C. T cell receptor-CD3 complex during early T cell differentiation: analysis of immature T cell acute lymphoblastic leukemias (T-ALL) at DNA, RNA, and cell membrane level. J Immunol 1987;138:1260-1269.
28. Van Dongen JJM, Wolvers-Tettero ILM, Wassenaar F, Borst J, Van den Elsen P. Rearrangement and expression of T-cell receptor delta genes in T-cell acute lymphoblastic leukemias. Blood 1989;74:334-342.
29. Huck S, Lefranc M-P. Rearrangements to the JP1, JP and JP2 segments in the human T-cell rearranging gamma gene (TRGγ) locus. FEBS Letters 1987;224:291-296.
30. Forster A, Huck S, Ghanem N, Lefranc M-P, Rabbitts TH. New subgroups in the human T cell rearranging Vγ gene locus. EMBO J 1987;6:1945-1950.
31. Chen Z, Font MP, Loiseau P, Bories JC, Degos L, Lefranc MP, Sigaux F. The human T-cell Vγ gene locus: cloning of new segments and study of Vγ rearrangements in neoplastic T and B cells. Blood 1988;72:776-783.
32. Hata S, Clabby M, Devlin P, Spits H, De Vries JE, Krangel MS. Diversity and organization of human T cell receptor δ variable gene segments. J Exp Med 1989;169:41-57.
33. Takihara Y, Reimann J, Michalopoulos E, Ciccone E, Moretta L, Mak TW. Diversity and structure of human T cell receptor δ chain genes in peripheral blood γ/δ-bearing T lymphocytes. J Exp Med 1989;169:393-405.
34. Borst J, Wicherink A, Van Dongen JJM, De Vries E, Comans-Bitter WM, Wassenaar F, Van den Elsen P. Non-random expression of T cell receptor γ and δ variable gene segments in functional T lymphocyte clones from human peripheral blood. Eur J Immunol 1989;19:1559-1568.
35. Triebel F, Hercend T. Subpopulations of human peripheral T gamma delta lymphocytes. Immunol Today 1989;10:186-188.
36. Casorati G, De Libero G, Lanzavecchia A, Migone N. Molecular analysis of human γ/δ^+ clones from thymus and peripheral blood. J Exp Med 1989;170:1521-1535.
37. Quertermous T, Strauss W, Murre C, Dialynas DP, Strominger JL, Seidman JG. Human T-cell γ genes contain N segments and have marked junctional variability. Nature 1986;322:184-187.
38. Hata S, Satyanarayana K, Devlin P, Band H, McLean J, Strominger JL, Brenner MB, Krangel MS. Extensive junctional diversity of rearranged human T cell receptor δ genes. Science 1988;240: 1541-1544.
39. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunological marker analysis of cells in the various hematopoietic differentiation stages and their malignant counterparts. In: Ruiter DJ, Fleuren GJ, Warnaar SO, eds. Application of monoclonal antibodies in tumor pathology. Dordrecht: Martinus Nijhoff, 1987:87-116.
40. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, second edition 1989.
41. Dialynas DP, Murre C, Quertermous T, Boss JM, Leiden JM, Seidman JG, Strominger JL. Cloning and sequence analysis of complementary DNA encoding an aberrantly rearranged human T-cell γ chain. Proc Natl Acad Sci USA 1986;83:2619-2623.

42. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of Immunoglobulin and T cell receptor genes. Part I: basic and technical aspects. Clin Chim Acta 1991;198:1-92
43. Duby AD, Seidman JG. Abnormal recombination products result from aberrant DNA rearrangement of the human T-cell antigen receptor β-chain gene. Proc Natl Acad Sci USA 1986;83:4890-4894.
44. Hata S, Brenner MB, Krangel MS. Identification of putative human T cell receptor δ complementary DNA clones. Science 1987;238:678-682.
45. Baer R, Boehm T, Yssel H, Spits H, Rabbitts TH. Complex rearrangements within the human Jδ-Cδ/Jα-Cα locus and aberrant recombination between Jα segments. EMBO J 1988;7:1661-1668.
46. Dariavach P, Lefranc M-P. First genomic sequence of the human T-cell δ2 gene (TRDV2). Nucleic Acids Res 1989;17:4880.
47. Hockett RD Jr, Nuñez G, Korsmeyer SJ. Evolutionary comparison of murine and human δ T-cell receptor deleting elements. New Biologist 1989;1:266-274.
48. Loh EY, Lanier LL, Turck CW, Littman DR, Davis MM, Chien Y-H, Weiss A. Identification and sequence of a fourth human T cell antigen receptor chain. Nature 1987;330:569-572.
49. Lefranc M-P, Rabbits TH. The human T-cell receptor γ (TRG) genes. Trends Biochem Sci 1989;14:214-218.
50. Lefranc M-P, Chuchana P, Dariavach P, Nguyen C, Huck S, Brockly F, Jordan B, Lefranc G. Molecular mapping of the human T cell receptor gamma (TRG) genes and linkage of the variable and constant regions. Eur J Immunol 1989;19:989-994.
51. Takihara Y, Champagne E, Ciccone E, Moretta L, Minden M, Mak TW. Organization and orientation of a human T cell receptor δ chain V gene segment that suggests an inversion mechanism is utilized in its rearrangement. Eur J Immunol 1989;19:571-574.
52. Macintyre E, D'Auriol L, Amesland F, Loiseau P, Chen Z, Boumsell L, Galibert F, Sigaux F. Analysis of junctional diversity in the preferential Vδ1-Jδ1 rearrangement of fresh T-acute lymphoblastic leukemia cells by *in vitro* gene amplification and direct sequencing. Blood 1989;74:2053-2061.
53. Lanier LL, Federspiel NA, Ruitenberg JJ, Phillips JH, Allison JP, Littman D, Weiss A. The T cell antigen receptor complex expressed on normal peripheral blood CD4$^-$, CD8$^-$ T lymphocytes: a CD3-associated disulfide-linked γ chain heterodimer. J Exp Med 1987;165:1076-1094.
54. Gouttefangeas C, Bensussan A, Boumsell L. Study of the CD3-associated T-cell receptors reveals further differences between T-cell acute lymphoblastic lymphoma and leukemia. Blood 1990;75:931-934.
55. Bottino C, Tambussi G, Ferrini S, Ciccone E, Varese P, Mingari MC, Moretta L, Moretta A. Two subsets of human T lymphocytes expressing γ/δ antigen receptor are identifiable by monoclonal antibodies directed to two distinct molecular forms of the receptor. J Exp Med 1988;168:491-505.

CHAPTER 2.3

UNRAVELLING HUMAN T-CELL RECEPTOR JUNCTIONAL REGION SEQUENCES*

Timo M. Breit and Jacques J.M. van Dongen

Department of Immunology, Erasmus University/University Hospital Dijkzigt, Rotterdam, The Netherlands.

ABSTRACT

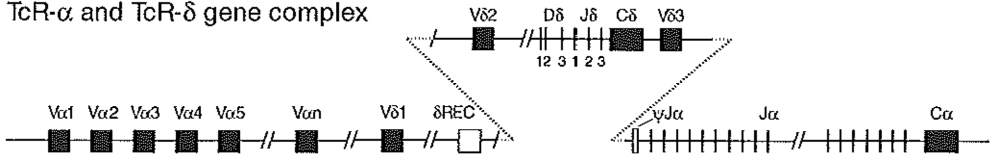
Careful analysis of functional Vδ2-Jδ1 and Vγ9-Jγ1.2 rearrangements of peripheral T-lymphocytes showed high frequencies of leucine and valine at a fixed position in the Vδ2-Jδ1 junctional regions. This phenomenon remained unnoticed in the numerous published junctional regions for over several years. Because comparable preferential motifs might also occur in junctional regions of other T-cell populations in health and disease, more precise analysis of junctional region diversity is needed. For this reason we describe general guidelines for identification of the various elements in TcR junctional regions: D-gene-derived nucleotides (in case of TcR-β and TcR-δ genes), P-region nucleotides, N-region nucleotides, and deletion of nucleotides by trimming of the rearranged gene segments. In addition, we summarized the known genomic germline sequences of rearranging TcR gene segments, which are necessary for proper application of the general guidelines. Subsequent analysis of the majority of published TcR junctional regions, allowed us to determine the composition and average insertion and deletion of nucleotides in genomic junctional regions. Because the protein junctional region instead of the genomic junctional region determines the actual specificity of TcR chains, the amino acid composition of the protein junctional regions of different types of TcR gene rearrangements was determined. This revealed some unexpected characteristics, such as the virtual absence of cysteine in all functional TcR junctional regions and increased or decreased frequencies of particular amino acid residues in specific TcR junctional regions. Application of the guidelines in combination with the summarized TcR germline sequences may contribute to uniformity in the analysis of junctional regions and may lead to important information concerning TcR specificity.

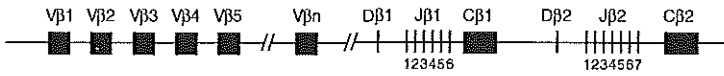
INTRODUCTION

Antigen-specific receptors of T cells are produced after functional rearrangements of V, D, and J gene segments in the four TcR gene complexes: TcR-α, β, γ and δ (1-3). The enormous diversity of TcR-αβ or TcR-γδ antigen specific receptors is accomplished by a large combinatorial diversity and an extended junctional diversity. The potential combinatorial diversity of the various TcR genes is determined by the number of different

---

* Published in: Thymus 1994;22:177-199.





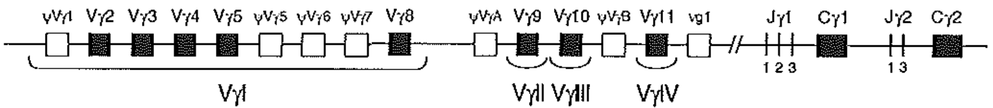


Figure 1. Schematic diagram of the germline configuration of the four human TcR gene complexes. The TcR-α gene complex consists of many Vα gene segments (>50), a long stretch of J gene segments (61) and one Cα gene segment. The TcR-δ locus is located within the TcR-α gene complex between the Vα and the Jα gene segments and consists of a few Vδ (~6), three Dδ, three Jδ, and one Cδ gene segments. Also indicated are the TcR-δ deleting elements δREC and ψJα (dotted boxes). The TcR-β gene complex contains many Vβ (~51) gene segments grouped in 24 families and two Cβ gene segments, which are preceded by one Dβ and six or seven Jβ gene segments. The TcR-γ gene complex consists of two Cγ gene segments, preceded by a two or three Jγ gene segments and a restricted number of Vγ gene segments: eight functional gene segments (solid boxes) and seven pseudo-gene segments (ψ; open boxes). The functional Vγ gene segments are grouped in four families (VγI, VγII, VγIII and VγIV).

V, (D) and J gene segments (Figure 1) (1,2). The junctional regions between V and J gene segments after joining by rearrangement processes (4) determine the junctional diversity and consist of D-gene-derived nucleotides in the case of TcR-β (5) and TcR-δ genes (6), regions of nucleotides forming a palindromic sequence with the juxtaposed nucleotides of an untrimmed gene segment (P-region nucleotides) (7,8), regions of randomly inserted nucleotides (N-region nucleotides) (9), and deletion of nucleotides by trimming the ends of the involved gene segments (9) (Figure 2).

In a recent study we analyzed the junctional regions of human peripheral blood TcR-$\gamma\delta^+$ T-lymphocytes bearing Vδ2-Jδ1-Cδ/Vγ9-Jγ1.2-Cγ1 chains and identified an invariant T nucleotide at a fixed position in 90% of the Vδ2-Jδ1 junctional regions of these polyclonal T-lymphocytes (10). Comparison and translation of a large series of published in frame Vδ2-Jδ1 junctional region sequences revealed that this T nucleotide is involved in the encoding of invariant amino acid residues (especially leucine and valine) at a fixed position in the Vδ2-Jδ1 junctional region (11-16). This distinct phenomenon remained unnoticed in the literature for over several years. Because comparable preferential motifs might also occur in junctional regions of other T-cell populations in health and disease, a more uniform approach for analyzing junctional regions is needed. For this reason we compared the results of the numerous studies which are performed to determine the actual diversity of rearranged TcR-α (17-20), TcR-β (19,21-28), TcR-γ (12-14,29-34) and TcR-δ

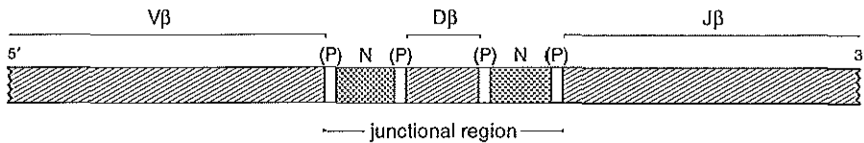


Figure 2. Schematic presentation of a TcR-β V-D-J gene rearrangement. Indicated are the germline Vβ, Dβ, and Jβ gene segments, as well as the potential P-regions (P), and the N-regions (N) at the junctions of the gene segments.

(6,12-16,29,33-45) genes.

Although in most TcR genes the combinatorial diversity is relatively easy to determine (2), determining the junctional diversity is far more complex. This is partly due to the fact that the junctional diversity can be examined at the level of DNA sequences or at the level of protein sequences. Confusing these two levels of examination may cause inadequate definition of the junctional region components or boundaries of V and J gene segments, which leads to incorrect conclusions towards the actual junctional diversity at the genomic level (20,22,24,27,32,41,46). To achieve congruity in determination of junctional diversity, we here describe general guidelines for unravelling TcR junctional region DNA sequences. To apply these guidelines properly, we summarized the available germline sequences of the rearranging TcR gene segments. The guidelines and germline sequences described here were used for unravelling a large series of published junctional regions of rearranged human TcR genes, to determine the composition of these junctional regions and to determine the junctional diversity at the DNA level as well as at the protein level.

METHODS

General guidelines to identify the components of TcR junctional regions

The 5′ side of a junctional region (3′ side of V gene segment)

To determine the exact 5′ side of a junctional region, the V-gene-derived nucleotides should be aligned with the corresponding germline V gene sequence, which continues up to the heptamer-nonamer RSS. The first nucleotide differing from the germline V gene sequence is to be considered the first nucleotide of the junctional region. So it is virtually impossible to determine the precise boundary between the V gene segment and the junctional region, if the 3′ germline sequence of the involved V gene segment is not known. For the TcR-γ and TcR-δ genes the complete 3′ germline V gene sequences are presented in Figure 3. The complete 3′ germline V gene sequences of most TcR-α and TcR-β genes are not yet available (17,47-55). Until all these complete germline sequences become available, we propose to arbitrarily define the 3′ side of Vα genes by assuming that the nucleotides encoding the conserved amino acid CA consensus represent the 3′ side of almost all Vα gene segments, deliberately ignoring, for now, the remaining (unknown) germline Vα sequences between the consensus sequence and the RSS (56). For Vβ genes the 3′ amino acid CASS consensus is assumed to define the 3′ side (56). These assumptions will probably lead to some overestimation of N-region nucleotides but makes junctional region studies more comparable.

The 3′ side of a junctional region (5′ side of J gene segment)

The 3′ side of a junctional region can be determined in a similar way as the 5′ side. Here the J-gene-derived nucleotides should be aligned with the corresponding germline J gene sequence, which starts from the heptamer-nonamer RSS. The first nucleotide differing from the germline sequence of the involved J gene

Name	Human germline sequence	P-region
	T-cell receptor Vγ	
ψVγ1	AGA CTG CAA AAT CTA ATT AAA AAT GAT TCT GGG TTC TAT TAC TGT GCC ACC TGG GAC AGG	cc
Vγ2	ATA CTG CGA AAT CTA ATT GAA AAT GAC TCT GGG GTC TAT TAC TGT GCC ACC TGG GAC GGG	cc
Vγ3	AGA CTG CAA AAT CTA ATT GAA AAT GAT TCT GGG GTC TAT TAC TGT GCC ACC TGG GAC AGG	cc
Vγ4	ATA CTG CGA AAT CTT ATT GAA AAT GAC TCT GGA GTC TAT TAC TGT GCC ACC TGG GAT GGG	cc
Vγ5	ATA CTA CGA AAT CTA ATT GAA AAT GAT TCT GGG GTC TAT TAC TGT GCC ACC TGG GGC AGG	cc
ψVγ5	AGA CTG CAA AAT CTA ATT GAA AAT GAT TCT GGG GTC TAT TAC TGT GCC ACC TGG GGC AGG	cc
ψVγ6	ATA CCT CCA AAA CTA AAT GAA AAT GCC TCT GGG GTC TAT TAC TGT GCC ACC TAG GAC AGG	cc
ψVγ7	ATA CTG CAA AAT CTA ATT GAA AAT GAT TCT GGA -TC TAT TAC TGT GCC ACC TGG GAC AGG	cc
Vγ8	ATA CTG GAA AAT CTA ATT GAA CGT GAC TCT GGG GTC TAT TAC TGT GCC ACC TGG GAT AGG	cc
Vγ9	ACC ATT CAC AAT GTA GAG AAA CAG GAC ATA GCT ACC TAC TAC TGT GCC TTG TGG GAG GTG	ca
Vγ10	ACC ATC AAG TCC GTA GAG AAA GAA GAC ATG GCC GTT TAC TAC TGT GCT GCG TGG TGG GTG GC	gc
Vγ11	AAA ATA AAG TTC TTA GAG AAA GAA GAT GAG GTG GTG TAC CAC TGT GCC TGC TGG ATT AGG CAC	gt
ψVγA	GCA GTA CTG AAG TTG GAG ACA GGC ATC GAG GGC ATG AAC TAC TGC ACA ACC TGG GCC CTG	ca
ψVγB	ACC ATA AAC TTC ATA GGA AAG GAA GAT GAG GCC ATT TAC TAC TGC ACT GCT TAG GAC C	gg
vg1	TACTTTGTGGTCAGACTTGTTAACTGGCGGGGGGAGGGGCTGGGTTGTGGCTTCAGTGTCTGTCCTC	ga
	T-cell receptor Vδ	
Vδ1	ACC ATT TCA GCC TTA CAG CTA GAA GAT TCA GCA AAG TAC TTT TGT GCT CTT GGG GAA CT	ag
Vδ2	AAG ATA CTT GCA CCA TCA GAG AGA GAT GAA GGG TCT TAC TAC TGT GCC TGT GAC ACC	gg
Vδ3	GTG ATC TCT CCA GTA AGG ACT GAA GAC AGT GCC ACT TAC TAC TGT GCC TTT AG	ct
Vδ4	GTC ATC TCC GCT TCA CAA CTG GGG GAC TCA GCA ATG TAT TTC TGT GCA ATG AGA GAG GG	cc
Vδ5	CAC ATT GTG CCC TCC CAG CCT GGA GAC TCT GCA GTG TAC TTC TGT GCA GCA AGC	gc
Vδ6	CAT ATC ATG GAT TCC CAG CCT GGA GAC TCA GCC ACC TAC TTC TGT GCA GCA AGA	tc
δREC	GCAACATCACTCTGTGTCTAGCACGTAGCCCAGAGGTTGCGGGCCCCATCCTCTCGTGTGAGGAGCC	gg

Figure 3. Germline sequences of human TcR-Vγ and Vδ gene segments with their potential P-region nucleotides. Presented are the 3′ germline sequences of functional Vγ, pseudo (ψ) Vγ and vestigial (vg1) gene segments derived from References (46,61,66-69). The 3′ germline sequences of Vδ gene segments and the upstream TcR-δ deleting element (δREC) were derived from References (36,50,70-73). Underlined triplets (TAG) represent stop codons. The vg1 and δREC gene segments are presented as a continuous sequences because both segments are severely degenerated as compared to functional gene segments.

segment is to be considered the last nucleotide of the junctional region. In principle, determining the boundary between the junctional region and the J gene segment should not be a problem, since the complete germline sequences of all TcR J gene segments are known (Figure 4).

D-gene-derived nucleotides

D-gene-derived nucleotides are identified by aligning the nucleotides of the junctional region with the germline sequences of Dβ or Dδ gene segments (Figure 5). However, identification of D-gene-derived nucleotides is often hampered by nucleotide deletion through exonucleic nibbling at both sides during the rearrangement process, which may extensively shorten the relatively small D gene segments. For identification

P-region	Human germline sequence	Name
	T-cell receptor Jα	
cc	GG TAC CGG GTT AAT AGG AAA CTG ACA TTT GGA GCC AAC ACT AGA GGA ATC ATG AAA CTC A	ψJα
ca	TG AAG ATC ACC TAG ATG CTC AAC TTT GGG AAG GGG ACT GAG TTA ATT GTG AGC CTG G	ψJα60
cc	GG AAG GAA GGA AAC AGG AAA TTT ACA TTT GGA ATG GGG ACG CAA GTG AGA GTG AAG CTA T	ψJα59
aa	TT TAA GAA ACC AGT GGC TCT AGG TTG ACC TTT GGG GAA GGA ACA CAG CTC ACA GTG AAT CCT G	Jα58
ta	TA ACT CAG GGC GGA TCT GAA AAG CTG GTC TTT GGA AAG GGA ACG AAA CTG ACA GTA AAC CCA T	Jα57
aa	T TAT ACT GGA GCC AAT AGT AAG CTG ACA TTT GGA AAA GGA ATA ACT CTG AGT GTT AGA CCA G	Jα56
gt	AC AAG TGC TGG TAA TGC TCC TGT TGG GGA AAG GGG ATG AGT ACA AAA ATA AAT CCA A	ψJα55
ta	TA ATT CAG GGA GCC CAG AAG CTG GTA TTT GGC CAA GGA ACC AGG CTG ACT ATC AAC CCA A	Jα54
ct	AG AAT AGT GGA GGT AGC AAC TAT AAA CTG ACA TTT GGA AAA GGA ACT CTC TTA ACC GTG AAT CCA A	Jα53
ag	CT AAT GCT GGT GGT ACT AGC TAT GGA AAG CTG ACA TTT GGA CAA GGG ACC ATC TTG ACT GTC CAT CCA A	Jα52
ct	AG ATG CGT GAC AGC TAT GAG AAG CTG ATA TTT GGA AAG GAG ACA TGA CTA ACT GTG AAG CCA A	ψJα51
ca	TG AAA ACC TCC TAC GAC AAG GTG ATA TTT GGG CCA GGG ACA AGC TTA TCA GTC ATT CCA A	Jα50
tc	G AAC ACC GGT AAC CAG TTC TAT TTT GGG ACA GGG ACA AGT TTG ACG GTC ATT CCA A	Jα49
ta	TA TCT AAC TTT GGA AAT GAG AAA TTA ACC TTT GGG ACT GGA ACA AGA CTC ACC ATC ATA CCC A	Jα48
ca	TG GAA TAT GGA AAC AAA CTG GTC TTT GGC GCA GGA ACC ATT CTG AGA GTC AAG TCC T	Jα47
ct	AG AAG AAA AGC AGC GGA GAC AAG CTG ACT TTT GGG ACC GGG ACT CGT TTA GCA GTT AGG CCC A	Jα46
ca	TG TAT TCA GGA GGA GGT GCT GAC GGA CTC ACC TTT GGC AAA GGG ACT CAT CTA ATC ATC CAG CCC T	Jα45
ta	TA AAT ACC GGC ACT GCC AGT AAA CTC ACC TTT GGG ACT GGA ACA AGA CTT CAG GTC ACG CTC G	Jα44
gt	AC AAT AAC AAT GAC ATG CGC TTT GGA GCA GGG ACC AGA CTG ACA GTA AAA CCA A	Jα43
ca	TG AAT TAT GGA GGA AGC CAA GGA AAT CTC ATC TTT GGA AAA GGC ACT AAA CTC TCT GTT AAA CCA A	Jα42
tc	G AAC TCA AAT TCC GGG TAT GCA CTC AAC TTC GGC AAA GGC ACC TCG CTG TTG GTC ACA CCC C	Jα41
gt	ACT ACC TCA GGA ACC TAC AAA TAC ATC TTT GGA ACA GGC ACC AGG CTG AAG GTT TTA GCA A	Jα40
ca	TG AAT AAT AAT GCA GGC AAC ATG CTC ACC TTT GGA GGG GGA ACA AGG TTA ATG GTC AAA CCC C	Jα39
ta	T AAT GCT GGC AAC AAC CGT AAG CTG ATT TGG GGA TTG GGA ACA AGC CTG GCA GTA AAT CCG A	Jα38
ca	T GGC TCT GGC AAC ACA GGC AAA CTA ATC TTT GGG CAA GGG ACA ACT TTA CAA GTA AAA CCA G	Jα37
ga	T CAA ACT GGG GCA AAC AAC CTC TTC TTT GGG ACT GGA ACG AGA CTC ACC GTT ATT CCC T	Jα36
tc	G ATA GGC TTT GGG AAT GTG CTG CAT TGC GGG TCC GGC ACT CAA GTG ATT GTT TTA CCA C	Jα35
ga	TCT TAT AAC ACC GAC AAG CTC ATC TTT GGG ACT GGG ACC AGA TTA CAA GTC TTT CCA A	Jα34
ca	TG GAT AGC AAC TAT CAG TTA ATC TGG GGC GCT GGG ACC AAG CTA ATT ATA AAG CCA G	Jα33
ca	TG AAT TAT GGC GGT GCT ACA AAC AAG CTC ATC TTT GGA ACT GGC ACT CTG CTT GCT GTC CAG CCA A	Jα32
cc	GG AAT AAC AAT GCC AGA CTC ATG TTT GGA GAT GGA ACT CAG CTG GTG GTG AAG CCC A	Jα31
ca	TG AAC AGA GAT GAC AAG ATC ATC TTT GGA AAA GGG ACA CGA CTT CAT ATT CTC CCC A	Jα30
cc	GG AAT TCA GGA AAC ACA CCT CTT GTC TTT GGA AAG GGC ACA AGA CTT TCT GTG ATT GCA A	Jα29
tg	CA TAC TCT GGG GCT GGG AGT TAC CAA CTC ACT TTC GGG AAG GGG ACC AAA CTC TCG GTC ATA CCA A	Jα28
ta	T AAC ACC AAT GCA GGC AAA TCA ACC TTT GGG GAT GGG ACT ACG CTC ACT GTG AAG CCA A	Jα27
cc	GG GAT AAC TAT GGT CAG AAT TTT GTC TTT GGT CCC GGA ACC AGA TTG TCC GTG CTG CCC T	Jα26
tg	CA GAA GGA CAA GGC TTC TCC TTT ATC TTT GGG AAG GGG ACA AGG CTG CTT GTC AAG CCA A	Jα25
ca	TG ACA ACT GAC AGC TGG GGG AAA TTG CAG TTT GGA GCA GGG ACC CAG GTT GTG GTC ACC CCA G	Jα24
ca	TG ATT TAT AAC CAG GGA GGA AAG CTT ATC TTC GGA CAG GGA ACG GAG TTA TCT GTG AAA CCC A	Jα23
aa	TT TCT TCT GGT TCT GCA AGG CAA CTG ACC TTT GGA TCT GGG ACA CAA TTG ACT GTT TTA CCT G	Jα22
ta	TAC AAC TTC AAC AAA TTT TAC TTT GGA TCT GGG ACC AAA CTC AAT GTA AAA CCA A	Jα21
ac	GT TCT AAC GAC TAC AAG CTC AGC TTT GGA GCC GGA ACC ACA GTA ACT GTA AGA GCA A	Jα20
gc	GC TAT CAA AGA TTT TAC AAT TTC ACC TTT GGA AAG GGA TCC AAA CAT AAT GTC ACT CCA A	Jα19
gg	CC GAC AGA GGC TCA ACC CTG GGG AGG CTA TAC TTT GGA AGA GGA ACT CAG TTG ACT GTC TGG CCT G	Jα18
ca	TG ATC AAA GCT GCA GGC AAC AAG CTA ACT TTT GGA GGA GGA ACC AGG GTG CTA GTT AAA CCA A	Jα17
cc	GG TTT TCA GAT GGC CAG AAG CTG CTC TTT GCA AGG GGA ACC ATG TTA AAG GTG GAT CTT A	Jα16
gg	CC AAC CAG GCA GGA ACT GCT CTG ATC TTT GGG AAG GGA ACC ACC TTA TCA GTG AGT TCC A	Jα15
at	ATT TAT AGC ACA TTC ATC TTT GGG AGT GGG ACA AGA TTA TCA GTA AAA CCT G	Jα14
ca	TG AAT TCT GGG GGT TAC CAG AAA GTT ACC TTT GGA ATT GGA ACA AAG CTC CAA GTC ATC CCA A	Jα13
cc	GG ATG GAT AGC AGC TAT AAA TTG ATC TTC GGG AGT GGG ACC AGA CTG CTG GTC AGG CCT G	Jα12
ca	TG AAT TCA GGA TAC AGC ACC CTC ACC TTT GGG AAG GGG ACT ATG CTT CTA GTC TCT CCA G	Jα11
at	ATA CTC ACG GGA GGA GGA AAC AAA CTC ACC TTT GGG ACA GGC ACT CAG CTA AAA GTG GAA CTC A	Jα10
cc	GGA AAT ACT GGA GGC TTC AAA ACT ATC TTT GGA GCA GGA ACA AGA CTA TTT GTT AAA GCA A	Jα9
ca	TG AAC ACA GGC TTT CAG AAA CTT GTA TTT GGA ACT GGC ACC CGA CTT CTG GTC AGT CCA A	Jα8
gt	AC TAT GGG AAC AAC AGA CTC GCT TTT GGG AAG GGG AAC CAA GTG GTG GTC ATA CCA A	Jα7
ca	T GCA TCA GGA GGA AGC TAC ATA CCT ACA TTT GGA AGA GGA ACC AGC CTT ATT GTT CAT CCG T	Jα6
ca	TG GAC ACG GGC AGG AGA GCA CTT ACT TTT GGG AGT GGA ACA AGA CTC CAA GTG CAA CCA A	Jα5
ca	TG TTT TCT GGT GGC TAC AAT AAG CTG ATT TTT GGA GCA GGG ACC AGG CTG GCT GTA CAC CCA T	Jα4
cc	G GGG TAC AGC AGT GCT TCC AAG ATA ATC TTT GGA TCA GGG ACC AGA CTC AGC ATC CGG CCA A	Jα3
ca	TG AAT ACT GGA GGA ACA ATT GAT AAA CTC ACA TTT GGG AAA GGG ACC CAT GTA TTC ATT ATA TCT G	Jα2
ac	G TAT GAA AGT ATT ACC TCC CAG TTG CAA TTT GGC AAA GGA ACC AGA GTT TCC ACT TCT CCC C	ψJα1

Figure 4A. Complete germline sequences of human TcR-Jα gene segments with their potential P-region nucleotides. Sequences are derived from References (48,71,74,82). Underlined triplets (TAG, TAA, and TGA) represent stop codons.

P-region	Human germline sequence	Name
	T-cell receptor Jβ	
ca	TG AAC ACT GAA GCT TTC TTT GGA CAA GGC ACC AGA CTC ACA GTT GTA G	Jβ1.1
ag	CT AAC TAT GGC TAC ACC TTC GGT TCG GGG ACC AGG TTA ACC GTT GTA G	Jβ1.2
ag	C TCT GGA AAC ACC ATA TAT TTT GGA GAG GGA AGT TGG CTC ACT GTT GTA G	Jβ1.3
tg	CA ACT AAT GAA AAA CTG TTT TTT GGC AGT GGA ACC CAG CTC TCT GTC TTG G	Jβ1.4
ta	T AGC AAT CAG CCC CAG CAT TTT GGT GAT GGG ACT CGA CTC TCC ATC CTA G	Jβ1.5
ag	C TCC TAT AAT TCA CCC CTC CAC TTT GGG AAT GGG ACC AGG CTC ACT GTG ACA G	Jβ1.6
ag	C TCC TAC AAT GAG CAG TTC TTC GGG CCA GGG ACA CGG CTC ACC GTG CTA G	Jβ2.1
cg	CG AAC ACC GGG GAG CTG TTT TTT GGA GAA GGC TCT AGG CTG ACC GTA CTG G	Jβ2.2
ct	AGC ACA GAT ACG CAG TAT TTT GGC CCA GGC ACC CGG CTG ACA GTG CTC G	Jβ2.3
ct	A GCC AAA AAC ATT CAG TAC TTC GGC GCC GGG ACC CGG CTC TCA GTG CTG G	Jβ2.4
gt	AC CAA GAG ACC CAG TAC TTC GGG CCA GGC ACG CGG CTC CTG GTG CTC G	Jβ2.5
ag	C TCT GGG GCC AAC GTC CTG ACT TTC GGG GCC GGC AGC AGG CTG ACC GTG CTG G	Jβ2.6
ag	C TCC TAC GAG CAG TAC TTC GGG CCG GGC ACC AGG CTC ACG GTC ACA G	Jβ2.7
	T-cell receptor Jγ	
at	AT ACC ACT GGT TGG TTC AAG ATA TTT GCT GAA GGG ACT AAG CTC ATA GTA ACT TCA CCT G	Jγ1.1
cc	GG CAA GAG TTG GGC AAA AAA ATC AAG GTA TTT GGT CCC GGA ACA AAG CTT ATC ATT ACA G	Jγ1.2
tc	G AAT TAT TAT AAG AAA CTC TTT GGC AGT GGA ACA ACA CTG GTT GTC ACA G	Jγ1.3
at	AT AGT AGT GAT TGG ATC AAG ACG TTT GCA AAA GGG ACT AGG CTC ATA GTA ACT TCG CCT G	Jγ2.1
tc	G AAT TAT TAT AAG AAA CTC TTT GGC AGT GGA ACA ACA CTT GTT GTC ACA G	Jγ2.3
	T-cell receptor Jδ	
gt	AC ACC GAT AAA CTC ATC TTT GGA AAA GGA ACC CGT GTG ACT GTG GAA CCA A	Jδ1
ag	CT TTG ACA GCA CAA CTC TTC TTT GGA AAG GGA ACA CAA CTC ATC GTG GAA CCA G	Jδ2
ag	C TCC TGG GAC ACC CGA CAG ATG TTT TTC GGA ACT GGC ATC AAA CTC TTC GTG GAG CCC C	Jδ3
gg	CC AGA CCC CTG ATC TTT GGC AAA GGA ACC TAT CTG GAG GTA CAA CAA C	Jδ4

Figure 4B. Complete germline sequences of human TcR-Jβ, Jγ, and Jδ gene segments with their potential P-region nucleotides. TcR-Jβ sequences are derived from Reference (5). TcR-Jγ sequences are derived from References (65,75-77). TcR-Jδ sequences are derived from refs (78,79,80,81).

of D-gene-derived nucleotides we propose the guideline that at least one third of the D gene segment should be present with a minimum of three successive nucleotides. This guideline is in line with assumptions made by other groups (6,11,13,25,57) and based on our own experience with TcR-δ gene rearrangements (29,34). Applying this guideline means that the minimum number of D-gene-derived nucleotides for recognition is: three successive nucleotides for TcR-Dδ1 (germline: 8 nucleotides) and TcR-Dδ2 (germline: 9 nucleotides), four successive nucleotides for TcR-Dβ1 (germline: 12 nucleotides) and TcR-Dδ3 (germline: 13 nucleotides), and five successive nucleotides for TcR-Dβ2 (germline: 16 nucleotides) (Figure 5). Misinterpretations may still

	P-region	Human germline sequence	P-region	# nucleotides for identification
		T-cell receptor β		
Dβ1	cc	GGGACAGGGGGC	gc	4
Dβ2	cc	GGGACTAGCGGGAGGG	cc	5
		T-cell receptor δ		
Dδ1	tc	GAAATAGT	ac	3
Dδ2	gg	CCTTCCTAC	gt	3
Dδ3	gt	ACTGGGGGATACG	cg	4

Figure 5. Germline sequences of human TcR-Dβ and Dδ gene segments with their potential P-region nucleotides. Indicated are also the minimal number of consecutive junctional region nucleotides for identification of a specific D gene segment in a gene rearrangement. Sequences are derived from References (5,6,79,80). Underlined sequences (TAG) represent a potential stop codon in a particular reading frame.

occur because of the chance that three (or four) random successive N-region nucleotides can be identical to three (or four) successive nucleotides of a D gene segment. Nevertheless, the guideline proposed here for identification of D-gene-derived nucleotides allows junctional diversity studies to be more comparable.

P-region nucleotides

To identify P-region nucleotides, basically three conditions have to be fulfilled, as proposed by Lafaille et al. (7): P-region nucleotides can only be found next to a rearranged gene segment, if that side of the gene segment is in its full coding sequence (i.e. without trimming); a P-region maximally consists of one or two nucleotides; and P-region nucleotides can be recognized by the palindromic nature of the P-region in combination with the juxtaposed nucleotides of the rearranged (untrimmed) gene segment. Because all P-region nucleotides are defined by the germline sequence of a gene segment, we have indicated the P-region nucleotides for every known TcR-V, D and J gene segment in Figures 3-5. Although recent publications indicate that P-regions of three or more nucleotides might occur, they only represent 5% of all P nucleotide inserts and may also be a result of a two-base P-region plus fortuitous N-region sequences (8). We therefore wish to stick to the original proposal of maximally two P-region nucleotides.

N-region nucleotides

N-region nucleotides are the nucleotides that cannot be appointed to either a V, (D), J gene segment or P-region (7,9). These nucleotides are randomly inserted by the enzyme TdT at the junctions of gene segments during the rearrangement process (9,58) and contribute largely to the total junctional diversity (2).

Assignment of junctional region nucleotides in the absence of N-region nucleotides

In case that N-region nucleotides are lacking in a junction of two gene segments, it may occur that nucleotides can be assigned to two different origins. In these ambiguous cases we propose an arbitrarily hierarchic order: firstly V-gene-derived, than J-gene-derived, subsequently D-gene-derived, and finally P-region nucleotides.

Deletion of nucleotides by trimming

Trimming of the involved V, (D) and J gene segments by exonucleic nibbling of the sides of the involved gene segments during the rearrangement process can be determined by comparing the germline sequences with the remaining sequences of the rearranged gene segments (9).

Reading frame status

To appoint a certain rearrangement to be 'in frame', it should be possible to read the functional V gene segment reading frame throughout the junctional region into the reading frame of the J gene segment without coming across any stop codons. The reading frames of the Vγ, Vδ and all J gene segments are indicated in

Figures 3 and 4. The reading frames of the Vα and Vβ gene segments should be in line with the amino acid CA and CASS consensus, respectively. D gene segments can in principle be read in all three reading frames, but the Dβ2 and Dδ1 gene segments contain a stop codon (TAG) in one reading frame (Figure 5). If the rearranged J gene segment is not in its correct reading frame or a stop codon is generated in the junctional region, than the rearrangement is appointed to be 'out of frame' and therefore non-functional. Incomplete rearrangements, such as V-D, D-D or D-J, are regarded to be non-functional as well.

Junctional region amino acids

Junctional region amino acid residues can easily be identified by aligning the protein sequence of the in-frame gene rearrangement with the germline protein sequences of V and J gene segments.

RESULTS AND DISCUSSION

Junctional regions of human TcR gene rearrangements

The general guidelines for unravelling junctional regions and the summarized TcR germline sequences were applied on previously published junctional regions sequences of human TcR-α (n = 132), TcR-β (n = 201), TcR-γ (n = 345), and TcR-δ (n = 779) gene rearrangements (6,12-45). Representative examples of junctional regions of each TcR gene are shown in Figures 6 and 7.

Human TcR-α gene rearrangements

Code		Vα	Vα/P/N-region	P	Jα		frame
HAP10	Vα1.1	AGTGACACAGCTGAGTACTTCTGTGCC	GTGAATGAAT		ACGACTACAAGCTCAGCTTTG	Jα20	+
HAP26	Vα2.1	AGTGATTCAGCCACCTACCTCTGTGCC	TTACG		AGATGGCCAGAAGCTGCTCT	Jα16	+
HAP05	Vα3.1	GCAGACACTGCTTCTTACTTCTGTGCT	ACGGATGG		GAACAGAGATGACAAGATCATCTTTG	Jα30	+
HAP08	Vα4.1	AGAGATGCTGCTGTGTACTACTGC	ATCAGAGCC		AATGCTGGTGGTACTAGCTATGGAA	Jα53	+
HAP35	Vα5.1	GCAGACTCAGCTACCTACCTCTGTGCT	CTAGCCCCATC		GTACAGCAGTGCTTCCAAGATAAT	Jα3	+
HAP01	Vα6.1	GGGGACTCAGCAATGTATTTCTGTGCA	AGTAGAGAGGGCT		CCGGTAACCAGTTCTATTTTGG	Jα49	+
HAP21	Vα7.1	AAAGACTCTGCCTCTTACTTCTGCGCT	GTTTTT		AACCAGGCAGGAACTGCTCTGATCT	Jα15	+
HAP41	Vα8.1	GAAGACTCGGCTGTCTACTTCTGTGCA	GCAAGTAGGAAGGAC		TCTGGGGGTTACCAGAAAGTTA	Jα13	+
HAP36	Vα9.1	GAAGACTCAGCCATGTATTACTGTGCT	CTAAGTG		TTTATAACCAGGGAGGAAAGCTTA	Jα23	+
HAP58	Vα10.1	GGTGATACAGGCCACTACCTCTGTGCA	GGCGTTT		CATCAGGAGGAAGCTACATACCTAC	Jα6	+
T078	δREC	GTGCGGGCCCCCATCCTCTCGTGTGAGG	GG		ACCGGGTTAATAGGAAACTGACAT	ψJα	0

Human TcR-γ gene rearrangements

Code		Vγ	P	N-region	P	Jγ		frame
pat8P	Vγ2	GTCTATTACTGTGCCACCTGGGACG		CGGAG		TGGTTCAAGATATTTG	Jγ1.1	+
patJB	Vγ2	GTCTATTACTGTGCCACCTGGG			C	GAATTATTATAAGAAACTCTTTGGCAG	Jγ1.3	+
patWC	Vγ3	GTCTATTACTGTGCCACCTGGGAC		TTCG		GTGATTGGATCAAGACGTTTG	Jγ2.1	+
patAV	Vγ3	GTCTATTACTGTGCCACCTGGG		CCC	C	GAATTATTATAAGAAACTCTTTGGCAG	Jγ2.3	+
patHZ	Vγ4	GTCTATTACTGTGCCACCTGGGATG		AGGCCCCCCACACCA		ATAAGAAACTCTTTGGCAG	Jγ2.3	+
patDD	Vγ5	GTCTATTACTGTGCCACCT		CTTTTA		TTTGGCAG	Jγ1.3	-
patEP	Vγ8	GTCTATTACTGTGCCACCTGGGA		AAAGAGGCTAACAGGGTTT		TTATTATAAGAAACTCTTTGGCAG	Jγ2.3	–
patIJ	Vγ9	ACCTACTACTGTGCCTTGTGGGAGGTG	CA	ATCTGGACCCAGGATTATTAAG		AAGAAACTCTTTGGCAG	Jγ1.3	+
patAV	Vγ9	ACCTACTACTGTGCCTTGTGGGAGGTG	C	TC		TTATTATAAGAAACTCTTTGGCAG	Jγ2.3	–
patJR	Vγ11	TACCACTGTGCCTGCTGGATTAGGCA		AAGGCCTCT		AATTATTATAAGAAACTCTTTGGCAG	Jγ2.3	-

Figure 6. Examples of junctional region nucleotide sequences in TcR Vα-Jα and Vγ-Jγ gene rearrangements. Precise assignment of the nucleotides in the Vα/junctional region boundary (Vα/P/N region) is not possible because of the incomplete information about Vα germline sequences (see text). Sequences are derived from References (17,29). Underlined sequences (TAA) represent stop codons.

Human TcR-β gene rearrangements

Code		Vβ	Vβ/P/N1	P	Dβ1	P	N2	P	Dβ2	P	N3	P	Jβ		frame
					GGGACAGGGGGC				GGGACTAGCGGGAGGG						
seq-1	Vβ1	TGTGCCAGC	G		CAGGGG						AAGGGG		ACACTGAAGCTTTCT	Jβ1.1	+
seq-3	Vβ2	TGCAGTGCTAG	T		GGGAC						GGATA	A	TAGCAATCAGCCCCAGCA	Jβ1.5	+
seq-9	Vβ4	TGCAGCGTTGAA	TT		GGACAG						AGG		CTACGAGCAGTACTT	Jβ2.7	+
seq-14	Vβ5	TGTGCCAGCAGC	CCCTTGGA						GGGAGG		TGACAT		TGAGCAGTT	Jβ2.1	+
seq-25	Vβ6	TGTGCCAGCAGC	TTATACAA									GT	ACCAAGAGACCCAGTACT	Jβ2.5	+
seq-26	Vβ6	TGTGCCAGCAGC	CAAAGACGGTCT									AG	CTCCTACGAGCAGTACTT	Jβ2.7	+
seq-31	Vβ7	TGCGCCAGCAGC	CAA		GGGAC						CGTTGGAA		GCTACACCT	Jβ1.2	+
seq-34	Vβ8	TGTGCCAGCAG	GCT		GGGACA						CCGGG	G	CTCTGGGGCCAACGTCCT	Jβ2.6	+
seq-35	Vβ9	TGTGCCAGCAGC	CTATCGGCGCCC									AG	CTCCTACGAGCAGTACTT	Jβ2.7	+
seq-36	Vβ12	TGTGCCAGCAGT	CT	C	GGGA		GC		AGCGGG		GGG		AATGAGCAGTT	Jβ2.1	+
seq-47	Vβ17	TGTGCCAGTA	CCCAAAGACGGTCT									AG	CTCCTACGAGCAGTACTT	Jβ2.7	+
seq-49	Vβ22	TGTGCCAGCAGC			ACAGGGGG						GGCGGGC		GGCTACACCT	Jβ1.2	+
seq-50	Vβ24	TGTGCCACCAGC	AGAGATCCCTCGG										ACAATGAGCAGTT	Jβ2.1	+

Human TcR-δ gene rearrangements

Code		Vδ	P	N1	P	Dδ1	P	N2	P	Dδ2	P	N3	P	Dδ3	P	N4	P	Jδ		frame
						GAAATAGT				CCTTCCTAC				ACTGGGGGATACG						
3yr-seq7	Vδ1	TTGGGGAACT	A	T						TTC		TA		GGGGGA		GAA		ACCGATAA	Jδ1	+
JH12	Vδ1	TTGGGG								CCT		C		CTGGGGGATACG		TCCCTTCGC		CTTTGACAGC	Jδ2	-
JH8	Vδ1	TTGGGGAA		GCCGGCCCGGCA									T	ACTGGGG		TTCCCTTG		CCTGGGAC	Jδ3	+
T053	Vδ2	CTGTGACACC	GG	ATGG						CTT		T	T	ACTGGGGGATA		GCCTAGG		CGATAA	Jδ1	+
K4	Vδ2	CTGTGACAC		G										CTGGGGGATA		AAGGGT		CTTTGACAGC	Jδ2	+
LB207	Vδ2	CTGTGA		CCG										ACTGGGGG		TT	AG	CTCCTGGGAC	Jδ3	+
T048	Vδ3	GTGCCTTTAG		TACACCCCGTG						TCC		CCCGCTCA		GGGAT		TTTCTCTCCCGCGCC		ATAA	Jδ1	-
T078	Vδ3	GTGCCTTT		CTTACTGCCGGGA						CTT		AGGA		GGGGA		G		GACAGC	Jδ2	-
KT041	Vδ3	GTGCCT		CCCTCAA						CCTA		A		ACTGGGGG		TCTGAGGACGTT	AG	CTCCTGGGAC	Jδ3	+
KT06A	Vδ4	TGAGAGAGG		AGA						CTAC	G	GAAC	T	ACTGGGGGATACG	C	TTAAGA		ACACCGATAA	Jδ1	+
KT08A	Vδ5	TGCAGCAAG		GC		AAA		GAA		CTTCCT		CCGGGGTCC		GGGGGATACG		AGGACCCGAA		ACACCGATAA	Jδ1	-
3yr-seq2	Vδ5	TGCAGCAAGC	GC	GGA										GGGATA		GGTG	G	CTTTGACAGC	Jδ2	-
KT05E	Vδ6	TGCAGCAAG		C		ATA		AGTCGCTGC								GT		ACACCGATAA	Jδ1	-
T062	δREC	GTGAGGAGCC	GG													ACGCCCCCCCGCGACAGA		ACACCGATAA	Jδ1	0

Figure 7. Examples of junctional region nucleotide sequences in TcR Vβ-(Dβ)-Jβ and Vδ-(Dδ)-Jδ gene rearrangements. Germline D sequences are double underlined and generated stop-codons are single underlined. Precise assignment of the nucleotides in the Vβ/junctional region boundary (Vβ/P/N1) is not possible because of the incomplete information about Vβ germline sequences (see text). Sequences are derived from References (27,29,35,37) and Breit et al. unpublished results (T048, T053, and T078).

Biased nucleotide insertion in N-region

To investigate the earlier noticed preference of the enzyme TdT to insert G nucleotides into junctional regions (9), we identified 'true' N-region nucleotides in junctional regions of human TcR rearrangements. Because of the uncertainty of V germline sequences, TcR-α and TcR-β gene rearrangements were excluded from this analysis. Furthermore, N-region nucleotides were analyzed only in out-of-frame TcR-γ junctional regions (n = 50) and TcR-δ junctional regions (n = 263) to exclude any possible bias caused by selection at the protein level. The relative N-region frequencies of the four nucleotides are given in Table 1. If TdT possesses a preference for a specific nucleotide, than the complementary nucleotide should also occur more frequently, because TdT would insert the particular nucleotide at the coding as well as the non-coding strand. Screening large numbers of non-selected N-region nucleotides (n = 3026) revealed higher frequencies of G (29%) and C (29%) nucleotides as compared to A (21%) and T(21%) nucleotides (Table 1). Because unrecognized D-gene derived nucleotides from the G-rich Dδ3 gene segment could lead to an overestimation of the G/C content of the TcR-δ N-regions, we also checked the fusion regions of a chromosome aberration caused by "illegitimate" V(D)J recombination. Even the randomly inserted fusion region nucleotides (n = 109) of *tal*-1 deletions, a chromosome aberration on 1p32 which is extremely homologous to TcR gene rearrangements (59 and references therein), revealed almost identical percentages of nucleotide insertion (Table 1). In total, G/C insertion was 58%, whereas A/T insertion was 42%, confirming the observation that TdT possesses a preference to insert G/C nucleotides in the junctional regions during rearrangement processes.

TABLE 1. Frequency of randomly inserted (N-region) nucleotides by TdT in junctional regions.

	n	Inserted nucleotides						
		G	C	G/C	A/T	A	T	(n)
out-frame TcR-γ junctional regions[a]	50	27.3%	29.8%	57.1%	42.9%	22.6%	20.4%	(319)
out-frame TcR-δ junctional regions[b]	263	28.8%	29.5%	58.3%	41.7%	21.1%	20.6%	(2707)
tal-1 deletion fusion regions[c]	109	28.7%	30.1%	58.8%	41.2%	20.1%	21.2%	(742)
Total	422	28.7%	29.6%	58.3%	41.7%	21.0%	20.7%	(3768)

a. Sequences derived from References 12,13,29,31,32 and 34.
b. Sequences derived from References 6,12,14-16,29,34,36,39,40and 42-45.
c. Sequences derived from Reference 59 and References therein.

Junctional diversity at the DNA level

Studying human TcR-α and TcR-β junctional diversity is difficult, because of the earlier mentioned incomplete information about germline Vα and Vβ gene sequences. Like in most other studies, we also arbitrarily defined the boundary between the V gene segment and the junctional region by assuming that the nucleotides encoding the conserved amino acid CA and CASS consensus represent the 3′ side of Vα and Vβ gene segments, respectively.

This lack of sequence information will not only cause overestimation of N-region nucleotides, but it also influences the identification of P-region nucleotides at the 3′ side of the Vα and Vβ gene segments. An additional problem in unravelling TcR-β junctional regions is the assignment of nucleotides to be either Dβ1 or Dβ2 gene derived, because these two gene segments are highly homologous (Figure 5). Furthermore, it should be stressed that a Vβ-Jβ2 junctional region potentially can contain a Dβ2 and/or a Dβ1 gene segment, whereas a Vβ-Jβ1 junctional region can only contain a Dβ1 gene segment (25,26). Dependent on the number of Dβ gene segments, Vβ-Jβ junctional regions can contain one to three N-regions, whereas Vα-Jα junctional regions only contain one N-region. Therefore, the mean size of the latter junctional regions is essentially smaller (8 nucleotides versus 16 nucleotides). The estimated mean size and the composition of junctional regions in complete TcR-α and TcR-β gene rearrangements as well as the deletion of the flanking sequences are summarized in Table 2.

Analyzing TcR-γ or TcR-δ junctional diversity is relatively easy, because all TcR-γ and TcR-δ germline sequences are known. Also, identification of Dδ gene derived nucleotides in the junctional regions is not hampered by homology between the three different Dδ gene segments (Figure 5). However, because of the small size of the Dδ gene segments and the fact that they are liable to undergo trimming at both sides, the Dδ gene segments in junctional regions easily disappear or become unidentifiable small, even after minor trimming by the recombinase enzyme complex. Because >80% of the peripheral γδ T-lymphocytes in man express a TcR consisting of Vγ9-Jγ1.2-Cγ1/Vδ2-Jδ1-Cδ chains and the fact that these cells probably are selected by peripheral expansion (15 and references therein,60), we analyzed these particular TcR gene rearrangements separately. The mean size of TcR-γ junctional regions (5 nucleotides) is significantly smaller than that of complete TcR-δ rearrangements (22 nucleotides), which is caused by the usage of Dδ gene segments. Except for a slightly reduced nucleotide deletion in the Vγ9-Jγ1.2 rearrangements, the junctional regions of all Vγ-Jγ rearrangements have a comparable size. Remarkably, nucleotide deletion of the Jγ sequences was twice that of Vγ sequences. Interestingly the mean size of Vδ2-Jδ1 junctional regions (20 nucleotides) is smaller than that of Vδ1-Jδ1 rearrangements (26 nucleotides). This may be explained by the fact that Vδ2-Jδ1 junctional regions in general only contain Dδ3 gene segments, whereas Vδ1-Jδ1 junctional regions contain Dδ2 as well as Dδ3 gene segments. All TcR-γ and TcR-δ junctional region characteristics are summarized in Table 2.

Junctional diversity at the protein level

Because the true specificity of TcR is determined by the expressed protein chains, we investigated the encoded amino acid sequences of human TcR gene rearrangements (Figure 8). Numerous in-frame junctional regions (n = 1144) of TcR-α, TcR-β, TcR-γ (Vγ9-Jγ1.2 and other Vγ-Jγ), and TcR-δ (Vδ2-Jδ1 and Vδ1-Jδ1) were analyzed by translating the DNA sequences into amino acid sequences. Apart from the average size and deletion of protein junctional regions, which are similar to that of the DNA junctional regions (Table 2), we also typified the amino acid contents of the protein junctional regions in the various TcR chains (Figure 9).

Some remarkable observations were: the high percentages of valine (V) and serine (S) residues in TcR-α gene junctional regions, which might be caused by yet unidentified Vα

TABLE 2. Insertion and deletion of junctional region nucleotides and amino acid residues in complete human TcR gene rearrangements[a].

		Nucleotides							Amino acids			
		Insertion				Deletion			Insertion	Deletion		
	n	N-region	P-region	D gene	Total	V gene	J gene	Total	Total	V gene	J gene	Total
TcR-α - Total	132[b]	8.1[c] (0-19)	0.1[c] (0-2)		8.2 (0-19)	0.1[c] (0-6)	3.7 (0-17)	3.8 (0-17)	3.1 (0-7)	0.1[d] (0-1)	1.3 (0-6)	1.4 (0-6)
TcR-β - Total	201[e]	10.3[c] (1-35)	0.3[c] (0-3)	5.0 (0-15)	15.6 (1-35)	1.0[c] (0-9)	4.0 (0-19)	5.0 (0-19)	5.6 (1-12)	0.4[d] (0-2)	1.2 (0-6)	1.6 (0-6)
TcR-γ - Vγ9-Jγ1.2	252[f]	4.3 (0-17)	0.3 (0-2)		4.6 (0-17)	1.9 (0-11)	3.8 (0-9)	5.7 (0-17)	1.7 (0-6)	0.7 (0-2)	0.8 (0-3)	1.4 (0-4)
- Vγ-Jγ other	43[f]	5.3 (0-18)	0.2 (0-2)		5.5 (0-18)	2.9 (0-12)	6.7 (1-15)	9.6 (2-20)	2.1 (0-6)	1.0 (0-5)	2.1 (0-5)	3.1 (1-7)
TcR-δ - Vδ2-Jδ1	336[g]	9.1 (0-29)	1.2 (0-5)	9.4 (0-21)	19.7 (7-40)	1.8 (0-7)	1.5 (0-9)	3.3 (0-11)	7.1 (3-13)	0.6 (0-2)	0.3 (0-3)	0.9 (0-4)
- Vδ1-Jδ1	180[g]	13.5 (2-34)	1.3 (0-6)	11.4 (0-24)	26.3 (8-55)	2.9 (0-13)	2.0 (0-9)	4.9 (0-17)	9.7 (3-19)	0.6 (0-3)	0.5 (0-3)	1.1 (0-4)

a. The mean number of nucleotides or amino acids are indicated with the range in parentheses.
b. Sequences derived form References 17-20.
c. Because of the incomplete information about germline Vα and Vβ sequences, the 3´ consensus CA and CASS amino acid sequences were assumed to represent the 3´ side of the Vα and Vβ gene segments, respectively. This might lead to an underestimation of P-region nucleotides and deleted nucleotides and an overestimation of N-region nucleotides.
d. Vα and Vβ gene deletion is determined from the 3´ consensus sequences.
e. Sequences derived from References 19 and 21-28.
f. Sequences derived from References 12-14 and 30-33.
g. Sequences derived from References 11-16,33,35-38,41,42,44,45 and 65.

		Vα	junctional region	Jα	
HAP10	Vα1.1	YFCA	VNEY	DYKLS	Jα20
HAP26	Vα2.1	YLCA	LR	DGQKL	Jα16
HAP05	Vα3.1	YFCA	TDG	NRDDKII	Jα30
HAP08	Vα4.1	YYC	IRA	NAGGTSY	Jα53
HAP35	Vα5.1	YLCA	LAPS	YSSASK	Jα3
HAP01	Vα6.1	YFCA	SREGS	GNQFY	Jα49
HAP21	Vα7.1	YFCA	VF	NQAGTAL	Jα15
HAP41	Vα8.1	YFCA	ASRKD	SGGYQK	Jα13
HAP36	Vα9.1	YYCA	LSV	YNQGGK	Jα23
HAP58	Vα10.1	YLCA	GVS	SGGSYI	Jα6

		Vγ	junctional region	Jγ	
patBP	Vγ2	TWD	AE	WFKI	Jγ1.1
patJB	Vγ2	TW	A	NYYKKLF	Jγ1.3
patWC	Vγ3	TWD	FG	DWIKT	Jγ2.1
patAV	Vγ3	TW	AP	NYYKKLF	Jγ2.3
patHZ	Vγ4	TWD	EAPHTN	KKLF	Jγ2.3
patIJ	Vγ9	LWEV	QSGPRIIK	KKLF	Jγ1.3

		Vβ	junctional region	Jβ	
seq-1	Vβ1	CAS	AGEGD	TEAFFG	Jβ1.1
seq-3	Vβ2	CSAS	GTDN	SNQPQHF	Jβ1.5
seq-9	Vβ4	CSVE	LDRG	YEQYFG	Jβ2.7
seq-14	Vβ5	CASS	PLEGGDI	EQFF	Jβ2.1
seq-25	Vβ6	CASS	LYKY	QETQYFG	Jβ2.5
seq-26	Vβ6	CASS	QRRSS	SYEQYFG	Jβ2.7
seq-31	Vβ7	CASS	QGTVGS	YTFG	Jβ1.2
seq-34	Vβ8	CAS	RLGHRG	SGANVLT	Jβ2.6
seq-35	Vβ9	CASS	LSAPS	SYEQYFG	Jβ2.7
seq-36	Vβ12	CASS	LGSSGG	NEQFF	Jβ2.1
seq-47	Vβ17	CAS	TQRRSS	SYEQYFG	Jβ2.7
seq-49	Vβ22	CASS	TGGAG	GYTFG	Jβ1.2
seq-50	Vβ24	CASS	RDPSD	NEQFF	Jβ2.1

		Vδ	junctional region	Jδ	
3yr-s 7	Vδ1	ALG	FLGGET	DKLIFG	Jδ1
JH8	Vδ1	ALGE	AGPAYWGSLA	WDTRQM	Jδ3
T053	Vδ2	ACDT	GWLLLGDSLG	DKLIFG	Jδ1
K4	Vδ2	ACDT	LGDKGS	LTAQLFF	Jδ2
LB207	Vδ2	ACD	RLGVS	SWDTRQM	Jδ3
KT041	Vδ3	YCA	SLNLNWGSEDVS	SWDTRQM	Jδ3
KT06A	Vδ4	AMRE	ETTELLGDTLKN	TDKLIFG	Jδ1

Figure 8. Examples of junctional region amino acid sequences in TcR V-J gene rearrangements. The sequences are translated from the in-frame junctional region nucleotide sequences in Figures 6 and 7.

germline sequences (48); the high frequencies of glycine (G) residues, which in TcR-β and TcR-δ gene junctional regions are partly caused by G nucleotide rich D gene segments, but were also present in elevated frequencies in TcR-α and TcR-γ junctional regions; the prominent valine and leucine (L) percentages in Vδ2-Jδ1 junctional regions, which are in line with the invariant presence of these amino acid residues at the relative second position in these junctional regions (10), but were surprisingly also frequently found in the co-expressed Vγ9-Jγ1.2 junctional regions. The latter observations are most likely caused by peripheral expansion of selected Vγ9/Vδ2 T-lymphocytes. Furthermore we observed: the virtual absence of cysteine (C) residues in all TcR junctional regions, which is probably caused by the fact that disulphide bonds mediated by junctional region cysteine residues may deform the immunoglobulin domain structure of the TcR molecule; the high frequency

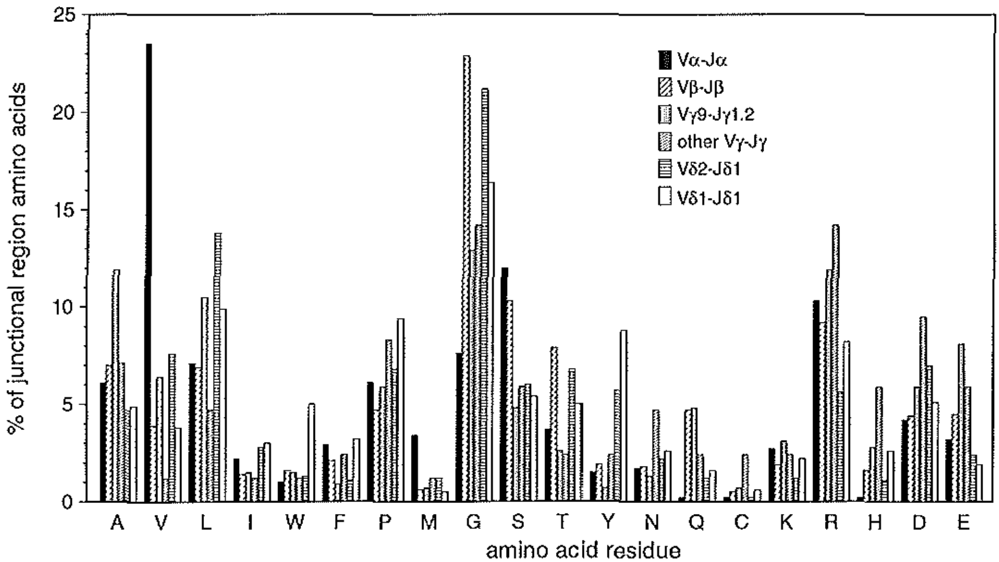


Figure 9. Relative frequency of amino acid residues in junctional regions of TcR gene rearrangements from peripheral T-lymphocytes. Indicated are TcR-α, TcR-β, TcR Vγ9-Jγ1.2, other TcR Vγ-Jγ, TcR Vδ2-Jδ1, and TcR Vδ1-Jδ1 gene rearrangements. The relative amino acid frequencies are determined by translation of junctional region sequences from References (12-28,30-33,35-38,65). The high percentage valine (V) in Vα-Jα junctional regions might be caused by germline Vα nucleotides (see text).

of arginine (R) in all TcR junctional regions, which might be important for interaction mechanisms between the TcR and the antigen-presenting MHC molecules; and the increased frequency of tryptophan (W) in Vδ1-Jδ1 junctional regions, which function remains to be elucidated. Overall, it can be concluded, that most amino acid residues are present in frequencies which are comparable for all TcR genes, whereas some exhibit non-random frequencies in specific TcR junctional regions (Figure 9).

CONCLUDING REMARKS

Although guidelines to unravel TcR junctional regions seem to be straightforward, several "common mistakes" are made in the interpretation of junctional regions. One such mistake is the assignment of nucleotides to be V- or J-gene-derived, only if these nucleotides belong to a *complete* codon of the V or J gene segment reading frame (22,24,27,32,46,61). By doing so, V- and/or J-gene-derived nucleotides are defined as junctional region nucleotides, causing overestimation of the total junctional region diversity. Additionally, by inaccurately defining the 3′ sides of V gene segments and 5′ sides of J gene segments, it is impossible to identify P-region nucleotides.

In their search for junctional diversity of human TcR-β gene rearrangements some investigators probably confused the *protein* junctional region with the *genomic* junctional region. This confusion may explain the above described incorrect definition of the 3′ sides of V gene segments and 5′ sides of J gene segments, resulting in an overestimation of

junctional region nucleotide insertion and underestimation of junctional region nucleotide deletion. Nevertheless, evaluation of junctional region diversity at the protein level is probably a better way of studying antigen receptor diversity, because it includes the newly formed amino acid residues at the V gene segment/junctional region and junctional region/J gene segment boundaries. Also, in this way, out-of-frame rearrangements are excluded and a more accurate estimation of the actual repertoire is achieved (13-15,17,19,20,22,23,25,27,30,33,38,42,57,62,63). Therefore, protein junctional region diversity should be analyzed *in addition* to the genomic junctional region diversity.

Incorrect identification of P-region nucleotides might occur by neglecting the guideline that P-region nucleotides can only be found next to a rearranged gene segment, if that side of the gene segment is in its full coding sequence (39). Such incorrect nucleotide assignments result in an overestimation of P-region nucleotides and underestimation of N-region nucleotides, but has no effect on the calculation of the total genomic junctional diversity or the translated protein junctional diversity.

Applying the guidelines for unravelling genomic junctional regions summarized here not only allows the identification of the various elements of TcR junctional regions, but also those of Ig junctional regions. However, like in TcR-α and TcR-β genes, the 3' germline sequences of most IgV genes are not yet known. In addition, the guidelines for identification of TcR D-gene-derived nucleotides cannot directly be applied to Ig DH gene derived nucleotides, because germline Ig DH gene segments are essentially longer (12-30 nucleotides) than TcR D gene segments (64). This, on the other hand, facilitates the positive recognition of DH-gene-derived nucleotides as compared to the shorter Dβ and Dδ gene segments. Therefore, in the case of IgH junctional regions, the guideline for identification of DH-gene-derived nucleotides can be modified to a maximum demand of seven successive aligned nucleotides for identification of DH gene segments with germline sequences containing more than 20 nucleotides.

The general guidelines for unravelling junctional region sequences described here may prevent mistakes in the identification of junctional region components and thus lead to a more accurate estimation of the actual junctional diversity. Applying these guidelines on numerous TcR gene rearrangements provided insight in the genomic junctional diversity of these genes, which is obviously influenced by preferential G/C insertion by the enzyme TdT and by selection at the protein level, either in the thymus or in the periphery. The selection processes are even more apparent when the amino acid contents of various types of junctional regions are evaluated and some residues appear to be present in higher or lower frequencies. To date junctional regions of TcR molecules with a presumed antigen specificity are predominantly investigated for amino acid motifs (62). However, typifying the occurrence of individual amino acid residues may also provide important information about specific junctional regions and selection processes. The guidelines described in this paper and the summary of germline sequences hopefully contribute to uniformity in the analysis of junctional regions and may initiate new insights in the complexity of receptor specificity.

Note added: The regions of TcR-α and TcR-β V gene segments are currently being sequenced and parts of the germline of the Vβ gene segments are already published (83).

ACKNOWLEDGEMENTS. The authors gratefully acknowledge Prof. Dr. R. Benner and Dr. H. Hooijkaas for their continuous support; Ms. I.L.M. Wolvers-Tettero and Ms. E.J. Mol for their technical assistance; Mr. T.M. van Os for excellent assistance in the preparation of the figures; and Ms. A.D. Korpershoek for her secretarial support.

REFERENCES

1. Toyonaga B, Mak TW. Genes of the T-cell antigen receptor in normal and malignant T cells. Ann Rev Immunol 1987;5:585-620.
2. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988;334:395-402.
3. Raulet DH. The structure, function, and molecular genetics of the γ/δ T cell receptor. Annu Rev Immunol 1989;7:175-207.
4. Schatz DG, Oettinger MA, Schlissel MS. V(D)J recombination: molecular biology and regulation. Annu Rev Immunol 1992;10:359-383.
5. Toyonaga B, Yoshikai Y, Vadasz V, Chin B, Mak TW. Organization and sequences of the diversity, joining, and constant region genes of the human T-cell receptor β chain. Proc Natl Acad Sci USA 1985;82:8624-8628.
6. Loh EY, Cwirla S, Serafini AT, Phillips JH, Lanier LL. Human T-cell-receptor δ chain: genomic organization, diversity, and expression in populations of cells. Proc Natl Acad Sci USA 1988;85:9714-9718.
7. Lafaille JJ, DeCloux A, Bonneville M, Takagaki Y, Tonegawa S. Junctional Sequences of T cell receptor γδ genes: implications for γδ T cell lineages and for a novel intermediate of V-(D)-J joining. Cell 1989;59:859-870.
8. Meier JT, Lewis SM. P nucleotides in V(D)J recombination: a fine-structure analysis. Mol Cell Biol 1993;13:1078-1092.
9. Alt FW, Baltimore D. Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-JH fusions. Proc Natl Acad Sci USA 1982;79:4118-4122.
10. Breit TM, Wolvers-Tettero ILM, Van Dongen JJM. Unique selection determinant in polyclonal Vδ2-Jδ1 junctional regions of human peripheral γδ T-lymphocytes. J Immunol 1994;152:2860-2864.
11. Loh EY, Elliott JF, Cwirla S, Lanier LL, Davis MM. Polymerase chain reaction with single-sided specificity: analysis of T cell receptor δ chain. Science 1989;243:217-220.
12. Tamura N, Holroyd KJ, Banks T, Kirby M, Okayama H, Crystal RG. Diversity in junctional sequences associated with the common human Vγ9 and Vδ2 gene segments in normal blood and lung compared with the limited diversity in a granulomatous disease. J Exp Med 1990;172:169-181.
13. De Libero G, Casorati G, Giachino C, Carbonara C, Migone N, Matzinger P, Lanzavecchia A. Selection by two powerful antigens may account for the presence of the major population of human peripheral γ/δ T cells. J Exp Med 1991;173:1311-1322.
14. Ohmen JD, Barnes PF, Uyemura K, Lu S, Grisso CL, Modlin RL. The T cell receptors of human γδ T cells reactive to *Mycobacterium tuberculosis* are encoded by specific V genes but diverse V-J junctions. J Immunol 1991;147:3353-3359.
15. Panchamoorthy G, McLean J, Modlin RL, Morita CT, Ishikawa S, Brenner MB, Band H. A predominance of the T cell receptor Vγ2/Vδ2 subset in human Mycobacteria-responsive T cells suggests germline gene encoded recognition. J Immunol 1991;147:3360-3369.
16. Uyemura K, Deans RJ, Band H, Ohmen J, Panchamoorthy G, Morita CT, Rea TH, Modlin RL. Evidence for clonal selection of γ/δ T cells in response to a human pathogen. J Exp Med 1991;174:683-692.
17. Yoshikai Y, Kimura N, Toyonaga B, Mak TW. Sequences and repertoire of human T cell receptor α chain variable region genes in mature T lymphocytes. J Exp Med 1986;164:90-103.
18. Klein MH, Concannon P, Everett M, Kim LDH, Hunkapiller T, Hood L. Diversity and structure of human T-cell receptor α-chain variable region genes. Proc Natl Acad Sci USA 1987;84:6884-6888.
19. Giegerich G, Pette M, Meinl E, Epplen JT, Wekerle H, Hinkkanen A. Diversity of human T cell receptor α and β chain genes expressed by human T cells specific for similar myelin basic protein peptide/major histocompatibility complexes. Eur J Immunol 1992;22:753-758.
20. Porcelli S, Yockey CE, Brenner MB, Balk SP. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood $CD4^{-}8^{-}$ α/β T cells demonstrates preferential use of several Vβ genes and an invariant TCR α chain. J Exp Med 1993;178:1-16.
21. Concannon P, Pickering LA, Kung P, Hood L. Diversity and structure of human T-cell receptor β-chain variable region genes. Proc Natl Acad Sci USA 1986;83:6598-6602.
22. Kimura N, Toyonaga B, Yoshikai Y, Triebel F, Debre P, Minden MD, Mak TW. Sequences and diversity of human T cell receptor β chain variable regions genes. J Exp Med 1986;164:739-750.
23. Leiden JM, Strominger JL. Generation of diversity of the β chain of the human T-lymphocyte receptor for antigen. Proc Natl Acad Sci USA 1986;83:4456-4460.
24. Tillinghast JP, Behlke MA, Loh DY. Structure and diversity of the human T-cell receptor β-chain variable region genes. Science 1986;233:879-883.
25. Bragado R, Lauzurica P, López D, López de Castro JA. T cell receptor Vβ gene usage in a human alloreactive response: shared structural features among HLA-B27-specific T cell clones. J Exp Med 1990;171:1189-1204.

26. Ferradini L, Roman-Roman S, Azocar J, Michalaki H, Triebel F, Hercend T. Studies on the human T cell α/β variable region genes: II. Identification of four additional Vβ subfamilies. Eur J Immunol 1991;21:935-942.
27. Rosenberg WMC, Moss PAH, Bell JI. Variation in human T cell receptor Vβ and Jβ repertoire: analysis using anchor polymerase chain reaction. Eur J Immunol 1992;22:541-549.
28. Van Kerckhove C, Russell GJ, Deusch K, Reich K, Bhan AT, DerSimonian H, Brenner MB. Oligoclonality of human intestinal intraepithelial T cells. J Exp Med 1992;175:57-63.
29. Breit TM, Wolvers-Tettero ILM, Hählen K, Van Wering ER, Van Dongen JJM. Extensive junctional diversity of γδ T-cell receptors expressed by T-cell acute lymphoblastic leukemias: implications for the detection of minimal residual disease. Leukemia 1991;5:1076-1086.
30. Delfau M-H, Hance AJ, Lecossier D, Vilmer E, Grandchamp B. Restricted diversity of Vγ9-JP rearrangements in unstimulated human γ/δ T lymphocytes. Eur J Immunol 1992;22:2437-2443.
31. Kohsaka H, Chen PP, Taniguchi A, Ollier WER, Carson DA. Regulation of the mature human T cell receptor γ repertoire by biased V-J gene rearrangement. J Clin Invest 1993;91:171-178.
32. Bender A, Heckl-Östreicher B, Grondal EJM, Kabelitz D. Clonal specificity of human γδ T cells: Vγ9+ T-cell clones frequently recognize *Plasmodium falciparum* merozoites, *Mycobacterium tuberculosis*, and group-A Streptococci. Int Arch Allergy Immunol 1993;100:12-18.
33. Flanagan BF, Wheatcroft NJ, Thornton SM, Christmas SE. T cell receptor junctional regions of Vγ9$^+$/Vδ2$^+$ T cell clones in relation to non-MHC restricted cytotoxic activity. Mol Immunol 1993;30:659-667.
34. Breit TM, Wolvers-Tettero ILM, Beishuizen A, Verhoeven M-AJ, Van Wering ER, Van Dongen JJM. Southern blot patterns, frequencies and junctional diversity of T-cell receptor δ gene rearrangements in acute lymphoblastic leukemia. Blood 1993;82:3063-3074.
35. Koning F, Knot M, Wassenaar F, Van den Elsen P. Phenotypical heterogeneity among human T cell receptor γ/δ-expressing clones derived from peripheral blood. Eur J Immunol 1989;19:2099-2105.
36. Takihara Y, Reimann J, Michalopoulos E, Ciccone E, Moretta L, Mak TW. Diversity and structure of human T cell receptor δ chain genes in peripheral blood γ/δ-bearing T lymphocytes. J Exp Med 1989;169:393-405.
37. Van der Stoep N, De Krijger R, Bruining J, Koning F, Van den Elsen P. Analysis of early fetal T-cell receptor δ chain in humans. Immunogenetics 1990;32:331-336.
38. Sioud M, Førre Ø, Natvig JB. T cell receptor δ diversity of freshly isolated T lymphocytes in rheumatoid synovitis. Eur J Immunol 1991;21:239-241.
39. McVay LD, Carding SR, Bottomly K, Hayday AC. Regulated expression and structure of T cell receptor γ/δ transcripts in human thymic ontogeny. EMBO J 1991;10:83-91.
40. Yokota S, Hansen-Hagge TE, Bartram CR. T-cell receptor δ gene recombination in common acute lymphoblastic leukemia: preferential usage of Vδ2 and frequent involvement of the Jα cluster. Blood 1991;77:141-148.
41. Bucht A, Söderström K, Hultman T, Uhlén M, Nilsson E, Kiessling R, Grönberg A. T cell receptor diversity and activation markers in the Vδ1 subset of rheumatoid synovial fluid and peripheral blood T lymphocytes. Eur J Immunol 1992;22:567-574.
42. Olive C, Gatenby PA, Serjeantson SW. Evidence for oligoclonality of T cell receptor δ chain transcripts expressed in rheumatoid arthritis patients. Eur J Immunol 1992;22:2587-2593.
43. Griesinger F, Grümayer ER, Van Ness B, Kersey JH. Rearrangement and diversification of T-cell receptor delta genes in acute lymphoblastic leukemia. Leukemia 1992;6:1054-1062.
44. Beldjord K, Beldjord C, Macintyre E, Even P, Sigaux F. Peripheral selection of Vδ1$^+$ cells with restricted T cell receptor δ gene junctional repertoire in the peripheral blood of healthy donors. J Exp Med 1993 178:121-127.
45. Forrester JM, Newman LS, Wang Y, King TE, Kotzin BL. Clonal expansion of lung Vδ1$^+$ T cells in pulmonary sarcoidosis. J Clin Invest 1993;91:292-300.
46. Huck S, Dariavach P, Lefranc M-P. Variable region genes in the human T-cell rearranging gamma (TRG) locus: V-J junction and homology with the mouse genes. EMBO J 1988;7:719-726.
47. Ikuta K, Ogura T, Shimizu A, Honjo T. Low frequency of somatic mutation in β-chain variable region genes of human T-cell receptors. Proc Natl Acad Sci USA 1985;82:7701-7705.
48. Yoshikai Y, Clark SP, Taylor S, Sohn U, Wilson BI, Minden MD, Mak TW. Organization and sequences of the variable, joining and constant region genes of the human T-cell receptor α-chain. Nature 1985;316:837-840.
49. Siu G, Strauss EC, Lai E, Hood LE. Analysis of a human Vβ gene subfamily. J Exp Med 1986;164:1600-1614.
50. Satyanarayana K, Hata S, Devlin P, Roncarolo MG, De Vries JE, Spits H, Strominger JL, Krangel MS. Genomic organization of the human T-cell antigen-receptor α/δ locus. Proc Natl Acad Sci USA 1988;85:8166-8170.
51. Wilson RK, Lai E, Kim LDH, Hood L. Sequence and expression of a novel human T-cell receptor β-chain variable gene segment subfamily. Immunogenetics 1990;32:406-412.
52. Li Y, Szabo P, Posnett DN. The genomic structure of human Vβ6 T cell antigen receptor genes. J Exp Med 1991;174:1537-1547.
53. Robinson MA. The human T cell receptor β-chain gene complex contains at least 57 variable gene segments: identification of six Vβ genes in four new gene families. J Immunol 1991;146:4392-4397.
54. Roman-Roman S, Ferradini L, Azocar J, Genevée C, Hercend T, Triebel F. Studies on the human T cell receptor α/β variable region genes: I. Identification of 7 additional Vα subfamilies and 14 Jα gene segments. Eur J Immunol 1991;21:927-933.
55. Charmley P, Wang K, Hood L, Nickerson DA. Identification and physical mapping of a polymorphic human T cell receptor Vβ gene with a frequent null allele. J Exp Med 1993;177:135-143.
56. Wilson RK, Lai E, Concannon P, Barth RK, Hood LE. Structure, organization and polymorphism of murine and human T-cell receptor α and β chain gene families. Immunol Rev 1988;101:149-172.

57. Macintyre E, d'Auriol L, Amesland F, Loiseau P, Chen Z, Boumsell L, Galibert F, Sigaux F. Analysis of junctional diversity in the preferential Vδ1-Jδ1 rearrangement of fresh T-acute lymphoblastic leukemia cells by in vitro gene amplification and direct sequencing. Blood 1989;74:2053-2061.
58. Desiderio SV, Yancopoulos GD, Paskind M, Thomas E, Boss MA, Landau N, Alt FW, Baltimore D. Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxytransferase in B cells. Nature 1984;311:752-755.
59. Breit TM, Mol EJ, Wolvers-Tettero ILM, Ludwig W-D, Van Wering ER, Van Dongen JJM. Site-specific deletions involving the *tal*-1 and *sil* genes are restricted to cells of the T-cell receptor αβ lineage: T-cell receptor δ gene deletion mechanism affects multiple genes. J Exp Med 1993;177:965-977.
60. Parker CM, Groh V, Band H, Porcelli SA, Morita C, Fabbi M, Glass D, Strominger JL, Brenner MB. Evidence for extrathymic changes in the T cell receptor γ/δ repertoire. J Exp Med 1990;171:1597-1612.
61. Quertermous T, Strauss W, Murre C, Dialynas DP, Strominger JL, Seidman JG. Human T-cell γ genes contain *N* segments and have marked junctional variability. Nature 1986;322:184-187.
62. Olive C, Gatenby PA, Serjeantson SW. Molecular characterization of the Vγ9 T cell receptor repertoire expressed in patients with rheumatoid arthritis. Eur J Immunol 1992;22:2901-2906.
63. Oksenberg JR, Panzara MA, Begovich AB, Mitchell D, Erlich HA, Murray RS, Shimonkevitz R, Sherritt M, Rothbard J, Bernard CCA, Steinman L. Selection for T-cell receptor Vβ-Dβ-Jβ gene rearrangements with specificity for a myelin basic protein peptide in brain lesions of multiple sclerosis. Nature 1993;362:68-70.
64. Ichihara Y, Matsuoka H, Kurosawa Y. Organization of human immunoglobulin heavy chain diversity gene loci. EMBO J 1988;7:4141-4150.
65. Shimonkevitz R, Colburn C, Burnham JA, Murray RS, Kotzin BL. Clonal expansions of activated γ/δ T cells in recent-onset multiple sclerosis. Proc Natl Acad Sci USA 1993;90:923-927.
66. Lefranc M-P, Forster A, Rabbitts TH. Rearrangement of two distinct T-cell γ-chain variable-region genes in human DNA. Nature 1986;319:420-422.
67. Bories J-C, Guglielmi P, Sigaux F, Bensussan A. Nucleotide sequence of a cDNA corresponding to a new human variable region of a functionally rearranged T cell receptor gamma chain. Nucleic Acids Res 1987;15:10059.
68. Chen Z, Font MP, Loiseau P, Bories JC, Degos L, Lefranc M-P, Sigaux F. The human T-cell Vγ gene locus: cloning of new segments and study of Vγ rearrangements in neoplastic T and B cells. Blood 1988;72:776-783.
69. Lefranc M-P, Chuchana P, Dariavach P, Nguyen C, Huck S, Brockly F, Jordan B, Lefranc G. Molecular mapping of the human T cell receptor gamma (TRG) genes and linkage of the variable and constant regions. Eur J Immunol 1989;19:989-994.
70. Guglielmi P, Davi F, d'Auriol L, Bories J-C, Dausset J, Bensussan A. Use of a variable α region to create a functional T-cell receptor δ chain. Proc Natl Acad Sci USA 1988;85:5634-5638.
71. De Villartay J-P, Hockett RD, Coran D, Korsmeyer SJ, Cohen DI. Deletion of the human-T-cell receptor δ-gene by a site-specific recombination. Nature 1988;335:170-174.
72. Hata S, Clabby M, Devlin P, Spits H, De Vries JE, Krangel MS. Diversity and organization of human T cell receptor δ variable gene segments. J Exp Med 1989;169:41-57.
73. Dariavach P, Lefranc M-P. First genomic sequence of the human T-cell receptor δ2 gene (TRDV2). Nucleic Acids Res 1989;17:4880.
74. Koop, BF, Row L , Wang K, Kuo CL, Seto D, Lenstra JA, Howard S, Shan W, Wilke E, Hood L. The human T-cell receptor Cα/Cδ region: organization, sequence and evolution of 97.6kb of DNA. Genomics 1994;19:478-493.
75. LeFranc M-P, Forster A, Baer R, Stinson MA, Rabbitts TH. Diversity and rearrangement of the human T cell rearranging γ genes: nine germ-line variable genes belonging to two subgroups. Cell 1986;45:237-246.
76. Quertermous T, Strauss WM, Van Dongen JJM, Seidman JG. Human T cell γ chain joining regions and T cell development. J Immunol 1987;138:2687-2690.
77. Huck S, Lefranc M-P. Rearrangements to the JP1, JP and JP2 segments in the human T-cell rearranging gamma gene (TCRγ) locus. FEBS Lett 1987;224:291-296.
78. Boehm T, Baer R, Lavenir I, Forster A, Waters JJ, Nacheva E, Rabbitts TH. The mechanism of chromosomal translocation t(11;14) involving the T-cell receptor Cδ locus on human chromosome 14q11 and a transcribed region of chromosome 11p15. EMBO J 1988;7:385-394.
79. Boehm T, Buluwela L, Williams D, White L, Rabbitts TH. A cluster of chromosome 11p13 translocations found via distinct D-D and D-D-J rearrangements of the human T cell receptor δ chain gene. EMBO J 1988;7:2011-2017.
80. Takihara Y, Tkachuk D, Michalopoulos E, Champagne E, Reimann J, Minden M, Mak TW. Sequence and organization of the diversity, joining, and constant region genes of the human T-cell δ-chain locus. Proc Natl Acad Sci USA 1988;85:6097-6101.
81. Davodeau F, Peyrat M-A, Hallet M-M, Vié H, Bonneville M. Characterization of a new functional TCR Jδ segment in humans: evidence for a marked conservation of Jδ sequences between humans, mice, and sheep. J Immunol 1994;153:137-142.
82. Koop BF, Row L, Wang K, Kuo CL, Seto D, Lenstra JA, Havard S, Shan W, Wilke E, Hood L. The human T-cell receptor Cα/Cδ region: organization, sequence and evolution of 97.6 kb of DNA. Genomics 1994;19:478-493.
83. Slightom JL, Siemieniak DR, Sieu LC, Koop BF, Hood L. Nucleotide sequence analysis of 77.7 kb of the human V(beta) T-cell receptor gene locus-direct primer-walking using cosmid template DNAs. Genomics 1994;20:149-168.

CHAPTER 2.4

EXTENSIVE JUNCTIONAL DIVERSITY OF $\gamma\delta$ T-CELL RECEPTORS EXPRESSED BY T-CELL ACUTE LYMPHOBLASTIC LEUKEMIAS: implications for the detection of minimal residual disease*

Timo M. Breit[1], Ingrid L.M. Wolvers-Tettero[1], Karel Hählen[2,3], Elisabeth R. van Wering[3], and Jacques J.M. van Dongen[1]

1. Department of Immunology, University Hospital Dijkzigt/Erasmus University, Rotterdam; 2. Department of Pediatrics, Subdivision of Hematology-Oncology, Sophia Children's Hospital/Erasmus University, Rotterdam; 3. Dutch Childhood Leukemia Study Group, The Hague, The Netherlands.

ABSTRACT

Rearrangements, junctional regions and expression of TcR-γ and TcR-δ genes were analyzed in thirteen TcR-$\gamma\delta^+$ T-ALL. All TcR-γ genes were rearranged except for one deleted allele and one allele in germline configuration. The expressed TcR-γ protein chains showed a preference for VγI (11/13), Jγ2.3 (7/13) and Cγ2 (8/13). Furthermore, the TcR-γ combinatorial repertoire appears to be even more limited by the fact that particular Vγ genes are preferentially used in TcR-γ1 or TcR-γ2 derived receptors, i.e. in disulfide-linked or non-disulfide-linked TcR-$\gamma\delta$ receptors. The combinatorial repertoire of the expressed TcR-δ genes was homogeneous, as all thirteen T-ALL expressed Vδ1-Dδ-Jδ1-Cδ protein chains. In contrast to the limited combinatorial repertoire of the TcR-γ and TcR-δ genes, the junctional diversity of both TcR genes was extensive due to insertion of N-region, P-region, and Dδ gene nucleotides in addition to deletion of nucleotides by trimming. Using PCR-mediated amplification and subsequent direct sequencing, we determined the junctional region sequences of all TcR-γ and TcR-δ rearrangements. Sequence analysis showed that in the TcR-γ junctional regions insertion varied from 0 to 25 nucleotides (average 8.0) and deletion from 1 to 27 nucleotides (average 8.7). In TcR-δ junctional regions, nucleotide insertion varied from 5 to 47 nucleotides (average 25.6) and deletion from 0 to 29 nucleotides (average 6.2) per junctional region. In general, the N-region nucleotides were the most prominent element in the junctional regions, i.e. 97% of the TcR-γ and 55% of the TcR-δ junctional regions. Dδ genes contributed significantly (40%) to the TcR-δ junctional diversity, whereas P-regions were hardly found in both TcR genes. Altogether, the junctional regions of TcR-δ genes were far more diverse than the junctional regions of TcR-γ genes. With respect to the new methods of detecting MRD in lymphoid malignancies utilizing PCR-mediated amplification of the junctional regions of TcR genes, our data indicate that this MRD-PCR analysis will generally be more sensitive if TcR-δ instead of TcR-γ junctional-region-specific probes are used.

* Published in: Leukemia 1991;5:1076-1086.

INTRODUCTION

Two types of antigen specific TcR exist: the classical TcR-$\alpha\beta$, which is expressed on the majority ($\geq$85%) of the mature lymphocytes in PB and the alternative TcR-$\gamma\delta$ which is expressed on only a small fraction ($\leq$15%) of the PB lymphocytes (1-4). The TcR molecule consists of two different glycoproteins (TcR-α and TcR-β or TcR-γ and TcR-δ), which generally are disulfide-linked and non-covalently associated with the signal-transducing CD3 protein complex (1-4). Each chain of the TcR heterodimer contains a variable antigen-recognizing region and a C region. The variable region is encoded by a V gene segment, a J gene segment and a junctional region linking the V and J gene segments together (1,3,4). This junctional region includes D gene segments in case of TcR-β and TcR-δ genes (1,3-6).

The TcR molecules of different T-lymphocytes are diverse, because different combinations of V (,D) and J gene segments and different junctional regions are obtained via gene rearrangement processes during early T-cell differentiation (1,3,4,7,8). TcR-α and TcR-β genes contain large series of functional V ($>$50 and $>$70, respectively) and J gene segments ($\sim$55 and 13, respectively) and two D gene segments in the case of TcR-β genes (5,9-13). However, the TcR-γ and TcR-δ genes contain only a few functional V (8 and $\sim$6, respectively) and J gene segments (5 and 3, respectively) and three D gene segments in the case of TcR-δ genes (6,14-23). The available V (,D) and J gene segments determine the potential combinatorial diversity. It is therefore obvious that TcR-$\alpha\beta$ has a far greater potential combinatorial diversity than TcR-$\gamma\delta$. The actual TcR-$\gamma\delta$ combinatorial diversity is even more limited by the fact that the available V and J gene segments are used in preferential patterns. For instance, $\sim$85% of the TcR-$\gamma\delta^+$ T-lymphocytes in PB express receptors consisting of a Vγ9-Jγ1.2-Cγ1 chain disulfide-linked to a Vδ2-Jδ1-Cδ chain (24-28), whereas $\sim$60% of the thymocytes express a Vδ1-Jδ1-Cδ chain, generally non-disulfide-linked to a TcR-γ chain (25,27,28). The presence or absence of the disulfide bond in TcR-$\gamma\delta$ is dependent on whether the TcR-γ chain is derived from the Cγ1 or Cγ2 gene segment, respectively (29-32).

The junctional region, which determines the junctional diversity, consists of D-gene-derived nucleotides in the case of TcR-β and TcR-δ genes (5,6,11), a region of a randomly inserted nucleotides, i.e. N-region nucleotides (10,11,33,34), a region of nucleotides forming a palindromic sequence with the juxtaposed nucleotides of an untrimmed gene segment i.e. P-region nucleotides (35), and deletion of nucleotides by trimming of the ends of the involved gene segments. Because it is difficult to distinguish Dδ nucleotides in the junctional region from N-region and P-region nucleotides, nucleotides are assumed to be 'Dδ-derived' if at least three successive junctional region nucleotides can be aligned with one of the three Dδ gene germline sequences (6,34). N-regions consist of nucleotides that are randomly inserted by the enzyme TdT at the junctions of gene segments during the rearrangement process (5,6,10,11,23,33,34,36-39). To identify the recently described P-regions, basically three conditions have to be fulfilled, as proposed by Lafaille *et al.* (35): P-region nucleotides can only be found next to a rearranging gene segment if that side of the gene segment is in its full coding sequence (i.e. without trimming); a P-region maximally consists of one or two nucleotides; and P-region nucleotides can be recognized by the palindromic nature of the P-region in combination with the juxtaposed nucleotides of the rearranging gene segment. Trimming of the rearranging gene segments resulting in deletion

of nucleotides is probably caused by exonucleolytic nibbling which occurs randomly at the ends of most involved gene segments (6,10,11,33-39). Because trimming can be extensive, this phenomenon considerably contributes to the total junctional diversity. In contrast to the combinatorial diversity, the junctional diversity of the TcR-γ and TcR-δ genes greatly exceeds that of the TcR-α and TcR-β genes. One may therefore speculate that the extensive junctional diversity of $\gamma\delta$ receptors compensates for their limited combinatorial diversity.

An analysis is presented of thirteen TcR-$\gamma\delta^+$ T-ALL, ten of which have previously been partly described (40). All thirteen TcR-$\gamma\delta^+$ T-ALL were studied for their phenotype by extensive marker analysis and for their combinatorial diversity by Southern blot analysis. In this study we focussed on the junctional diversity of the rearranged TcR-γ and TcR-δ genes, which were analyzed by PCR-mediated amplification and direct sequencing of the junctional regions.

MATERIALS AND METHODS

Cell samples

Cell samples were obtained from thirteen different TcR-$\gamma\delta^+$ T-ALL patients at initial diagnosis. MNC were isolated from PB and BM by Ficoll-Paque (density: 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. The cell samples were frozen and stored in liquid nitrogen. Immunophenotypic and genotypic data of the TcR-$\gamma\delta^+$ T-ALL have been described previously (40), except for patients JR, PB and JM. The TcR-$\gamma\delta^+$ cell line PEER was used as a positive control for PCR-mediated amplification and direct sequencing of the junctional regions (30,41).

Immunologic marker analysis

The MNC of the T-ALL patients were analyzed for the nuclear expression of TdT, for the cell membrane expression of the T-cell markers CD1 (66IIC7), CD2 (Leu-5b), CD3 (Leu-4), CD4 (Leu-3a), CD5 (Leu-1), CD6 (OKT17), CD7 (3A1), and CD8 (Leu-2a), for the HLA-DR antigen and for reactivity with the McAb, WT31 (anti-TcR-$\alpha\beta$), BMA031 (anti-TcR-$\alpha\beta$), 11F2 (anti-TcR-$\gamma\delta$), TCRδ1 (anti-TcR-δ), Ti-γA (anti-TcR-Vγ9), δTCS1 (anti-TcR-Vδ1), and BB3 (anti-TcR-Vδ2) (24-28,40,42). The rabbit anti-TdT antiserum was purchased from Supertechs (Bethesda, MD, USA); the McAb of the Leu series, anti-HLA-DR, WT31 and 11F2 were obtained from Becton Dickinson (San Jose, CA, USA); the CD1 antibody was obtained from Monosan/Sanbio (Nistelrode, The Netherlands); OKT17 from Ortho Diagnostic Systems (Raritan, NJ, USA); the 3A1 hybridoma from the American Type Culture Collection (Rockville, MD, USA); TCRδ1 and δTCS1 were obtained from T Cell Sciences (Cambridge, MA, USA). McAb BMA031, Ti-γA and BB3 were kindly provided by Dr. R. Kurrle (Behring, Marburg, Germany), Dr. T. Hercend (Villejuif, France), and Dr. L. Moretta (Genova, Italy), respectively. Immunofluorescence stainings were performed as described and evaluated with Zeiss fluorescence microscopes (Carl Zeiss, Oberkochen, Germany) and/or a FACScan flowcytometer (Becton Dickinson) (42).

Southern blot analysis

DNA was isolated from frozen MNC as described previously (8,43). A 15 μg sample was digested with the appropriate restriction enzymes (Pharmacia), size-fractionated in 0.7% agarose gels and transferred to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) as described (8,43). The TcR-γ gene rearrangements were analyzed by use of the VγI, Jγ1.2, Jγ1.3, Jγ2.1 and Cγ probes (14,15) in *Bam*HI, *Bgl*II, *Eco*RI and *Kpn*I digests. The configuration of the TcR-δ genes was analyzed by use of the Vδ1, Vδ2, Vδ3, Dδ1, Dδ3, Jδ1, Jδ2 and Cδ probes (6,20,21,44,45) in *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII and *Kpn*I digests.

PCR amplification analysis

PCR was essentially performed as described previously (43). A 0.1 μg sample of DNA, 25 pmol of the 5′ and the 3′ oligonucleotide primer and 1 unit of *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA) were used in each PCR reaction. The oligonucleotide primers are listed in Table 1. These oligonucleotides were synthesized according to published TcR-δ gene sequences (6,22,46-48) and TcR-γ gene sequences (14,15,17,49) on a 381A DNA synthesizer (Applied Biosystems, Forster City, CA, USA) using the solid-phase phosphotriester method. The PCR reaction mixture was incubated at 94°C for 3 min, at 55°C for 2 min and at 72°C for 3 min in a thermal cycler (Perkin-Elmer Cetus). Following this initial cycle, denaturing, annealing and extension steps were performed for another 29 cycles at 94°C for 1 min, at 55°C for 1 min and at 72°C for 3 min, respectively. After the last cycle an additional extension step of 72°C for 7 min was executed. Short Vδ1-3′S ↔ Jδ1-5′BF PCR products were size-fractionated by 10% PAGE and visualized by ethidium bromide staining.

Direct sequencing analysis

One μl of the original PCR product, 5 pmol of the limiting primer, 250 pmol of the opposite primer and 5 units of *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus) were used in each asymmetric PCR reaction of 500 μl. The reaction mixture was incubated for a total of 25 to 30 cycles with the above-described regular temperature cycles. After the asymmetric amplification, the PCR product was precipitated twice in 50% ethanol (plus 0.1 vol. 2 M NaAc, pH 5.6). The dried pellet was resolved in 22 μl H_2O, half of which was used in the sequence reaction. Fifty pmol sequence primer was used in each reaction (sequence primers are indicated in Table 1). The sequence reactions were performed with the T7-sequencing kit (Pharmacia)

TABLE 1. Oligonucleotide primers for PCR and direct sequencing analysis of the TcR-γ and TcR-δ genes.

Code[a]	PCR/Seq[b]	Sequence[c]	Reference[d]
TcR-δ genes			
Vδ1-5′XBg	PCR	GAAGATCTAGACTCAAGCCCAGTCATCAGTATCC	46
Vδ1-3′S	PCR/Seq	CGCGTCGACGCCTTAACCATTTCAGCCTTAC	46
Vδ2-5′S	PCR	CGCGTCGACCAAACAGTGCCTGTGTCAATAGG	47
Vδ2-3′S	Seq	CGCGTCGACCTGGCTGTACTTAAGATACTTGC	47
Vδ3-5′S	PCR	CGCGTCGACCAGACGGTGGCGAGTGGC	22
Vδ3-3′S	Seq	CGCGTCGACTTGGTGATCTCTCCAGTAAGG	22
Dδ1-5′S	PCR	CGCGTCGACTCCATGTTCAAATAGATATAGTATT	6
Dδ2-seq	Seq	CGGGTGGTGATGGCAAAGTGCC	
Dδ3-3′XBg	PCR/Seq	GTAGATCTAGAAATGGCACTTTTGCCCCTGCAG	46
Jδ1-5′BF	PCR	CACGGGATCCTTTTCCAAGGATGAG	46
Jδ1-3′XBg	PCR	GAAGATCTAGACCTCTTCCCAGGAGTCCTCC	46
Jδ1-seq	Seq	CCTTAGATGGAGGATGCCTTAACC	46
δREC-5′X	PCR	TGCTCTAGATCTTCAAGGGTCGAGACTGTC	48
δREC-seq	Seq	ATGAAATTTATGAACACATGCTGAGG	48
TcR-γ genes			
VγI-5′XBg	PCR	GAAGATCTAGACAGGCCGACTGGGTCATCTGC	14
VγI-seq	Seq	AACTCCAGGGTTGTGTTGGAATCA	14
VγII-5′XBg	PCR	GAAGATCTAGACAGCCCGCCTGGAATGTGTGG	49
VγII-3′S	Seq	CGCGTCGACCATTCACAAGGTAGAGAAACAGG	49
VγIV-5′XBg	PCR	CCTGAAAGATCTATTTCTAGACCAGC	17
VγIV-3′	Seq	GGGAGGAAACAGAGGCAGAGCG	17
Jγ1.1/2.1-3′XBg	PCR/Seq	GAAGATCTAGACTTACCAGGTGAAGTTACTATAAGC	15
Jγ1.2-3′	PCR	AAGAAAACTTACCTGTAATGATAAGC	14
Jγ1.3/2.3-3′X	PCR	CTAGTCTAGACCGTATATGCACAAAGCCAGATC	49
Jγ1.3/2.3-5′	Seq	AATGTTGTATTCTTCCGATACTTACC	49

a. 5′ and 3′ indicate the location of the oligonucleotide primer within the gene segment concerned.
b. Oligonucleotide primers used in PCR or sequence analysis are indicated PCR and Seq, respectively.
c. The underlined sequences represent the aspecific sequences of the oligonucleotide primers, which include or generate artificial restriction sites; *Bam*HI (B), *Bgl*II (Bg), *Fok*I (F), *Sal*I (S) and *Xba*I (X).
d. Sequence information used to design the oligonucleotide primers was derived from the indicated literature references, or from our own sequence data in case of Dδ2-seq primer.

following the manufactor's instructions using ^{35}S radiolabeling, and run on a normal, denaturing 8% polyacrylamide sequence gel.

All TcR-junctions were sequenced in both directions. Similar rearrangements on both alleles of one patient (e.g. two Vδ1-Jδ1 rearrangements or a VγI-Jγ1.3 plus VγI-Jγ2.3 rearrangement) resulted in a mixed PCR product as well as mixed sequences. In such cases, we unraveled the mixed sequences by matching information about sequences read from opposite directions. This is based on the fact that a mixture of two junctional region sequences, read from opposite directions, align in a different way if these junctional sequences differ in size, or if they differ in trimming of nucleotides at the ends of the involved gene segments. By taking advantage of this approach, cloning was unnecessary in all our patients, which included three double Vδ1-Jδ1 rearrangements and five double VγI-Jγ1.3/2.3 rearrangements.

RESULTS

Phenotype of the T-ALL

The T-ALL cells of patients JR, PB and JM expressed TcR-γδ (McAb 11F2 and TCRδ1) and the associated CD3 molecule (Leu-4) on the cell membrane. Absence of staining with McAb WT31 and BMA031 showed that they did not express a TcR-αβ. The three T-ALL were positive for TdT and only one patient (JM) was positive for CD1 (66IIC7). The three patients differed in their CD4/CD8 phenotype. Patient JR demonstrated a $CD4^{+}/CD8^{-}$ phenotype, patient PB a $CD4^{-}/CD8^{+}$ phenotype and patient JM a $CD4^{+}/CD8^{+}$ phenotype. All three T-ALL expressed Vδ1 (δTCS1) and did not express Vγ9 (Ti-γA) or Vδ2 (BB3). Detailed information concerning the marker analysis of these three patients and the other ten TcR-$\gamma\delta^{+}$ T-ALL is summarized in Table 2 and Reference 40, respectively.

TcR gene rearrangement studies by Southern blot analysis

TcR-γ gene rearrangements in patients JR, PB and JM involved the Jγ2.3 gene segment on four alleles and the Jγ1.1 gene segment on one allele. The sixth TcR-γ allele (patient PB) was deleted as consequence of an iso(7q) chromosomal aberration (A. Hagemeijer, personal communication), implying the loss of the complete p-arm of chromosome 7, which contains the TcR-γ gene (50,51). In patient JR a VγIV (Vγ11)-Jγ2.3 rearrangement was detected, which was the only TcR-γ rearrangement using a Vγ gene segment not derived from the VγI or VγII gene families. Of the six rearranged TcR-δ genes of patients JR, PB and JM, four rearrangements were Vδ1-Jδ1, one was Vδ2-Jδ1 and one Vδ2-Dδ3. All patients had at least one Vδ1-Jδ1 rearrangement, which confirmed the Vδ1 expression in these patients as detected with the McAb δTCS1. The TcR-γ gene and TcR-δ gene configurations of patients JR, PB and JM are summarized in Table 2 and the information concerning the other ten patients has been described previously (40).

PCR analysis of the size of the Vδ1-Jδ1 junctional regions

For the PCR analysis of the Vδ1-Jδ1 junctional regions of the thirteen T-ALL, the junctions were amplified using the internal Vδ1-3′s and the internal Jδ1-5′BF primer. The short PCR products of ~130 bp obtained were analyzed by 10% PAGE (Figure 1). PAGE revealed significant differences in size between the PCR products, which can only be explained by differences in size of the junctional regions. The PCR products were between ~115 bp (patient EP) and ~155 bp (patient MA), which implies that the Vδ1-Jδ1 junctional regions of the T-ALL differed in size up to ~40 bp. PAGE also clearly showed the predicted

TABLE 2. Immunologic marker analysis and configuration of TcR-γ and TcR-δ genes in three TcR-γδ+ T-ALL[a].

Patient	(Cell Sample)	JR (BM)	PB (PB)	JM (PB)
Immunologic markers[b]				
TdT		+	+	69%
HLA-DR		(L243)	–	18%–
CD1	(66IIC7)	–	–	+
CD2	(Leu-5b)	+	+	+
CD3	(Leu-4)	+	+	+
CD4	(Leu-3A)	23%	–	+
CD5	(Leu-1)	+	+	+
CD6	(OKT17)	–	+	17%
CD7	(3A1)	+	+	+
CD8	(Leu-2A)	–	+	69%
TcR-αß	(WT31)	–	–	NT
TcR-αß	(BMA031)	–	–	–
TcR-γδ	(11F2)	+	+	60%
TcR-δ	(TCRδ1)	+	+	+
TcR-V gene expression[b]				
Vγ9	(Ti-γA)	–	–	–
Vδ1	(δTCS1)	+	+	+
Vδ2	(BB3)	–	–	–
TcR-γ gene configuration[c]				
Jγ1.1 gene segment		D/D	Vγ3/D[d]	D/D
Jγ1.2 gene segment		D/D	G/D[d]	D/D
Jγ1.3 gene segment		D/D	G/D[d]	D/D
Jγ2.1 gene segment		D/D	G/D[d]	D/D
Jγ2.3 gene segment		Vγ4/Vγ11	G/D[d]	Vγ4/Vγ8
TcR-δ gene configuration[c]				
Dδ3 gene segment		D/D	D/D	Vδ2/D
Jδ1 gene segment		Vδ1/Vδ1	Vδ1/Vδ2	G/Vδ1
Jδ2 gene segment		G/G	G/G	G/G
Jδ3 gene segment		G/G	G/G	G/G
Cδ gene segment		G/G	G/G	G/G

a. Detailed information about the other ten TcR-γδ+ T-ALL has been published previously (40). Abbreviations used in this table: PB, peripheral blood; BM, bone marrow; NT, not tested.

b. Immunologic marker analysis: +, ≥75% of the cells are positive; –, ≤15% of the cells are positive; percentages positivity between 15% and 75% are indicated.

c. Southern blot analysis: Interpretation of the results using the five described TcR-γ probes and the eight described TcR-δ probes. G, gene segment in germline configuration; D, deletion of the in volved gene segment.

d. Deletion of this TcR-γ allele was caused by an i(7q) chromosomal aberration (A. Hagemeijer, personal communication).

double Vδ1-Jδ1 rearrangements in patients HZ and MA, but not in patient JR (Figure 1). This indicated that the difference in the sizes of the Vδ1-Jδ1 junctional regions of patient JR was maximally one or two nucleotides. The estimated differences in size of the Vδ1-Jδ1 junctional regions in patient HZ was ~8 bp and in patient MA ~35 bp.

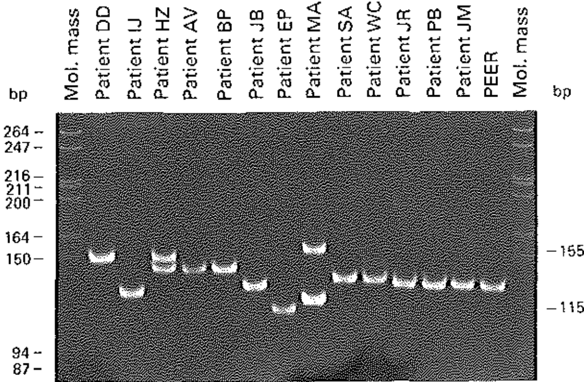


Figure 1. PAGE analysis of short Vδ1-Jδ1 PCR products of the thirteen TcR-$\gamma\delta^+$ T-ALL. The PCR-mediated amplification of the Vδ1-Jδ1 rearrangements was performed using the Vδ1-3′S and the Jδ1-5′BF oligonucleotide primers (Table 1). The PCR products of the double Vδ1-Jδ1 rearrangements in patients HZ and MA are clearly visible. In patient JR the two PCR products show as one band as a result of co-migration (see text). Using the molecular mass markers, the size of the PCR products, indicative for the relative size of the junctional regions, could be measured. The PCR products were between ~115 bp (patient EP) and ~155 bp (patient MA).

TcR-γ junctional region sequences

Direct sequencing of the TcR-γ gene PCR products revealed the junctional region sequences of all TcR-γ rearrangements. Table 3 shows the actual sequences of the TcR-γ junctional regions and Table 5 summarizes the characteristics of the junctional regions in the TcR-$\gamma\delta^+$ T-ALL. From these data it was possible to deduce which of the two rearranged TcR-γ alleles in each patient was in-frame and therefore coding for the expressed TcR-γ protein (Table 6). In only one T-ALL (patient JB) the junctional regions of both rearranged TcR-γ genes appeared to be in-frame. Sequence analysis of large parts of the involved Vγ and Jγ gene segments in this patient did not uncover any mutations leading to a stopcodon. Therefore we could not discriminate between the functional and non-functional allele in patient JB.

The sizes of the junctional regions differed from 0 (patient MA) to maximally 25 nucleotides (patient JM) with an average of 8.0 nucleotides. Only two out of 24 rearrangements did not have any randomly inserted N-region nucleotides, i.e. Vγ8-Jγ2.3 in patient MA and Vγ2-Jγ1.3 in patient JB. The latter junctional region consisted of only one P-region nucleotide. Because Dγ gene segments do not exist, the TcR-γ junctional region can only be composed of N-region and P-region nucleotides. However, hardly any P-region nucleotides were found (5 in total), because extensive trimming at the ends of the V and J gene segments had occurred in most cases. Trimming resulted in deletion of 1 to 27 nucleotides (average 8.7 nucleotides) per junction. Total trimming of the Jγ gene segments (131 deleted nucleotides) was almost twice as much as in case of the Vγ gene segments (77 deleted nucleotides). There were only five complete Vγ and three complete Jγ gene segments (i.e. without trimming). Remarkably, all three rearranged Vγ9 gene segments experienced no trimming (Table 3).

TcR-δ junctional region sequences

The TcR-δ junctional region sequences of all TcR-δ rearrangements in the thirteen TcR-$\gamma\delta^+$ T-ALL are shown in Table 4 (Figure 2). Table 5 summarizes the characteristics of the TcR-δ junctional regions. All patients had only one in-frame TcR-δ gene rearrangement. In all thirteen T-ALL this functional rearrangement was a Vδ1-Jδ1 rearrangement (Table 6). Patient HZ appeared to have two in-frame Vδ1-Jδ1 rearrangements, but one was non-functional due to a generated TAA stopcodon in the junctional region. Two stopcodons

TABLE 3. Junctional region sequences of TcR-γ rearrangements in thirteen TcR-γδ$^+$ T-ALL[a].

Patient		VγI	N region	Jγ1.1/2.1	
	Vγ2	................CG..			
	Vγ3	................C...			
	Vγ4	.................G..			
	Vγ5	..............GC...		...CC.C..G....T.....	Jγ1.1
	Vγ8	ACTGTGCCACCTGGGATAGG		ATAGTAGTGATTGGATCAAG	Jγ2.1
BP	Vγ2	ACTGTGCCACCTGGGACG	CGGAG	TGGTTCAAG	Jγ1.1
JB	Vγ2	ACTGTGCCACCTGGGAC	CT	GTTCAAG	Jγ1.1
WC	Vγ3	ACTGTGCCACCTGGGAC	TTCG	GTGATTGGATCAAG	Jγ2.1
PB	Vγ3	ACTGTGCCACCTGG	T	CTGGTTGGTTCAAG	Jγ1.1
				Jγ1.3/2.3	
				....	Jγ1.3
				GAATTATTATAAGAAACTCT	Jγ2.3
PEER	Vγ8	ACTGTGCCACCTGG	AAGG	ATTATTATAAGAAACTCT	Jγ2.3
DD	Vγ5	ACTGTGCCACCT	CTTTTA	T	Jγ1.3
IJ	Vγ2	ACTGTGCCACCTGGGACG	CACCTACTC	TAAGAAACTCT	Jγ2.3
HZ	Vγ3	ACTGTGCCACCTGGGACAGG	TCCATT	ATTATTATAAGAAACTCT	Jγ2.3
HZ	Vγ4	ACTGTGCCACCTGGGATG	AGGCCCCCCACACCA	ATAAGAAACTCT	Jγ2.3
AV	Vγ3	ACTGTGCCACCTGGG	CCCC	GAATTATTATAAGAAACTCT	Jγ2.3
BP	Vγ4	ACTGTGCCACCTGGG	CGTCCCCTAAAA	TAtaaGAAACTCT	Jγ2.3
JB	Vγ2	ACTGTGCCACCTGGG	C	GAATTATTATAAGAAACTCT	Jγ1.3
EP	Vγ8	ACTGTGCCACCTGGGA	CTCAGGCCCGGG	TAAGAAACTCT	Jγ2.3
EP	Vγ8	ACTGTGCCACCTGGGA	AAAGAGGCTAACAGGGTTT	TTATTAtaaGAAACTCT	Jγ2.3
MA	Vγ8	ACTGTGCCACCTGGGA	CTTCCGAGT	AATTATTATAAGAAACTCT	Jγ2.3
MA	Vγ8	ACTGTGCCACCTGGGA		TTATTATAAGAAACTCT	Jγ2.3
SA	Vγ3	ACTGTGCCACCTGGGACG	AATGTA	ATAAGAAACTCT	Jγ2.3
SA	Vγ8	ACTGTGCCACCTGGGATA	CCCC	ATTAtaaGAAACTCT	Jγ2.3
JR	Vγ4	ACTGTGCCACCTGGGATGGG	ACTACAACTA	ATTATAAGAAACTCT	Jγ2.3
JM	Vγ4	ACTGTGCC	TCTGAACAATCCAAACAAGACCACG	GAATTATTATAAGAAACTCT	Jγ2.3
JM	Vγ8	ACTGTGCCACCTGGGAT	GGA	AATTATTATAAGAAACTCT	Jγ2.3
		VγII			
	Vγ9	ACTGTGCCTTGTGGGAGGTG			
PEER	Vγ9	ACTGTGCCTT	CCGGCCCG	AAGAAACTCT	Jγ2.3
DD	Vγ9	ACTGTGCCTTGTGGGAGGTG	TGT	AATTATTATAAGAAACTCT	Jγ1.3
IJ	Vγ9	ACTGTGCCTTGTGGGAGGTG	CAATCTGGACCCAGGATTATTAAG	AAGAAACTCT	Jγ1.3
AV	Vγ9	ACTGTGCCTTGTGGGAGGTG	CTC	TTATTAtaaGAAACTCT	Jγ2.3
		VγIV			
	Vγ11	GTGCCTGCTGGATTAGGCAC			
JR	Vγ11	GTGCCTGCTGGATTAGGCA	AAGGCCTCT	AATTATTATAAGAAACTCT	Jγ2.3

a. Sequences of the junctional regions are aligned with the known (here underlined) Vγ and Jγ germline sequences (14,15,17,49). Underlined nucleotides at the end of N-region represent P-region nucleotides. Identification P-region nucleotides is described in the Introduction (36). Sequences in lower case characters (taa) are stopcondons when read in the correct reading frame. Characteristics of the sequences are summarized in Table 5.

b. The TcR-γ gene rearrangement in patient WC has previously been described erroneously as Vγ3-Jγ1.1 on the basis of Southern blot analysis (40). This misinterpretation was caused by a high percentage (~40%) of non-leukemic cells.

(TAA, TAG) were also found in the junctional region of the out-of-frame Vδ1-Jδ1 rearrangement in patient MA.

The TcR-δ junctional regions varied from 5 to 47 nucleotides (average 25.6 nucleotides). Calculation of the relative sizes of the Vδ1-Jδ1 junctional regions (insertion minus deletion by trimming), revealed a maximum difference of 39 nucleotides, which was in line with the results of the PAGE analysis. By analogy, the differences of the double Vδ1-Jδ1 rearrangements could be calculated: eight nucleotides in patients HZ, 33 nucleotides in patient MA, and one nucleotide in patient JR, confirming the results of the PAGE analysis. Only one junctional region (Vδ1-Jδ1 in patient EP) consisted solely of two P-region and three Dδ3 gene nucleotides. All other junctions contained N-region and Dδ gene nucleotides, except for the Vδ2-Jδ1 rearrangement in patient JB, which did not

TABLE 4. Junctional region sequences of TcR-δ rearrangements in thirteen TcR-γδ+ T-ALL[a].

Patient	Vδ1	N1	Dδ1	N2	Dδ2	N3	Dδ3	N4	Jδ1
	TCTTGGGGAACT		GAAATAGT		CCTTCCTAC		ACTGGGGGATACG		ACACCGATAAAC
PEER	TCTTGGG	ACGGGGGTGA					GGGGA	CTCCAGG	ACACCGATAAAC
DD	TCTTGGGGAA	A			CTTC	AACCC	GGGGA	CCGTTCATACCAAC/ CACCCTCTACGGGGG	CACCGATAAAC
IJ	TCTTGGGGAACT		→GAA		TTC←	ATTGTAC	GGGGG		CGATAAAC
HZ	TCTTGGGGAACT	ATTCGGTC			CCTAC	GGAta	aCTGGGGGATACG	TGTGGAGTA	ACACCGATAAAC
HZ	TCTTGGGGAAC	CTGTGGG	TAG			GGTCTCGCCCGT	ACTGGGGG	TCTT	CACCGATAAAC
AV	TCTTGGGGA	T	AAA	CC	TCC	CCGGCCAGAGAGCT	ACTGGG	ACGATGGGC	CCGATAAAC
BP	TCTTGGG			AACGGCAAG	CCTTCC	CCAAAATTT	ACTGGGGG	TCCTT	ACACCGATAAAC
JB	TCTTGGGGAACT		AAA	GAGGG			TGGGGG	CCCCGT	ACACCGATAAAC
EP	TCTTGGGGAACT						ACG	GT	ACACCGATAAAC
MA	TCTTGGGGA	TCAAGGAG	GAA	GAA	CTt	aaTGGCC	GGGGG	GGGTAtagTCGGCTGGAA	ACACCGATAAAC
MA	TCTTGGGGA						GGGATAC	TTCCCCG	ACACCGATAAAC
SA	TCTTGGGGA				CCTT	ATCTCTCATACAA	ACTGGGGGATACG	CGGTG	AAC
WC	TCTTGGGG	GCAA			CTTCCTAC	GATC	GGGGATA	GAAGCGGA	CGATAAAC
JR	TCTTGGGGAA	TGGAG	TAGT		TTCC	CCTTGG	CTGGG	AAGGGTAAG	AC
JR	TCTTGGGGAA	GGAGG			TCC	GTT	ACTGGGGGATAC	TGAGGT	TAAAC
PB	TCTTGGGGAACT	CCC	AGT			G	CTGGGGGA	CTTGT	ACACCGATAAAC
JM	TCTTGGG	CCAACC			CCTAC	TGA	TGGGGGA	GCGT	ACACCGATAAAC
	Vδ2								
	GCCTGTGACACC								
JB	GCCTGTG							TGTGT	ACACCGATAAAC
PB	GCCTGTG	TTACTA			CTTC	TTCTT	CTGGGGGAT	TGAG	CCGAtaaAC
JM	GCCTGTGAC	C	AGT	CC	TCCT	T	ACTGGGGGATACG		
	Vδ3								
	CTGTGCCTTTAG								
SA	CTGTGCCT	ATàààCCTTATTTG[b]			TTCCT		ACTGGGGGA	CCAT	CGAtaaAC
	δREC								
	CTGTGAGGAGCC								
BP	CTGTGAGGAGCC	GG					ACG	CCCCCCCGCGACAGA	ACACCGATAAAC
					Dδ2				
					[d]CATTGTGCCTTCCTAC				
DD					CATTGTG	AATCC	ACG	AT	ACACCGATAAAC
IJ					CATTG	GGCCATCA	GGGGGAT	TGAAGT	ACACCGATAAAC
AV					CAT	.GCCTTTGGAT	TGGGGGATACG	GC	[e]
WC					CATTGTGCCTTCCTAC	GTCTT	ACTGGGGGATAC	AAAT	AAC

a. Sequences of the junctional regions are aligned with the know (here underlined) Vδ, Dδ and Jδ1 germline sequences (6,22,46-48). Only if at least three successive nucleotides corresponded with a Dδ germline sequence were the nucleotides assumed to be derived from the involved Dδ gene segment. N-regions are indicated at the top (N) but it was not possible to appoint sequences to a certain N-region if no intermediate Dδ nucleotides were present. Underlined nucleotides at the ends of the N-regions represent P-region nucleotides. Identification of P-region nucleotides is described in the Introduction (35). Horizontal arrows (→←) indicate the generated *Eco*RI restriction site in the Vδ1-Jδ1 junctional region of patient IJ (40). Sequences in small characters (taa and tag) are stopcodons when read in the correct reading frame. Characteristics of the sequences are summarized in Table 5.

b. Although the ààà sequence of the Vδ3-Jδ1 rearrangement of patient SA could be aligned with the Dδ1 germline sequence, Southern blot data showed that Dδ1 was not used in this rearrangement.

d. The CATTGTG sequence at the 5′ site of the Dδ2 sequence represents the heptamer recognition sequence (6).

e. The Jδ1 gene segment of the Dδ2-Jδ1 rearrangement of patient AV had lost 16 bp due to trimming during the rearrangement event.

TABLE 5. Characteristics of the junctional regions of TcR-γ and TcR-δ rearrangements in thirteen TcR-γδ$^+$ T-ALL.

	TcR-γ junctional region[a]						TcR-δ junctional region[b]						
Patient	Rearrangement	In[c] frame	Junctional[d] nucleotides	N-region[e] nucleotides	P-region[f] nucleotides	Deleted[g] nucleotides	Rearrangement	In[c] frame	Junctional[d] nucleotides	Dδ gene[h] nucleotides	N-region[e] nucleotides	P-region[f] nucleotides	Deleted[g] nucleotides
DD	Vγ9-Jγ1.3	+	3	3	0	1	Vδ1-Jδ1	+	44	9	35	0	3
	Vγ5-Jγ1.3	–	6	6	0	27	Dδ2-Jδ1	0	10	3	6	1	9
IJ	Vγ9-Jγ1.3	+	24	22	2	10	Vδ1-Jδ1	+	18	11	7	0	4
	Vγ2-Jγ2.3	–	9	9	0	11	Dδ2-Jδ1	0	21	7	12	2	11
HZ	Vγ4-Jγ2.3	+	15	15	0	10	Vδ1-Jδ1	+	34	11	21	2	2
	Vγ3-Jγ2.3	–	6	6	0	2	Vδ1-Jδ1	–	40	18	20	2	0
AV	Vγ3-Jγ2.3	+	4	3	1	5	Vδ1-Jδ1	+	38	12	25	1	6
	Vγ9-Jγ2.3	–	3	2	1	3	Dδ2-Jδ1	0	23	11	12	0	29
BP	Vγ2-Jγ1.1	+	5	5	0	13	Vδ1-Jδ1	+	37	14	20	3	5
	Vγ4-Jγ2.3	–	12	12	0	12	δREC-Jδ1	0	20	3	14	3	0
JB	Vγ2-Jγ1.1	+	2	2	0	16	Vδ1-Jδ1	+	20	9	9	2	0
	Vγ2-Jγ1.3	+	1	0	1	5	Vδ2-Jδ1	–	5	0	3	2	5
EP	Vγ8-Jγ2.3	+	12	12	0	13	Vδ1-Jδ1	+	5	3	0	2	0
	Vγ8-Jγ2.3	–	19	19	0	7	Deleted						
MA	Vγ8-Jγ2.3	+	0	0	0	7	Vδ1-Jδ1	+	14	7	7	0	3
	Vγ8-Jγ2.3	–	9	9	0	5	Vδ1-Jδ1	–[i]	47	11	36	0	3
SA	Vγ3-Jγ2.3	+	6	6	0	10	Vδ1-Jδ1	+	35	17	16	2	12
	Vγ8-Jγ2.3	–	4	4	0	7	Vδ3-Jδ1	–	32	14	18	0	8
WC	Vγ3-Jγ2.1	+	4	4	0	9	Vδ1-Jδ1	+	31	15	15	1	8
	Germline						Dδ2-Jδ1	0	21	12	6	3	9
JR	Vγ4-Jγ2.3	+	10	10	0	5	Vδ1-Jδ1	+	29	15	13	1	9
	Vγ11-Jγ2.3	–	9	9	0	2	Vδ1-Jδ1	–	33	13	20	0	12
PB	Vγ3-Jγ1.1	+	1	1	0	12	Vδ1-Jδ1	+	20	11	7	2	0
	Deleted						Vδ2-Jδ1	–	28	13	15	0	8
JM	Vγ8-Jγ2.3	+	3	3	0	4	Vδ1-Jδ1	+	25	12	11	2	5
	Vγ4-Jγ2.3	–	25	25	0	12	Vδ2-Dδ3	0	11	7	3	1	3
Average no. of nucleotides per rearranged allele			8.0	7.8	0.2	8.7			25.6	10.3	14.0	1.3	6.2

a. The actual sequences of the TcR-γ junctional regions are described in Table 3.
b. The actual sequences of the TcR-δ junctional regions are described in Table 4.
c. +, in-frame rearrangement; –, out-of-frame rearrangement; 0, incomplete rearrangement.
d. Junctional region nucleotides: sum of (Dδ gene,) N-region and P-region nucleotides.
e. N-region nucleotides: random inserted nucleotides of all N-regions.
f. P-region nucleotides: nucleotides recognized as fulfilling the conditions for P-regions as described in the Introduction (35).
g. Deleted nucleotides: total loss of nucleotides per junctional regions as caused by trimming at 5′ and 3′ sides of the rearranging V and J gene segments.
h. D gene nucleotides: nucleotides which align with a Dδ gene segment. Rules for alignment are described in the Introduction.
i. This Vδ1-Jδ1 rearrangement appeared to be in-frame with respect to the number of inserted and deleted nucleotides but was actually out-of-frame due to the generation of a TAA stopcodon in the junctional region (Table 4).

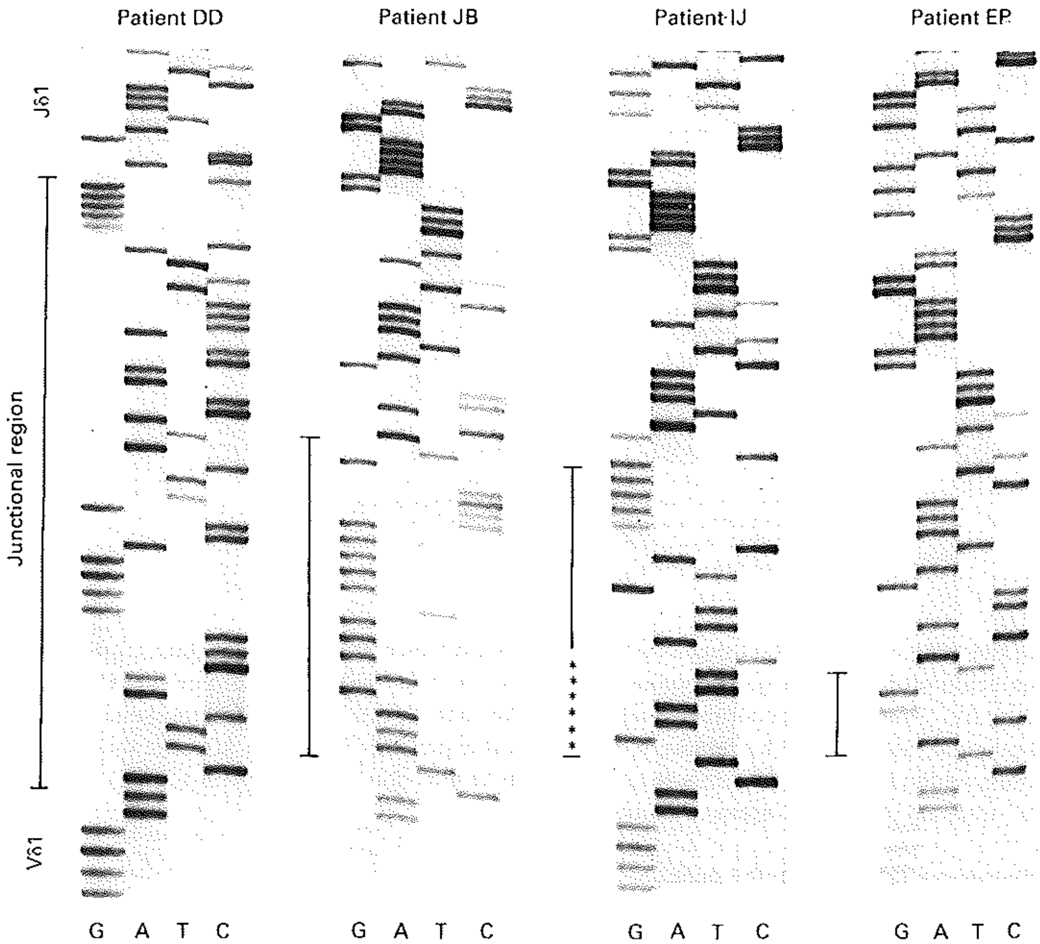


Figure 2. Direct sequence analysis of the Vδ1-Jδ1 junctional region PCR products of patients DD, JB, IJ and EP using the Vδ1-3′s sequence primer (Table 1). The Vδ1 region, the junctional regions and the Jδ1 region are indicated (cf. Table 4). The generated *Eco*RI site (GAATTC) in the junctional region of patient IJ is indicated by asterisks. The G-A-T-C sequence radiographs should be read from 5′ (bottom) to 3′ (top).

contain any Dδ gene nucleotides. Dδ1, Dδ2 and Dδ3 gene segment derived nucleotides were found in 8, 18 and 24 of the 25 TcR-δ gene rearrangements, respectively. One, two or three P-region nucleotides were found in 17 out of 25 rearrangements. As for the distribution of the junctional region nucleotides: 40% were Dδ gene, 55% N-region and 5% P-region derived nucleotides.

Trimming of the ends of the involved Vδ gene segments (50 deleted nucleotides) and Jδ gene segments (71 deleted nucleotides) was less extensive than in the TcR-γ gene rearrangements and resulted in deletion of 0 to 29 nucleotides (average 6.2 nucleotides) per junction. Six Vδ and twelve Jδ1 gene segments were complete (i.e. without trimming). Trimming of the Dδ gene segments did occur, but it was difficult to determine the degree

TABLE 6. Expressed TcR-γδ in TcR-γδ⁺ T-ALL[a].

Patient	TcR-γ chain	TcR-δ chain	Disulfide bond
DD	Vγ9-Jγ1.3-Cγ1	Vδ1-Jδ1-Cδ	+
IJ	Vγ9-Jγ1.3-Cγ1	Vδ1-Jδ1-Cδ	+
HZ	Vγ4-Jγ2.3-Cγ2	Vδ1-Jδ1-Cδ	–
AV	Vγ3-Jγ2.3-Cγ2	Vδ1-Jδ1-Cδ	–
BP	Vγ2-Jγ1.1-Cγ1	Vδ1-Jδ1-Cδ	+
JB	Vγ2-Jγ1.?-Cγ1[b]	Vδ1-Jδ1-Cδ	+
EP	Vγ8-Jγ2.3-Cγ2	Vδ1-Jδ1-Cδ	–
MA	Vγ8-Jγ2.3-Cγ2	Vδ1-Jδ1-Cδ	–
SA	Vγ3-Jγ2.3-Cγ2	Vδ1-Jδ1-Cδ	–
WC	Vγ3-Jγ2.1-Cγ2	Vδ1-Jδ1-Cδ	–
JR	Vγ4-Jγ2.3-Cγ2	Vδ1-Jδ1-Cδ	–
PB	Vγ3-Jγ1.1-Cγ1	Vδ1-Jδ1-Cδ	+
JM	Vγ8-Jγ2.3-Cγ2	Vδ1-Jδ1-Cδ	–

a. Expression of rearranged genes is based on McAb stainings with Ti-γA (Vγ9) and δTCS1 (Vδ1), as well as sequence data of the junctional regions.

b. The junctional regions of both Vγ2-Jγ1.1 and Vγ2-Jγ1.3 rearrangements of patient JB were in frame.

of trimming, due to the fact that extensive trimming will delete the whole Dδ gene segment, as occurred to the Dδ2 gene segments in the Dδ2-Jδ1 rearrangements of patients DD, IJ and AV. Finally, the generated *Eco*RI restriction site in the Vδ1-Jδ1 rearrangement of patient IJ, as predicted by Southern blot and PCR analysis in Reference 40, was found in the junctional region and was a result of joining of a 3′-trimmed Dδ1 gene segment to a 5′-trimmed Dδ2 gene segment (Figure 2).

Expressed TcR-γ and TcR-δ genes

The combined immunofluorescence and sequence data allowed us to deduce which TcR-γ and TcR-δ genes encoded for the expressed TcR-γ and TcR-δ protein chains in all TcR-γδ⁺ T-ALL (Table 6). Only in patient JB was it not possible to determine which TcR-γ gene was expressed, because both rearranged TcR-γ genes appeared to be in-frame, i.e. Vγ2 rearranged either to a Jγ1.1 or a Jγ1.3 gene segment (Table 6). The expressed TcR-γ gene generally consisted of a VγI variable region (11/13), a Jγ2.3 joining region (7/13) and a Cγ2 constant region (8/13). No other Vγ gene family was used except for VγII (Vγ9) in two patients. Interestingly, Vγ2 and Vγ9 genes were only used in combination with Jγ1-Cγ1 gene segments, whereas Vγ4, Vγ8 and three out of four Vγ3 genes were used in combination with Jγ2-Cγ2 gene segments, suggesting a preferential Vγ gene usage in TcR-γ1 and TcR-γ2 derived receptors. The expression of the TcR-δ chain was uniform, since all thirteen T-ALL expressed a Vδ1-Dδ-Jδ1-Cδ protein chain.

DISCUSSION

A group of thirteen TcR-γδ⁺ T-ALL was analyzed phenotypically and genotypically in this study and in a previously described study (40). The TcR-γδ⁺ T-ALL had a

heterogeneous CD4/CD8 phenotype, since all possible combinations of CD4 and CD8 expression were found in our series of TcR-$\gamma\delta^+$ T-ALL, with a preference for the $CD4^+/CD8^-$ phenotype in eight out of thirteen T-ALL. Also the CD1 antigen was expressed heterogeneously (seven out of thirteen) in contrast to TdT expression, which was observed in all T-ALL.

The expressed TcR-γ protein chains as determined by combined Southern blot and PCR analysis showed a preference for VγI (11/13), Jγ2.3 (7/13) and Cγ2 (8/13) usage. Expressed Vγ2 and Vγ9 genes were only observed in combination with Jγ1-Cγ1 gene segments, whereas expressed Vγ4, Vγ8 and most Vγ3 genes were observed in combination with Jγ2-Cγ2 genes (Table 6). This suggests that, in TcR-$\gamma\delta^+$ T-ALL, particular Vγ genes are preferentially used in TcR-γ1 or TcR-γ2 derived receptors, i.e. in disulfide-linked or non-disulfide-linked TcR-$\gamma\delta$ receptors (25,27,32). The preferential usage of particular TcR-γ gene segments was not as explicit as the preferential TcR-δ gene usage, since all thirteen T-ALL expressed a Vδ1-Jδ1-Cδ protein chain. Although there was no selection in our series of thirteen TcR-$\gamma\delta^+$ T-ALL patients, it is peculiar that they all expressed a Vδ1-Jδ1-Cδ chain, since in the literature non-Vδ1 expressing TcR-$\gamma\delta^+$ T-ALL are also described (52).

The combinatorial diversity of normal TcR-$\gamma\delta^+$ cells is limited by the restricted combinatorial repertoire and by preferential rearrangements. This limited combinatorial diversity is reflected by the preferential expression of a Vγ9-Jγ1.2-Cγ1 chain disulfide-linked to a Vδ2-Jδ1-Cδ chain by ~85% of the TcR-$\gamma\delta^+$ T-lymphocytes in normal PB (25-28). However, our T-ALL did not express the Vγ9/Vδ2 receptor, but predominantly expressed a VγI-Jγ2.3-Cγ2 chain non-disulfide-linked to a Vδ1-Jδ1-Cδ chain. This TcR phenotype resembles that of the majority of TcR-$\gamma\delta^+$ thymocytes (26-28). This, together with the TdT expression indicates that the TcR-$\gamma\delta^+$ T-ALL most probably have a thymic origin.

In sharp contrast to the limited combinatorial diversity are the extensive TcR-γ and TcR-δ junctional diversities, generated by insertion of N-region, P-region and/or Dδ gene derived nucleotides as well as by deletion of nucleotides by trimming (6,7,23,35-39). In the TcR-γ junctional regions insertion varied from 0 to 25 nucleotides (average 8.0 nucleotides) and deletion from 1 to 27 nucleotides (average 8.7 nucleotides) per junction. This resulted in TcR-γ junctional regions with a great diversity, but this diversity was still limited as compared to that of the TcR-δ genes. The nucleotide insertions and deletions in the TcR-δ genes varied from 5 to 47 nucleotides (average 25.6 nucleotides) and 0 to 29 nucleotides (average 6.2 nucleotides) per junctional region, respectively.

In general, the N-region nucleotides were the most prominent element in the junctional diversity. They constituted 97% of the TcR-γ and 55% of the TcR-δ junctional regions. In TcR-δ genes the Dδ-gene-derived nucleotides comprised 40% of the junctional regions but, because of their template-dependent nature, the impact on the junctional diversity was less than that of the randomly inserted N-region nucleotides. This also applies to the P-region nucleotides, which contribution to diversity was further diminished by the fact that they contributed only 3 and 5% of the TcR-γ and TcR-δ junctional regions, respectively. Although deletion of nucleotides does not induce the same effect on the junctional diversity as insertion of nucleotides, trimming also contributes substantially to the total junctional diversity. For instance, on average there were more nucleotides deleted (average 8.7 nucleotides) than inserted (average 8.0 nucleotides) in the TcR-γ junctions. Trimming in TcR-δ junctions (average 6.2 nucleotides) was less profound than in TcR-γ junctions, but

this was fully compensated by the extra diversity of Dδ gene usage. Noticeable, trimming of both Vγ and Vδ gene segments never affected the conserved TGT triplet, which codes for the cysteine residue that is involved in the intrachain disulfide bond of the V domain (14,47,49). However, incomplete Dδ2-Jδ1 rearrangements may be caused by extensive trimming which affects the 5' heptamer recognition sequence (patients IJ and HZ) (6), thereby preventing further Vδ to Dδ2-Jδ1 rearrangement.

Recently, new methods have been described to detect minimal residual disease (MRD) in lymphoid malignancies utilizing PCR-mediated amplification of the junctional regions of rearranged Immunoglobulin (Ig) or TcR genes of leukemic cells (53-59). This is based on the fact that junctional regions of rearranged Ig or TcR genes vary enormously. This implies that the junctional regions are different in each lymphocyte or clone of lymphocytes. It is therefore assumed that junctional regions of rearranged Ig and TcR genes in lymphoid malignancies can be regarded as 'tumor-specific' markers. The limited combinatorial diversity of the TcR-γ and TcR-δ genes allows simple determination of the rearranged gene segments by Southern blot analysis, which is useful for choosing the correct primers for the MRD-PCR analysis (8,16-18,60,61). However, one should be aware that the restriction patterns in Southern blot analysis can be disturbed by generation of restriction sites in the junctional region, like the *Eco*RI site in the Vδ1-Jδ1 rearrangement in patient IJ (40). Furthermore it is evident that the TcR-δ junctional regions, because of their size, are more accessible for the design of a leukemia-specific oligonucleotide probe than the smaller TcR-γ junctional regions. In our series of TcR-$\gamma\delta^+$ T-ALL, only two TcR-γ rearrangements, compared with nineteen TcR-δ rearrangements, contained at least twenty nucleotides. Consequently, PCR-mediated detection of MRD via TcR-δ junctional regions will be applicable in a larger proportion of the leukemia patients than via TcR-γ. Furthermore, in many patients a TcR-δ junctional region probe will have a higher specificity due to the larger size of the junctional region (average >25 nucleotides), which will lead to lower detection limits of the MRD-PCR analysis.

ACKNOWLEDGMENTS. The authors gratefully acknowledge Prof. Dr. R. Benner, Dr. H. Hooijkaas and Dr. H.J. Adriaansen for their continuous support; Dr. R. Kurrle (Behring, Marburg, Germany), Dr. T. Hercend (Villejuif, France) and Dr. L. Moretta (Genova, Italy) for kindly providing the BMA031, Ti-γA and BB3 antibodies, Dr. T.H. Rabbitts, Dr. T. Quertermous, Dr. P. van de Elsen, Dr. S.J. Korsmeyer and Dr. M.-P. Lefranc for kindly providing the TcR-γ and TcR-δ probes; Mr. T.M. van Os for excellent assistance in the preparation of the figures; and Ms. A.D. Korpershoek for her secretarial support; Dr. D. Campana, Dr. J.C. Kluin-Nelemans, Dr. R.J. van de Griend and Dr. C.E. van der Schoot for kindly providing TcR-$\gamma\delta^+$ T-ALL cell samples; The Dutch Childhood Leukemia Study Group (DCLSG) kindly provided three leukemia cell samples. Board members of the DCLSG are J.P.M. Bökkerink, M.V.A. Bruin, P.J. van Dijken, W.A. Kamps, E.F. van Leeuwen, F.A.E. Nabben, A. Postma, J.A. Rammeloo, I.M. Risseeuw-Appel, G.A.M. de Vaan, E.Th. van 't Veer-Korthof, A.J.P. Veerman, F.C. de Waal, M. van Weel-Sipman, and R.S. Weening.

REFERENCES

1. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988;334:395-402.
2. Borst J, Van Dongen JJM, Bolhuis RLH, Peters PJ, Hafler DA, De Vries E, Van de Griend RJ. Distinct molecular forms of human T cell receptor γ/δ detected on viable T cells by a monoclonal antibody. J Exp Med 1988;167:1625-1644.

3. Raulet DH. The structure, function, and molecular genetics of the γ/δ T cell receptor. Ann Rev Immunol 1989;7:175-207.
4. Van Dongen JJM, Comans-Bitter WM, Wolvers-Tettero ILM, Borst J. Development of human T lymphocytes and their thymus-dependency. Thymus 1990;16:207-234.
5. Toyonaga B, Yoshikai Y, Vadasz V, Chin B, Mak TW. Organization and sequences of the diversity, joining, and constant region genes of the human T-cell receptor β chain. Proc Natl Acad Sci USA 1985;82:8624-8628.
6. Loh EY, Cwirla S, Serafini AT, Phillips JH, Lanier LL. Human T-cell-receptor δ chain: genomic organization, diversity, and expression in populations of cells. Proc Natl Acad Sci USA 1988;85:9714-9718.
7. Blackwell TK, Alt FW. Molecular characterization of the lymphoid V(D)J recombination activity. J Biol Chem 1989;264:10327-10330.
8. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of Immunoglobulin and T cell receptor genes. Part I: basic and technical aspects. Clin Chim Acta 1991;198:1-92
9. Yoshikai Y, Clark SP, Taylor S, Sohn U, Wilson BI, Minden MD, Mak TW. Organization and sequences of the variable, joining and constant region genes of the human T-cell receptor α-chain. Nature 1985;316:837-840.
10. Yoshikai Y, Kimura N, Toyonaga B, Mak TW. Sequences and repertoire of human T cell receptor α chain variable region genes in mature T lymphocytes. J Exp Med 1986;164:90-103.
11. Concannon P, Pickering LA, Kung P, Hood L. Diversity and structure of human T-cell receptor β-chain variable region genes. Proc Natl Acad Sci USA 1986;83:6598-6602.
12. Kimura N, Toyonaga B, Yoshikai Y, Du R-P, Mak TW. Sequences and repertoire of the human T cell receptor α and β chain variable region genes in thymocytes. Eur J Immunol 1987;17:375-383.
13. Klein MH, Concannon P, Everett M, Kim LDH, Hunkapiller T, Hood L. Diversity and structure of human T-cell receptor α-chain variable region genes. Proc Natl Acad Sci USA 1987;84:6884-6888.
14. LeFranc M-P, Forster A, Baer R, Stinson MA, Rabbitts TH. Diversity and rearrangement of the human T cell rearranging γ genes: nine germ-line variable genes belonging to two subgroups. Cell 1986;45:237-246.
15. Quertermous T, Strauss WM, Van Dongen JJM, Seidman JG. Human T cell γ chain joining regions and T cell development. J Immunol 1987;138:2687-2690.
16. Forster A, Huck S, Ghanem N, Lefranc M-P, Rabbitts TH. New subgroups in the human T cell rearranging Vγ gene locus. EMBO J 1987;6:1945-1950.
17. Lefranc M-P. The human T-cell rearranging gamma (TRG) genes and the gamma T-cell receptor. Biochimie 1988;70:901-908.
18. Chen Z, Font MP, Loiseau P, Bories JC, Degos L, Lefranc MP, Sigaux F. The human T-cell Vγ gene locus: cloning of new segments and study of Vγ rearrangements in neoplastic T and B cells. Blood 1988;72:776-783.
19. Takihara Y, Tkachuk D, Michalopoulos E, Champagne E, Reimann J, Minden M, Mak TW. Sequence and organization of the diversity, joining, and constant region genes of the human T-cell δ-chain locus. Proc Natl Acad Sci USA 1988;85:6097-6101.
20. Baer R, Boehm T, Yssel H, Spits H, Rabbitts TH. Complex rearrangements within the human Jδ-Cδ/Jα-Cα locus and aberrant recombination between Jα segments. EMBO J 1988;7:1661-1668.
21. Van Dongen JJM, Wolvers-Tettero ILM, Wassenaar F, Borst J, Van den Elsen P. Rearrangement and expression of T-cell receptor delta genes in T-cell acute lymphoblastic leukemias. Blood 1989;74:334-342.
22. Hata S, Clabby M, Devlin P, Spits H, De Vries JE, Krangel MS. Diversity and organization of human T cell receptor δ variable gene segments. J Exp Med 1989;169:41-57.
23. Takihara Y, Reimann J, Michalopoulos E, Ciccone E, Moretta L, Mak TW. Diversity and structure of human T cell receptor δ chain genes in peripheral blood γ/δ-bearing T lymphocytes. J Exp Med 1989;169:393-405.
24. Triebel F, Faure F, Mami-Chouaib F, Jitsukawa S, Griscelli A, Genevée C, Roman-Roman S, Hercend T. A novel human Vδ gene expressed predominantly in the TiγA fraction of γ/δ^+ peripheral lymphocytes. Eur J Immunol 1988;18:2021-2027.
25. Bottino C, Tambussi G, Ferrini S, Ciccone E, Varese P, Mingari MC, Moretta L, Moretta A. Two subsets of human T lymphocytes expressing γ/δ antigen receptor are identifiable by monoclonal antibodies directed to two distinct molecular forms of the receptor. J Exp Med 1988;168:491-505.
26. Borst J, Wicherink A, Van Dongen JJM, De Vries E, Comans-Bitter WM, Wassenaar F, Van den Elsen P. Non-random expression of T cell receptor γ and δ variable gene segments in functional T lymphocyte clones from human peripheral blood. Eur J Immunol 1989;19:1559-1568.
27. Triebel F, Hercend T. Subpopulations of human peripheral T gamma delta lymphocytes. Immunol Today 1989;10:186-188.
28. Casorati G, De Libero G, Lanzavecchia A, Migone N. Molecular analysis of human γ/δ^+ clones from thymus and peripheral blood. J Exp Med 1989;170:1521-1535.
29. LeFranc M-P, Forster A, Rabbitts TH. Genetic polymorphism and exon changes of the constant regions of the human T-cell rearranging gene γ. Proc Natl Acad Sci USA 1986;83:9596-9600.
30. Littman DR, Newton M, Crommie D, Ang S-L, Seidman JG, Gettner SN, Weiss A. Characterization of an expressed CD3-associated Ti γ-chain reveals Cγ domain polymorphism. Nature 1987;326:85-88.
31. Krangel MS, Band H, Hata S, McLean J, Brenner MB. Structurally divergent human T cell receptor γ proteins encoded by distinct Cγ genes. Science 1987;237:64-67.
32. Van Dongen JJM, Wolvers-Tettero ILM, Seidman JG, Ang S-L, Van de Griend RJ, De Vries EFR, Borst J. Two types of gamma T cell receptors expressed by T cell acute lymphoblastic leukemias. Eur J Immunol 1987;17:1719-1728.
33. Quertermous T, Strauss W, Murre C, Dialynas DP, Strominger JL, Seidman JG. Human T-cell γ genes contain *N* segments and have marked junctional variability. Nature 1986;322:184-187.

34. Loh EY, Elliott JF, Cwirla S, Lanier LL, Davis MM. Polymerase chain reaction with single-sided specificity: analysis of T cell receptor δ chain. Science 1989;243:217-220.
35. Lafaille JJ, DeCloux A, Bonneville M, Takagaki Y, Tonegawa S. Junctional Sequences of T cell receptor γδ genes: implications for γδ T cell lineages and for a novel intermediate of V-(D)-J joining. Cell 1989;59:859-870.
36. Elliott JF, Rock EP, Patten PA, Davis MM, Chien Y-H. The adult T-cell receptor δ-chain is diverse and distinct from that of fetal thymocytes. Nature 1988;331:627-631.
37. Hata S, Satyanarayana K, Devlin P, Band H, McLean J, Strominger JL, Brenner MB, Krangel MS. Extensive junctional diversity of rearranged human T cell receptor δ genes. Science 1988;240: 1541-1544.
38. Huck S, Dariavach P, Lefranc M-P. Variable region genes in the human T-cell rearranging gamma (TRG) locus: V-J junction and homology with the mouse genes. EMBO J 1988;7:719-726.
39. Macintyre E, D'Auriol L, Amesland F, Loiseau P, Chen Z, Boumsell L, Galibert F, Sigaux F. Analysis of junctional diversity in the preferential Vδ1-Jδ1 rearrangement of fresh T-acute lymphoblastic leukemia cells by in vitro gene amplification and direct sequencing. Blood 1989;74:2053-2061.
40. Breit TM, Wolvers-Tettero ILM, Hählen K, Van Wering ER, Van Dongen JJM. Limited combinatorial repertoire of γδ T-cell receptors expressed by T-cell acute lymphoblastic leukemias. Leukemia 1991;5:116-124.
41. Loh EY, Lanier LL, Turck CW, Littman DR, Davis MM, Chien Y-H, Weiss A. Identification and sequence of a fourth human T cell antigen receptor chain. Nature 1987;330:569-572.
42. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunological marker analysis of cells in the various hematopoietic differentiation stages and their malignant counterparts. In: Ruiter DJ, Fleuren GJ, Warnaar SO, eds. Application of monoclonal antibodies in tumor pathology. Dordrecht: Martinus Nijhoff 1987:87-116.
43. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning, a laboratory manual, 2nd edn. Cold Spring Harbor: Cold Spring Harbor Laboratory, 1989.
44. Boehm T, Baer R, Lavenir I, Forster A, Waters JJ, Nacheva E, Rabbitts TH. The mechanism of chromosomal translocation t(11;14) involving the T-cell receptor Cδ locus on human chromosome 14q11 and a transcribed region of chromosome 11p15. EMBO J 1988;7:385-394.
45. Hata S, Brenner MB, Krangel MS. Identification of putative human T cell receptor δ complementary DNA clones. Science 1987;238:678-682.
46. Satyanarayana K, Hata S, Devlin P, Grazia Roncarolo M, De Vries JE, Spits H, Strominger JL, Krangel MS. Genomic organization of the human T-cell antigen-receptor α/δ locus. Proc Natl Acad Sci USA 1988;85:8166-8170.
47. Dariavach P, Lefranc M-P. First genomic sequence of the human T-cell receptor δ2 gene (TRDV2). Nucleic Acids Res 1989;17:4880.
48. Hockett RD Jr, Nuñez G, Korsmeyer SJ. Evolutionary comparison of murine and human δ T-cell receptor deleting elements. New Biologist 1989;1:266-274.
49. Lefranc M-P, Forster A, Rabbitts TH. Rearrangement of two distinct T-cell γ-chain variable-region genes in human DNA. Nature 1986;319:420-422.
50. Murre C, Waldmann RA, Morton CC, Bongiovanni KF, Waldmann TA, Shows TB, Seidman JG. Human γ-chain genes are rearranged in leukaemic T cells and map to the short arm of chromosome 7. Nature 1985;316:549-552.
51. Stern M-H, Lipkowitz S, Aurias A, Griscelli C, Thomas G, Kirch IR. Inversion of chromosome 7 in ataxia telangiectasia is generated by a rearrangement between T-cell receptor β and T-cell receptor γ genes. Blood 1989;74:2076-2080.
52. Gouttefangeas C, Bensussan A, Boumsell L. Study of the CD3-associated T-cell receptors reveals futher differences between T-cell acute lymphoblastic lymphoma and leukemia. Blood 1990;75:931-934.
53. D'Auriol L, Macintyre E, Galibert F, Sigaux F. *In vitro* amplification of T cell γ gene rearrangements: a new tool for the assessment of minimal residual disease in acute lymphoblastic leukemias. Leukemia 1989;3:155-158.
54. Hansen-Hagge TE, Yokota S, Bartram CR. Detection of minimal residual disease in acute lymphoblastic leukemia by *in vitro* amplification of rearranged T-cell receptor δ chain sequences. Blood 1989;74:1762-1767.
55. Yamada M, Wasserman R, Lange B, Reichard BA, Womer RB, Rovera G. Minimal residual disease in childhood B-lineage lymphoblastic leukemia: persistence of leukemic cells during the first 18 months of treatment. New Engl J Med 1990;323:448-455.
56. Jonsson OG, Kitchens RL, Scott FC, Smith RG. Detection of minimal residual disease in acute lymphoblastic leukemia using immunoglobulin hypervariable region specific oligonucleotide probes. Blood 1990;76:2072-2079.
57. Campana D, Yokota S, Coustan-Smith E, Hansen-Hagge TE, Janossy G, Bartram CR. The detection of residual acute lymphoblastic leukemia cells with immunologic methods and polymerase chain reaction: a comparative study. Leukemia 1990;4:609-614.
58. Macintyre EA, D'Auriol L, Duparc N, Leverger G, Galibert F, Sigaux F. Use of oligonucleotide probes directed against T cell antigen receptor gamma delta variable-(diversity)-joining junctional sequences as a general method for detecting minimal residual disease in acute lymphoblastic leukemias. J Clin Invest 1990;86:2125-2135.
59. Yokota S, Hansen-Hagge TE, Ludwig W-D, Reiter A, Raghavachar A, Kleihauer E, Bartram CR. Use of polymerase chain reactions to monitor minimal residual disease in acute lymphoblastic leukemia patients. Blood 1991;77:331-339.
60. Loiseau P, Guglielmi P, Le Paslier D, Macintyre E, Gessain A, Bories J-C, Flandrin G, Chen Z, Sigaux F. Rearrangements of the T cell receptor δ gene in T acute lymphoblastic leukemia cells are distinct from those occurring in B lineage acute lymphoblastic leukemia and preferentially involve one Vδ gene segment. J Immunol 1989;142:3305-3311.
61. Griesinger F, Greenberg JM, Kersey JH. T cell receptor gamma and delta rearrangements in hematologic malignancies: relationship to lymphoid differentiation. J Clin Invest 1989;84:506-516.

CHAPTER 2.5

UNIQUE SELECTION DETERMINANT IN POLYCLONAL Vδ2-Jδ1 JUNCTIONAL REGIONS OF HUMAN PERIPHERAL γδ T-LYMPHOCYTES*

Timo M. Breit, Ingrid L.M. Wolvers-Tettero and Jacques J.M. van Dongen

Department of Immunology, Erasmus University/University Hospital Dijkzigt, Rotterdam, The Netherlands.

ABSTRACT

Human peripheral γδ T-lymphocytes are characterized by the preferential expression of a TcR consisting of a Vδ2-Jδ1-Cδ chain and a Vγ9-Jγ1.2-Cγ1 chain, which are virtually absent on thymocytes. Here we report the identification of a unique selection determinant that is located in the polyclonal Vδ2-Jδ1 junctional regions of peripheral γδ T-lymphocytes. The selection determinant was discovered by the presence of an invariant T nucleotide at the relative second position of the Vδ2-Jδ1 junctional regions of peripheral polyclonal γδ T-lymphocytes. Comparison of published sequences from peripheral γδ T-lymphocytes confirmed the presence of this invariant T nucleotide (90%) in healthy individuals and in patients with various diseases. Translation of the relative first codon of the Vδ2-Jδ1 junctional regions revealed strikingly high frequencies of the homologous hydrophobic amino acids leucine (46%), valine (35%), and isoleucine (5%) at this position. The invariant T nucleotide was absent in polyclonal thymocytes and out-of-frame Vδ2-Jδ1 junctional regions, which proves that selection occurred at the protein level and not at the genomic level. No selection determinant could be identified in Vγ9-Jγ1.2 junctional regions, but the frequently occurring invariable, so-called canonical junctional region provided evidence for biased recombination processes. Although the obtained data do not allow discrimination between thymic selection and/or peripheral antigen-driven expansion, the identification of a strong selection determinant consisting of only one amino acid at a fixed position in Vδ2-Jδ1 junctional regions of virtually all peripheral polyclonal Vδ2/Vγ9 T-lymphocytes provides a new perception of TcR specificity and selection processes at the TcR protein level.

INTRODUCTION

TcR molecules obtain their enormous antigen recognizing diversity by recombination of V, D, and J gene segments of the TcR-α, TcR-β, and TcR-γ, TcR-δ gene complexes (1). In addition to this combinatorial diversity, TcR have an extensive junctional diversity caused by deletion and random insertion of nucleotides (N-regions) at the V-(D)-J junctional regions

* Published in: J Immunol 1994;152:2860-2864.

during the recombination processes (1,2). However, in humans the majority of the $\gamma\delta$ PB T-lymphocytes express a TcR consisting of a Vδ2-Jδ1-Cδ chain and a Vγ9-Jγ1.2-Cγ1 chain (3-6). In contrast to their limited combinatorial diversity, these $\gamma\delta$ T-cells have an extensive junctional diversity (7,8). The function of the Vδ2/Vγ9 T-lymphocytes and the nature of their selection are poorly understood, although it is assumed that selection by peripheral expansion plays an important role, because this Vδ2-Vγ9 TcR is almost absent on CD3$^+$ thymocytes (9-11). In experiments to study clonality of a TcR-$\gamma\delta^+$ T-lymphocytosis in a patient with granulocytopenia by use of PCR analysis and subsequent direct sequencing of the Vδ2-Jδ1 and Vγ9-Jγ1.2 junctional regions (12), we discovered an invariant T nucleotide in the polyclonal Vδ2-Jδ1 junctional regions of PB MNC from a healthy control. This observation lead to the identification of a unique selection determinant that is located in the polyclonal Vδ2-Jδ1 junctional regions of human peripheral $\gamma\delta$ T-lymphocytes. In this study we investigated whether this selection determinant originates from biased recombination processes at the DNA level or from selection at the TcR protein level.

MATERIALS AND METHODS

Cell samples

MNCs were isolated from PB of ten healthy volunteers by Ficoll-Paque (density 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. Thymocytes were obtained from thymus samples of ten children undergoing cardiac surgery. The thymic samples were minced with scissors in RPMI 1640 medium containing 10% fetal calf serum and were flushed through a nylon gauze filter with 100 μm openings. All human cell samples were obtained with the approval of the Committee of Medical Ethics of the Erasmus University/University Hospital Dijkzigt, Rotterdam, The Netherlands.

DNA and RNA isolation

DNA was isolated from PB-MNC and thymocytes as described previously (13,14). RNA was isolated using the method as described by Chromczinski and Sacchi (15). cDNA was obtained by RT of a 1.0 μg RNA sample using *Superscript* reverse transcriptase (Gibco-BRL, Berlin, Germany) for 30 min at 37°. The oligonucleotide primer used for the RT reaction was Cδ-3´s (cgcgtcgACTTCAAAGTCAGTGGAGTGCAC).

PCR-sequencing analysis

PCR and direct sequencing analyses were performed as described previously (16). A 1.0 μg sample of DNA or a 1/8 of the RT reaction product was used in the initial PCR reaction. The oligonucleotide primers used for the initial PCR reaction were Vδ2-5´s (17) and Cδ-5´s (cgcgtcgACCAGACAAGCGACATTTGTTCC) for amplifying cDNA, or Vδ2-5´s and Jδ1-3´XBg, or VγII-5´XBg and Jγ1.2-3´ for amplifying DNA (17). For the asymmetric PCR reaction, oligonucleotide primers Vδ2-5´s and Jδ1-5´BF or VγII-5´XBg and Jγ1.2-3´ were used (17). Direct sequence reactions were performed with the sequence primers Vδ2-3´s, Jδ1-5´BF, or VγII-3´s (17).

RESULTS AND DISCUSSION

Vδ2-Jδ1 junctional regions

PCR and subsequent sequencing analysis performed on Vδ2-Jδ1 junctional regions of polyclonal PB-MNC from ten healthy individuals showed in all experiments an invariant T nucleotide in the otherwise random junctional regions (Figure 1A). There was no significant difference between analyses performed on DNA or cDNA. This curious finding prompted

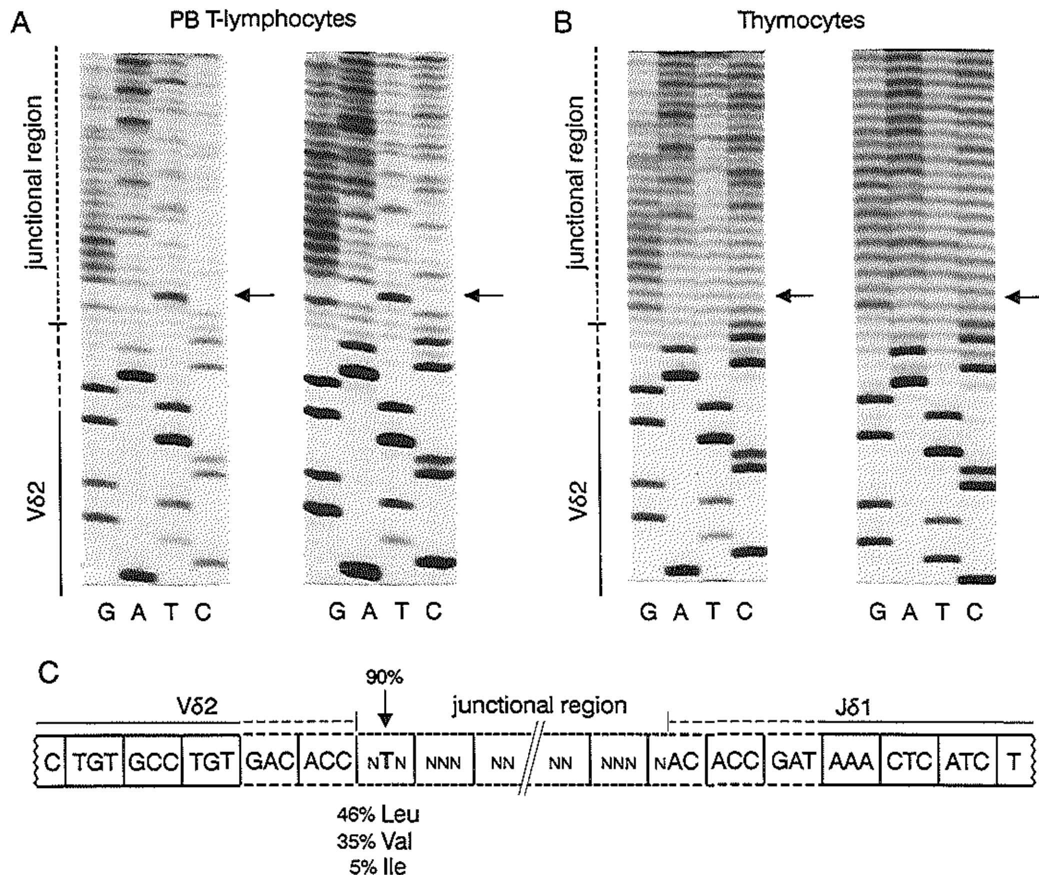


Figure 1. Invariant T nucleotide coding for Leu, Val, or Ile in polyclonal Vδ2-Jδ1 junctional regions of peripheral γδ T-lymphocytes. **(A)** PCR-sequencing analysis of polyclonal PB-MNC from two healthy individuals. The arrows indicate the invariant T-nucleotide at the relative second position of the otherwise random Vδ2-Jδ1 junctional regions. **(B)** PCR-sequencing analysis of polyclonal thymocytes from two thymi. The arrows indicate the absence of the invariant T nucleotide in these polyclonal junctional regions. **(C)** Schematic presentation of the Vδ2-Jδ1 junctional region in peripheral γδ T-lymphocytes. The Vδ2 and Jδ1 germline sequences are given. N represent random junctional region nucleotides. The arrow indicates the relative second position of the junctional region with the invariant T nucleotide (90%). The percentages Leu, Val and Ile found in the relative first codon of these junctional regions are indicated.

us to check the available literature for this invariant T nucleotide in the sequences of Vδ2-Jδ1 junctional regions. For this purpose we evaluated over 300 published Vδ2-Jδ1 junctional regions derived from peripheral T-lymphocytes of healthy individuals and patients (7,8,18-22) (Figure 2). Indeed, 90% of the in-frame junctional regions contained the invariant T nucleotide at the relative second nucleotide position of the junctional region (Table 1). There was also a slight preference in the relative first junctional region nucleotide position for C (38%) and G (40%) nucleotides. In the relative third nucleotide position, only a preference for G nucleotides (42%) was found (Table 1). All these observations were on average equal between individuals regardless of the "peripheral" cell source, e.g. PB-MNC of normal individuals (7,8,19,20), PB-MNC of sarcoidosis patients (8), mycobacterium-stimulated T-cells (19,20,22), or bronchoalveolar lymphocytes (22).

We assumed that the preferential occurrence of specific nucleotides in the Vδ2-Jδ1 junctional regions is caused by selection at the protein level rather than by biased recombination processes. To prove protein selection, PCR-sequencing experiments were performed using polyclonal thymocytes. The far majority (>98%) of thymocytes are $CD3^-$ or TcR-$\alpha\beta^+$ (9,23) and therefore most Vδ2-Jδ1 rearrangements in these cells are not expressed and thus unselected at the protein level. In PCR-sequencing analysis of polyclonal thymocytes derived from ten different thymi we did not find the invariant T nucleotide at DNA or cDNA level (Figure 1B). Also, in T-ALL, which is presumed to originate from cortical thymocytes, the invariant T nucleotide was not present (Figure 2) (17). Finally, the published out-of-frame Vδ2-Jδ1 junctional region sequences (n = 76) (8,19-22) also did not contain the invariant T nucleotide (Figure 2 and Table 1). This proves that the presence

	Vδ2	junctional region	Jδ1	Vδ2	junctional region	Jδ1
germline sequence	TGTGACACC		ACACCGATAAA	CACDT		TDKLI
in-frame PB T-lymphocytes		↓			↓	
PT1	TGTGACACC	TTACTGGGGGACCCGT	ACACCGATAAA	CACDT	LLGDPY	TDKLI
PT2	TGTGAC	CCCGTACTGCCCGTCC	CCGATAAA	CACD	PVLPVP	DKLI
PT3	TGTGACA	ACCTGCTGGGGGATACATATG	ACACCGATAAA	CACD	NLLGDTYD	TDKLI
PT5	TGTGAC	CTACTGGGGGATACG	ACCGATAAA	CACD	LLGDT	TDKLI
PT7	TGTGACACC	TTATCTGGGGGATACGAGGGTCGG	ACCGATAAA	CACDT	LSGGYEGR	TDKLI
in-frame T-ALL		↓			↓	
RB	TGTGACACC	GGCCAAGTGGGGATACCCCCATT	CACCGATAAA	CACDT	GQVGIPPF	TDKLI
RH	TGTGACACC	TCTGGGGGATGTAGG	-9	CACDT	SGGCR	-1
Ho	TGTGA	TCGACCTACGTA	CGATAAA	CACD	RPTY	DKLI
CM	TGTGACACC	GGATGGCTTTTACTGGGGGATAGCCTAGG	CGATAAA	CACDT	GWLLLGDSLG	DKLI
JN	TGTGACACC	TTGGGTGGGGGATACTCTT	ACACCGATAAA	CACDT	LGGGYSY	TDKLI
out-of-frame PB T-lymphocytes		↓				
43	TGTGACACC	GAGGGGATACTGGGGGATACTTAACG	ACCGATAAA			
2	TGTGACACC	GTTGGGGGCGT	ACACCGATAAA			
4	TGTGACAC	GTCCCACGGGGGACGGCGGGAA	CGATAAA			
5	TGTGACACC	GGCCGGGGGCCTTCTCGTTACTGGT	ACCGATAAA			
15	TGTGACAC	GAGGAACTGGGGGCCAACACTT	ACACCGATAAA			

Figure 2. Representative examples of Vδ2-Jδ1 junctional region sequences. DNA and protein sequences of Vδ2-Jδ1 junctional regions aligned relative to the Vδ2 segment. The arrows indicate the relative second nucleotide of the DNA junctional region or the relative first amino acid of the protein junctional region. The invariant T nucleotide and preferential Leu and Val (bold) are present in in-frame Vδ2-Jδ1 rearrangements of peripheral T-lymphocytes (7), but absent in T-ALL (representing the malignant counterparts of cortical thymocytes) (17) and out-of-frame PB T-lymphocytes (19).

TABLE 1. Relative frequencies (%) of nucleotides and amino acids at the relative first codon of the Vδ2-Jδ1 junctional region[a].

	1st nucleotide				2nd nucleotide				3rd nucleotide				amino acid residue					
	G	A	T	C	G	A	T	C	G	A	T	C	Leu	Val	Ile	Ala Trp Pro Met	Gly Thr	Other
In-frame Vδ2-Jδ1 of peripheral T-lymphocytes (n=306)	39.9	9.8	12.7	37.6	3.9	1.3	89.9	4.9	41.5	25.8	15.0	17.7	46.4	34.7	5.2	8.5	2.6	2.6
Out-of-frame Vδ2-Jδ1 of all T-cells (n=76)	30.3	11.8	30.3	27.6	32.9	11.8	31.6	23.7	34.2	17.1	30.3	18.4	NR[b]	NR	NR	NR	NR	NR

a. Sequences derived from References 7,8,19-22.
b. NR, not relevant.

of the invariant T nucleotide at the relative second position of Vδ2-Jδ1 junctional regions of peripheral T-lymphocytes is caused by selection at the protein level.

Translating the relative first codon of the Vδ2-Jδ1 junctional regions of peripheral T-lymphocytes revealed strikingly high percentages of leucine (46%), valine (35%), and isoleucine (5%), which are biochemically comparable amino acids (Figure 1 and Table 1). Other amino acids with some biochemical or steric homology (such as alanine, tryptophan, proline, methionine, glycine, or threonine) were found at very low frequencies, whereas the remaining eleven amino acids were rarely observed at this position in peripheral T-lymphocytes (Figure 2 and Table 1). These data prove that the homologous hydrophobic leucine, valine, and isoleucine residues at the relative first position of protein Vδ2-Jδ1 junctional regions represent an unique selection determinant for peripheral Vδ2/Vγ9 T-lymphocytes. Recently, Davodeau *et al.* (24) also identified this selection determinant in 26 of 31 selected Vδ2/Vγ9 blood T-cell clones.

Attempts to identify additional invariant nucleotides or preferential amino acids in polyclonal Vδ2-Jδ1 junctional regions (e.g. on a relatively fixed distance to the Jδ1 gene segments) by PCR-sequencing analysis or sequence comparison were unsuccessful, except for the observation that cysteine residues are virtually absent in junctional regions of functional Vδ2-Jδ1 rearrangements. This negative selection for cysteine residues is also observed in Vδ1-Jδ1 junctional regions (25,26) and may be true for all Ig/TcR junctional region sequences as to avoid disruption of the receptor domains by incorrect disulfide bonds.

Vγ9-Jγ1.2 junctional regions

PCR-sequencing analysis and sequence comparison of the Vγ9-Jγ1.2 junctional regions (8,20,22,27,28), which in combination with the Vδ2-Jδ1 junctional regions form the TcR antigen recognition elements on peripheral γδ T-lymphocytes, did not elucidate any obvious selection determinant relative to either the Vγ9 or Jγ1.2 segment, except for the absence of cysteine residues. The only remarkable observation was that in almost all individuals analyzed a so-called canonical junctional region was found (Figure 3). Canonical junctional regions lack any random nucleotide insertion and always join the Vγ to the Jγ gene segment in an identical manner. Canonical junctional regions are frequently observed in preferential TcR-γ rearrangements in mice (29,30). Recently, it was proven that this

```
      Vγ9  TGTGCCTTGTGGGAGGT GCA CCT
     Jγ1.2          GCCCATGG GCA AGAGTTGGGCAAA
 canonical  TGTGCCTTGTGGGAGGT GCA AGAGTTGGGCAAA
              C  A  L  W  E  V   Q   E  L  G  K
```

Figure 3. Canonical Vγ9-Jγ1.2 junctional region. Extending the germline sequences of the Vγ9 and Jγ1.2 gene segments with the corresponding P nucleotides (underlined) (31), reveals overlapping nucleotides around the junctions (**GCA**) after alignment. Via this trinucleotide homology, preferential recombination occurs, resulting in in-frame Vγ9-Jγ1.2 canonical junctional regions that are analogous to the canonical TcR-γ junctional regions in mice (32,33).

phenomenon is caused by biased recombination processes via homologous sequences at the ends (including P-region nucleotides) of the rearranging gene segments (31-33). Our data suggest that this is also true for humans because the Vγ9 and Jγ1.2 gene segments are joined together preferentially at the sequence **GCA**, which is present in the Vγ9 gene segment extended with two P-region nucleotides and within the Jγ1.2 gene segment (Figure 3). Therefore, the preferential TcR-γ junctional regions are probably not caused by selection at the protein level but by biased DNA recombination processes.

Antigen specificity

Antigen specificity depending on only one junctional region amino acid has been described for pigeon cytochrome-c antigen (34). The mutually exchangeable junctional region amino acids were an asparagine in combination with TcR-Vβ3 and an aspartic acid in combination with TcR-Vβ1 or Vβ16. Recently, it was shown that in TcR-β junctional regions of T-lymphocytes in brain plaques of patients with multiple sclerosis, five motifs are present in the protein junctional region of preferential Vβ5.2-Jβ rearrangements (35). Although not discussed by Oksenberg *et al.* (35), their data also revealed an invariant T nucleotide at the relative second nucleotide position of the Vβ2.5-Jβ junctional region (71%), and a preferential leucine at the relative first amino acid position of the junctional region (69%). Homologous to our findings, the various preferential amino acids were located at the relative first amino acid position of the junctional region, i.e. four to five amino acid residues C-terminal from the highly conserved 3′ cysteine of the TcR-V domain. Therefore, this relative position may play a crucial role in antigen specificity of TcR.

It still remains unclear which mechanisms and antigens select the peripheral Vδ2/Vγ9 T-lymphocytes, although it has been suggested that they are selected by a superantigen (9,11,19). If this assumption is correct, then our results indicate that determinants for superantigen selection are not only located in germline-encoded protein sequences, but also in the junctional region of the TcR protein chains.

Conclusion

Here we have identified a strong selection determinant consisting of a single amino acid residue at a fixed position in functional Vδ2-Jδ1 junctional regions of polyclonal Vδ2/Vγ9 T-lymphocytes that represent the majority of the peripheral γδ T-cells. The selection determinants present in the germline-encoded Vδ2, Jδ1, Vγ9, and Jγ1.2 segments of the TcR-γδ protein chains have to be identified in further studies. Although our data do not allow discrimination between thymic selection and/or peripheral antigen-driven

expansion, the highly invariant motif in the protein junctional region of virtually all peripheral Vδ2/Vγ9 T-lymphocytes in healthy individuals and different patient groups provide a new perception of TcR specificity and selection processes at the protein level.

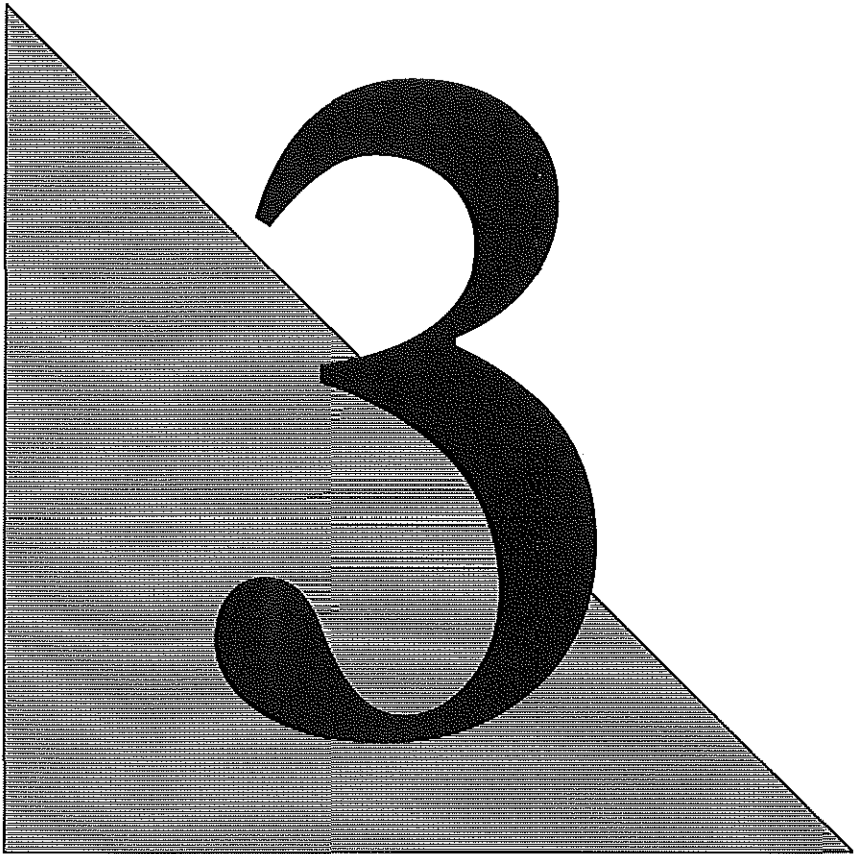
ACKNOWLEDGMENTS. We thank Prof. Dr. R. Benner, Dr. A. Beishuizen, Dr. H. Hooijkaas and Ms. E.J. Mol for their continuous support and Mr. T.M. van Os and Ms. A.D. Korpershoek for assistance in preparation of the manuscript.

REFERENCES

1. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988;334:395-402 and erratum 1988;335:744.
2. Schatz DG, Oettinger MA, Schlissel MS. V(D)J recombination: molecular biology and regulation. Annu Rev Immunol 1992;10:359-383.
3. Triebel F, Faure F, Graziani M, Jitsukawa S, Lefranc M-P, Hercend T. A unique V-J-C-rearranged gene encodes a γ protein expressed on the majority of CD3^{+} T cell receptor-α/β^{-} circulating lymphocytes. J Exp Med 1988;167:694-699.
4. Triebel F, Faure F, Mami-Chouaib F, Jitsukawa S, Griscelli A, Genevée C, Roman-Roman S, Hercend T. A novel human Vδ gene expressed predominantly in the TiγA fraction of γ/δ^{+} peripheral lymphocytes. Eur J Immunol 1988;18:2021-2027.
5. Borst J, Wicherink A, Van Dongen JJM, De Vries E, Comans-Bitter WM, Wassenaar F, Van den Elsen P. Non-random expression of T cell receptor γ and δ variable gene segments in functional T lymphocyte clones from human peripheral blood. Eur J Immunol 1989;19:1559-1568.
6. Casorati G, De Libero G, Lanzavecchia A, Migone N. Molecular analysis of human γ/δ^{+} clones from thymus and peripheral blood. J Exp Med 1989;170:1521-1535.
7. Loh EY, Elliott JF, Cwirla S, Lanier LL, Davis MM. Polymerase chain reaction with single-sided specificity: analysis of T cell receptor δ chain. Science 1989;243:217-220.
8. Tamura N, Holroyd KJ, Banks T, Kirby M, Okayama H, Crystal RG. Diversity in junctional sequences associated with the common human Vγ9 and Vδ2 gene segments in normal blood and lung compared with the limited diversity in a granulomatous disease. J Exp Med 1990;172:169-181.
9. Parker CM, Groh V, Band H, Porcelli SA, Morita C, Fabbi M, Class D, Strominger JL, Brenner MB. Evidence for extrathymic changes in the T cell receptor γ/δ repertoire. J Exp Med 1990;171:1597-1612.
10. Rust CJJ, Verreck F, Vietor H, Koning F. Specific recognition of staphylococcal enterotoxin A by human T cells bearing receptors with the Vγ9 region. Nature 1990;346:572-574.
11. Lefranc M-P, Bonneville M (eds.). 33rd Forum in Immunology: γδ T-cells. Res Immunol 1990;141:579-695.
12. Van Oostveen JW, Breit TM, De Wolf JTM, Brandt RMP, Smit JW, Van Dongen JJM, Borst J, Melief CJM. Polyclonal expansion of T-cell receptor $\gamma\delta^{+}$ T-lymphocytes associated with neutropenia and thrombocytopenia. Leukemia 1992;6:410-418.
13. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning, a laboratory manual, 2nd edn. Cold Spring Harbor: Cold Spring Harbor Laboratory, 1989.
14. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of immunoglobulin and T cell receptor genes. Part I: basic and technical aspects. Clin Chim Acta 1991;198:1-91.
15. Chomczinski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-159.
16. Breit TM, Mol EJ, Wolvers-Tettero ILM, Ludwig W-D, Van Wering ER, Van Dongen JJM. Site-specific deletions involving the *tal*-1 and *sil* genes are restricted to cells of the T cell receptor αβ lineage: T cell receptor δ gene deletion mechanism affects multiple genes. J Exp Med 1993;177:965-977.
17. Breit TM, Wolvers-Tettero ILM, Beishuizen A, Verhoeven M-AJ, Van Wering ER, Van Dongen JJM. Southern blot patterns, frequencies and junctional diversity of T-cell receptor δ gene rearrangements in acute lymphoblastic leukemia. Blood 1993;82:3063-3074.
18. Takihara Y, Reimann J, Michalopoulos E, Ciccone E, Moretta L, Mak TW. Diversity and structure of human T cell receptor δ chain genes in peripheral blood γ/δ-bearing T lymphocytes. J Exp Med 1989;169:393-405.

19. Panchamoorthy G, McLean J, Modlin RL, Morita CT, Ishukawa S, Brenner MB, Band H. A predominance of the T cell receptor Vγ2/Vδ2 subset in human mycobacteria-responsive T cells suggests germline gene encoded recognition. J Immunol 1991;147:3360-3369.
20. De Libero G, Casorati G, Giachino C, Carbonara C, Migone N, Matzinger P, Lanzavecchia A. Selection by two powerful antigens may account for the presence of the major population of human peripheral γ/δ T cells. J Exp Med 1991;173:1311-1322.
21. Uyemura K, Deans RJ, Band H, Ohmen J, Panchamoorthy G, Morita CT, Rea TH, Modlin RL. Evidence for clonal selection of γ/δ T cells in response to a human pathogen. J Exp Med 1991;174:683-692.
22. Ohmen JD, Barnes PF, Uyemura K, Lu S, Grisso CL, Modlin RL. The T cell receptors of human γδ T cells reactive to *mycobacterium tuberculosis* are encoded by specific V genes but diverse V-J junctions. J Immunol 1991;147:3353-3359.
23. Van Dongen JJM, Comans-Bitter WM, Wolvers-Tettero ILM, Borst J. Development of human T lymphocytes and their thymus-dependency. Thymus 1990;16:207-234.
24. Davodeau F, Peyrat MA, Hallet MM, Houde I, Vie H, Bonneville M. Peripheral selection of antigen receptor junctional features in a major human γδ subset. Eur J Immunol 1993;23:804-808.
25. Bucht A, Söderström K, Hultman T, Uhlén M, Nilson E, Kiessling R, Grönberg A. T cell receptor diversity and activation markers in the Vδ1 subset of rheumatoid synovial fluid and peripheral blood T lymphocytes. Eur J Immunol 1992;22:567-574.
26. Forrester JM, Newman LS, Wang Y, King Jr TE, Kotzin BL. Clonal expansion of lung Vδ1$^+$ T cells in pulmonary sarcoidosis. J Clin Invest 1993;91:292-300.
27. Delfau M-H, Hance AJ, Lecossier D, Vilmer E, Grandchamp B. Restricted diversity of Vγ9-JP rearrangements in unstimulated human γ/δ T lymphocytes. Eur J Immunol 1992;22:2437-2443.
28. Bender A, Heckl-Östreicher B, Grondal EJM, Kabelitz D. Clonal specificity of human γδ T cells: Vγ9$^+$ T-cell clones frequently recognize *Plasmodium flaciparum merozoites, Mycobacterium tubercolosis*, and group-A streptococci. Int Arch Allergy Immunol 1993;100:12-18.
29. Sim, G-K, Augustin A. Dominant expression of the T cell receptor BALB invariant δ (BID) chain in resident pulmonary lymphocytes is due to selection. Eur J Immunol 1991;21:859-861.
30. Sim G-K, Augustin A. Extrathymic positive selection of γδ T cells: Vγ4Jγ1 rearrangements with "GxYS" junctions. J Immunol 1991;146:2439-2445.
31. Lafaille JJ, DeCloux A, Bonneville M, Takagaki Y, Tonegawa S. Junctional sequences of T cell receptor γδ genes: implications for γδ T cell lineages and for a novel intermediate of V-(D)-J joining. Cell 1989;59:859-870.
32. Itohara S, Mombaerts P, Lafaille J, Lacomini J, Nelson A, Clarke AR, Hooper ML, Farr A, Tonegawa S. T cell receptor δ gene mutant mice: independent generation of αβ T cells and programmed rearrangements of γδ TcR genes. Cell 1993;72:337-348.
33. Asarnow DM, Cado D, Raulet DH. Selection is not required to produce invariant T-cell receptor γ-gene junctional sequences. Nature 1993;362:158-160.
34. Engel I, Hedrick SM. Site-directed mutations in the VDJ junctional region of a T cell receptor β chain cause changes in antigenic peptide recognition. Cell 1988;54:473-484.
35. Oksenberg JR, Panzara MA, Begovich AB, Mitchell D, Erlich HA, Murray RS, Schimonkevitz R, Sherritt M, Rothbard J, Bernard CCA, Steinman L. Selection for T-cell receptor Vβ-Dβ-Jβ gene rearrangements with specificity for a myelin basic protein peptide in brain lesions of multiple sclerosis. Nature 1993;362:68-70.

REARRANGEMENTS AND DELETIONS OF THE HUMAN TcR-δ GENE



REARRANGEMENTS AND DELETIONS OF THE HUMAN TcR-δ GENE

3.1 Regulation of TcR-δ gene rearrangements in human T-cell differentiation 85

3.2 Southern blot patterns, frequencies and junctional diversity of T-cell receptor δ gene rearrangements in acute lymphoblastic leukemia 95
Published in: Blood 1993;82:3063-3074.

3.3 Two pathways of sequential TcR-δ gene rearrangements in human thymocytes 113
Submitted for publication.

3.4 Rearrangements of the human TcR-δ deleting elements, δREC and $\psi J\alpha$ 129
Published in: Immunogenetics 1994;40:70-75.

3.5 Human T-cells with an active V(D)J recombinase complex for TcR-δ gene deletion 137
Submitted for publication.

CHAPTER 3.1

REGULATION OF TcR-δ GENE REARRANGEMENTS IN HUMAN T-CELL DIFFERENTIATION

Timo M. Breit, Martie C.M. Verschuren, and Jacques J.M. van Dongen

Department of Immunology, Erasmus University/University Hospital Dijkzigt, Rotterdam, The Netherlands.

Introduction

The discovery of the TcR-δ gene in 1987 ended a long search by numerous immunologists for antigen specific receptors on T-cells. The first TcR chain, TcR-β, was found in 1984 by subtractive hybridization techniques (1), as was in the same year the TcR-α chain (2). However, it turned out that the latter TcR chain could not be the TcR-α chain, because it lacked certain consensus glycosylation sites (3). Shortly thereafter, the correct TcR-α chain was identified and the original candidate TcR-α chain was renamed TcR-γ (4,5). Although models in which TcR-γ pairs with either a TcR-α or TcR-β chain were proposed, the discoveries of small subpopulations of peripheral $CD3^+$/TcR-$\alpha\beta^-$ T-lymphocytes (6-11) and γ-chain-containing heterodimer receptors on murine fetal thymocytes earlier in the ontogeny than TcR-αβ expression (12,13), lead to the theory of a fourth TcR-chain, called TcR-δ (6,9). Again by the subtractive hybridization approach the human TcR-δ chain was discovered (14). Independently, the murine TcR-δ gene was isolated about the same time as a rearranging gene located just upstream of the Jα gene segments (15,16). The human and murine TcR-δ genes are quite homologous (reviewed in 17,18).

The human TcR-δ gene

The human TcR-δ gene complex is located on chromosome 14q11 and exists of three commonly used Vδ gene segments (Vδ1, Vδ2, and Vδ3), three Dδ gene segments, four Jδ gene segments and a single Cδ region (Figure 1) (17-20).

The Vδ1 and Vδ2 gene segments are located upstream of the Cδ region, whereas the Vδ3 is found in an inverse orientation just downstream of the Cδ region. Rearrangements involving the Vδ1 or Vδ2 gene segment use the so-called looping-out mechanism in which

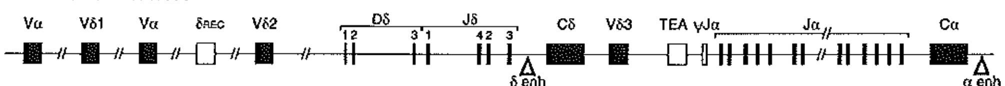


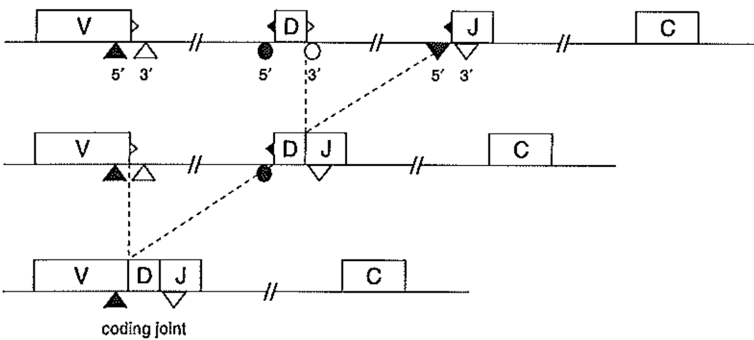
Figure 1. The TcR-α/δ gene complex. Schematic representation of the TcR-δ gene complex within the TcR-α gene complex. Indicated also are the TcR-δ deleting elements, δREC and ψJα, the T early alpha (TEA) element, the TcR-δ gene enhancer (δ enh), and the TcR-α gene enhancer (α enh).

the DNA between the rearranging gene segments is deleted (21). Rearrangements involving the Vδ3 gene segment use an inversional mechanism that inverse the DNA between the rearranging gene segments and preserve the coding joint as well as the signal joint of the rearrangement (Figure 2) (21).

The three Dδ gene segments are located upstream of the Jδ gene segments, and are relatively small (8,9, and 13 nucleotides) (19). The RSS of the Dδ gene segments contain a 12 bp spacer (upstream) or 23 bp spacer (downstream). Because the Vδ and Jδ gene segments have a RSS with a 23 bp spacer and a 12 bp spacer, respectively, potential TcR-δ rearrangements are: Vδ-Jδ, Vδ-Dδ-Jδ, Vδ-Dδ-Dδ-Jδ, and Vδ-Dδ1-Dδ2-Dδ3-Jδ.

Recently the fourth Jδ gene segment was discovered (20). However, the Jδ1 and Jδ3

Deletional rearrangement



Inversional rearrangement

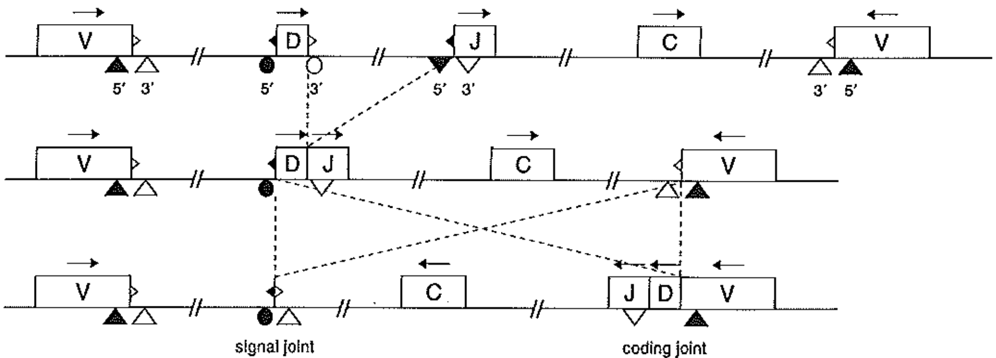


Figure 2. Hypothetical elements in the regulation of V(D)J recombination. **Left**; normal rearrangement using a looping-out mechanism that deletes the DNA sequences between the rearranging gene segments. The regulatory elements belonging to the rearranging side of a gene segment are also deleted. After rearrangement, new combinations of regulatory elements are formed. **Right**; rearrangement using a inversional mechanism that inverts the DNA sequences between the rearranging gene segments. Because the signal joint is incorporated in the genome, the 5′ D regulatory element remains intact but is combined with a 3′ V regulatory element. This combination is probably not capable of rearranging to the upstream located V gene segment.

gene segments are most frequently used in TcR-δ gene rearrangements, whereas use of the Jδ2 and Jδ4 gene segments is less frequently observed (17,18,20). The homology between the human, murine, and ovine Jδ gene segments both on the DNA and protein level is an indication for strong selective pressure on the configuration of the Jδ gene segments (20). The TcR-δ germline sequences are reviewed in Chapter 2.3 (19).

The TcR-δ gene enhancer is located in the Jδ3-Cδ intron (Figure 1) (22). This makes the TcR-δ gene different from the other TcR genes as they contain enhancers that are located 3' of their C regions (Figure 1) (23-25).

TcR-δ gene rearrangement

The TcR-δ gene complex contains only a few gene segments. This allows easy identification of TcR-δ gene rearrangements (Chapter 3.2) (28), and therefore a lot of studies have been performed to determine TcR-δ gene rearrangements in a wide range of cell sources. However, TcR-δ gene deletions by TcR-α gene rearrangements severely hamper studies of TcR-δ gene rearrangements in normal differentiating thymocytes. Recently, the first studies were published in which a partial human TcR-δ gene complex was introduced as a transgene in mice (26,27). The most important pathways of TcR-δ gene rearrangements in early human thymocyte differentiation are described in Chapter 3.3 (and references therein). In the majority of fetal thymocytes the Vδ2 gene segment rearranges to the Dδ3 gene segment, which in turn rearranges to the Jδ3 or Jδ2 gene segment (Vδ2-Dδ3-Jδ3). In post-natal thymocytes the major rearrangement pathway starts with rearrangement of the Dδ2 to Dδ3 gene segment. This Dδ2-Dδ3 gene segment rearranges to the Jδ1 gene segment after which linkage to the Vδ1 gene segment will complete the rearrangement (Vδ1-Dδ2-Dδ3-Jδ1). Although a lot is known about the ordered TcR-δ gene rearrangements, still little is known about the regulation of the sequential pathways of TcR-δ gene rearrangements (27). Except for the TcR-δ enhancer, the RSS of the TcR-δ gene segments, and the promoter sequences of Vδ gene segments, no regulatory elements have been found yet (21,22,29), but it can be expected that several will be detected in the next few years.

Regulation of TcR-δ gene rearrangement

Although, except for the mentioned sequences, no regulatory elements are defined yet in the TcR-δ gene, we can speculate as to where these elements are likely to be found. In this light, it is interesting to note that a signal joint that for instance originates from the inversional Vδ3-Dδ2 rearrangement and is incorporated in the genome, seems not able to further rearrange to another (upstream located) Vδ gene segment (Figure 2). Because Vδ3 gene segments use an inversional recombination mechanism to rearrange to other TcR-δ gene segments, the 5' RSS of the Dδ2 gene segment and all sequences upstream will be left intact. Hence, the newly formed signal joint resembles a Dδ gene segment, because it exists of a nonamer-12 bp spacer-heptamer-heptamer-23 bp-nonamer sequence (Figure 2). The only difference is the lack of nine Dδ2 nucleotides between the heptamer sequences (Figure 2). There are several likely explanations for the phenomenon of this repressed Vδ rearrangement: because the Cδ region is inverted, the TcR-δ enhancer is also orientated in the opposite direction. This might prevent further rearrangements, if the TcR-δ enhancer works unidirectional, which is in general not the case with enhancers. Above all, the

enhancer orientation probably does not make any difference because Vδ → Dδ rearrangement is TcR-δ enhancer independent (27). But there might also be another explanation: the sequence downstream of, or even the Dδ sequence itself, may play an important role in the regulation of recombination. Because there are also regulatory (= promoter) sequences located upstream of the Dδ2 gene segment, an attractive explanation remains; sequences at both sides of a Dδ gene segment (or any rearranging gene segment for that matter) might be important in the regulation of the rearrangement processes. Because a rearrangement of a gene segment will always replace one side of a gene segment with one side of the other rearranging gene segment, the newly formed coding joint, as well as signal joint of a rearrangement will have another combination of upstream and downstream signals than any original set of signals in the germline TcR-δ gene (Figure 2). This will allow discrimination of germline and rearranged gene segments by the recombinase complex. Although there is no hard evidence to support this theory, it still seems an attractive model for regulation of ordered Ig and TcR rearrangements, and might be worthwhile to investigate.

TcR-δ gene deletion

A curious feature of the TcR-δ gene is its location within the TcR-α gene (Figure 1) (17,18,21,28). Because of this, TcR-α rearrangement will delete the TcR-δ gene. It is obvious that the TcR-α/δ locus plays a crucial role in the bifurcation of the αβ and γδ lineages (17,18,21,29). This theory is supported by the observation that the TcR-δ gene is flanked by the so-called TcR-δ deleting elements, δREC and ψJα (Figure 1). De Villartay et al. (30) discovered in 1988 a rearrangement that was prominently present in human thymus cell samples. This δREC-ψJα rearrangement turned out to be a non-functional rearrangement that is not transcribed (30,31). Except for the Vδ1 gene segment all TcR-δ gene segments lie between the δREC and ψJα gene segments and will therefore be deleted after rearrangement of these deleting elements to each other. The junctional regions of δREC-ψJα rearrangements rarely contain Dδ gene segments and therefore resemble TcR-α gene rearrangements (32). The regulation of this particular rearrangement seems to be depending partly on the TcR-δ enhancer and partly on the TcR-α enhancer. Although no definitive proof has been provided, it appears that the δREC gene segment is regulated by the TcR-δ enhancer, whereas the ψJα gene segment is regulated by the TcR-α enhancer. Transcription of the so-called TEA (T early alpha element which is located just upstream of the ψJα gene segment) results, after splicing to Cα, in a sterile transcript (Figure 1). This TEA transcription seems to initiate rearrangement to the ψJα gene segment (33-35). It is curious though, that this TEA element activates the ψJα gene segment (which is the most 5′ of the 61 Jα gene segments) for recombination, and is then deleted by the δREC-ψJα rearrangement. It is obvious that transcription of the TEA element may be the trigger for a thymocyte to enter the αβ lineage, but we have to wait for the TEA knock-out mice to find out the significance of this step in T-cell differentiation.

There are still several questions left with regard to the TcR-δ deleting elements. The δREC-ψJα rearrangement is present as a preferential rearrangement in post-natal thymocytes (30-32). However, the major pathway of TcR-δ gene rearrangements in post-natal thymocytes ends in Vδ1-Dδ2-Dδ3-Jδ1 rearrangements. This and all other rearrangements involving the Vδ1 gene segment delete the downstream located δREC gene segment. Thus,

δREC-ψJα rearrangements are definitively not a consequential step of the major pathway of post-natal TcR-δ gene rearrangements. The remaining question therefore is: what is deleted by the δREC-ψJα gene rearrangement? Is it: TcR-δ genes in germline configuration, (in)complete TcR-δ gene rearrangements, or both? Of interest is also the question what triggers TcR-α gene rearrangement after δREC-ψJα rearrangement and TEA deletion. It might be just activation by the TcR-α enhancer, or it may be that the joined δREC and ψJα gene segments provide a new signal for TcR-α rearrangement (Figure 2). But that does not solve the problem that Vδ1-Jδ1 rearrangements cannot be deleted by δREC-ψJα and an active TEA element remains on the same allele until TcR-α gene rearrangement. It might be that the deletion caused by Vδ1 rearrangement is enough to bring the Vα gene segments within reach of the TcR-α enhancer so that TcR-α rearrangements can occur.

Regulation of the TcR-δ gene deletion

One of the most intriguing features of the δREC-ψJα rearrangement is the great preference in which these two gene segments rearrange to each other despite several other active gene segments (Chapters 3.3 and 3.4). Although δREC can potentially rearrange to all Dδ, Jδ, and Jα gene segments, it still rearranges preferentially to the ψJα gene segment and it is difficult to believe that this is solely based on the fact that ψJα is the most 5′ Jα gene segment. With distance being the only condition, one would expect a lot of δREC-(Dδ)-Jδ gene rearrangements. Therefore, other regulatory mechanisms probably play an important role in this particular rearrangement. This is supported by the observation that the ψJα gene segment is able to preferentially rearrange to the δREC gene segment, which is surrounded by Vδ and Vα gene segments. As proposed before, the RSS of a certain gene segment might be flanked by regulatory sequences, which may play a role in gene segment recognition (Figure 2). It might also be that the RSS itself is involved in this regulation. The heptamer and nonamer sequences in a RSS are highly conserved and it is not to be expected that they play any role other than being the "core" sequence of a RSS. This is not the case for the 12 or 23 bp spacers that separate them, which show such a limited homology, that for long it has been assumed that their entire role was, to determine the space between a heptamer and a nonamer (36). However, along with the increasing data about RSS sequences, rose the appreciation of the importance of spacers as regulatory elements in V(D)J recombination (reviewed in 21, 37). Conserved homologies, although sometimes cryptic, between genes and categories of gene segments indicate an important role in gene recognition. In line with these observations, we have searched for homologies in the RSS of the δREC and ψJα gene segments (31). It was surprisingly easy to assign almost all nucleotides of both spacers to consensus sequences. It turned out that the 23 bp spacer of the δREC RSS could be divided in three short sequence motifs, which might represent binding-sites for DNA-binding proteins (Figure 3). Comparison with spacer sequences of related gene segments revealed some interesting points. The first sequence (I in Figure 3) was also detected in the Vδ2 RSS, the Vδ3 RSS, and in some breakpoints of chromosomal translocations involving the TcR-δ gene (see Chapter 4.1). The second sequence (II in Figure 3) was completely present in the ψJα RSS and inversively located in the Vδ3 RSS. The last sequence (III in Figure 3) was found by alignment of the RSS involved in a homologous deletion rearrangement in the Igκ gene (discussed below) (38). This sequence was also observed in the Dδ3-3′ RSS. Hence the RSS of the δREC gene

```
RSS            heptamer |........spacer.......| nonamer
Motif                I        II         III              I   II  III
consensus      CACAGTGATGCAGGGGCACCTGCNNCCCGTACAAAAACC

RSS δREC       ---G--------TA-------CGAC-----G-CT-----    +   +   +

RSS Vδ2        ---CC--C-----CTCT--T-CTGAG-A-CT--------    +   -   -
RSS Vδ3 →      ---TA---------T--C-AG-AAGT-ATA---C----A    +   -   -
RSS Jκ-Cκ      ---------A--AATAATG···········C--CT--GG    +   -   -

Spacer Vδ3 ←   ··TATGAC-T-CT----------AT··············    -   +   -
RSS ψJα        -----GA····GT--------TT·······---------    -   +   -

RSS Kde        -------TGCGCT-CCA------TG-----G--G----T    -   +   +

RSS Dδ3-3´     -------C-A--AAAC-TA-A-AGA--T----------T    -   -   +

RSS Vδ1        -------T-TG-A-T-ATAG-AAAAG-AAA---------    -   -   -
```

Figure 3. Consensus sequences in the spacer of the δREC RSS. Three consensus sequences (I-III) are identified in the spacer of the δREC RSS after alignment with RSS of related gene segments. On the right is indicated which of the three sequences are present in the aligned spacer. Several combinations of these sequences are present and they may represent a regulatory mechanism for V(D)J recombination. -, nucleotide is identical to the consensus sequence; ·, gap for alignment.

segment is formed by at least five sequences that may represent protein binding sites. We cannot formally rule out the possibility that these sequences are present just because these gene segments are evolutionary related, but the fact that for instance the Vδ1 RSS does not contain any of the conserved sequences does not support this suggestion (Figure 3). Although no effort was made to align the sequences (just) outside the RSS, it can be expected that other homologous binding sites can be found. For now, it is uncertain whether these short sequences represent regulatory elements in the complex system of V(D)J recombination and if so, what their role might be. But it can be hypothesized, that (combinations) of proteins for a specific gene segment (or family of gene segments) regulate the ordered manner of TcR gene rearrangements.

Because the δREC-ψJα rearrangement occurs in a particular stage of the thymocyte differentiation, we expect that there are specific proteins or specific combinations of proteins that define this differentiation stage. Research to identify the involved protein(s) has been initiated and the first progress was the identification of monoclonal cell populations (T-ALL and T-cell lines) that actively rearrange the δREC to ψJα gene segment (Chapter 3.5).

The δREC-ψJα homologue in the Igκ gene

As with many characteristics of TcR genes and gene rearrangements, the TcR-δ deleting elements have a homologue in the Ig genes: the Igκ deleting element (Kde). Although not obligatory, the Igκ genes are generally deleted before Igλ rearrangement (39,40). This deletional rearrangement involves on the one hand the Kde gene segment that is located ~24 kb downstream of the Cκ region, and on the other hand an isolated RSS in the Jκ-Cκ intron, or a RSS of a Vκ gene segment (38,41-44). Thus either the Cκ or Jκ-Cκ region is deleted. Deletion of one lineage specific gene before

rearranging another gene belonging to a different lineage is just one similarity between δREC-ψJα and Kde rearrangements. Another, more surprising homology was observed in the RSS of δREC and Kde, both of which have a RSS with a 23 bp spacer (Figure 3). Although no cross-lineage δREC-ψJα rearrangements or Kde rearrangements are observed in B and T-cells, respectively (Beishuizen, personal communication), their RSS share a unique sequence adjacent to the nonamer (sequence III in Figure 3), at which position other gene segments such as Jα contain an extremely homologous Jα specific sequence (45). But also the Jκ-Cκ intron RSS showed some homology with the spacer sequence (I in Figure 3) adjacent to the heptamer. This homology may imply that both deletional rearrangements are sharing some unique motifs and may therefore use homologous V(D)J recombinase complexes.

Concluding remarks and speculations

The TcR-δ gene is unique by its location within the TcR-α gene and by the presence of the TcR-δ deleting elements, δREC and ψJα. The ordered fashion of TcR-δ gene rearrangement, TcR-δ gene deletion (δREC-ψJα rearrangement), and TcR-α rearrangement shows an important role for the TcR-α/δ locus in lineage commitment of maturing thymocytes.

The sequence homologies in the RSS of related gene segments such as δREC, ψJα, Kde, and Vδ2/3 provide new leads in the search for regulatory elements in TcR gene rearrangement processes. They might also provide new insight in the "true" nature of TcR-δ gene segments. It is peculiar that the RSS of the δREC, Vδ2, and Vδ3 gene show homology, whereas those of the Vδ1 and Jδ gene segments do not. It is also strange that the Vδ1 gene segment lies "outside" the δREC-ψJα rearrangement. These facts and the point that Vδ1 is more homologous to Vα gene segments than Vδ2 or Vδ3 (17,21,46), lead us to the speculation that rearrangement of the Vδ1 gene segment may represent an intermediate form of Vα → Jδ (Vδ1-Jδ1) between Vδ → Jδ (Vδ2-Jδ1) and Vα → Jα (Vαn-Jαn) rearrangements. By defining as such a new TcR-γδ class, which is characterized by a Vα alike (Vδ1) gene segment in the TcR-δ chain, alternative points of view may arise. Because "true" TcR-$\gamma\delta^+$ cells contain a TcR-δ gene rearrangement that can be deleted by the specifically deleting δREC-ψJα rearrangement, they may use a form of peripheral negative selection. Hence, there maybe a difference in the thymic selection processes of "true" TcR-$\gamma\delta^+$, Vδ1 TcR-$\gamma\delta^+$, and TcR-$\alpha\beta^+$ thymocytes. We did try to prove negative selection of TcR-$\gamma\delta^+$ T-lymphocytes in the periphery by δREC-ψJα rearrangement, but our efforts thus far were severely hampered not only by the peripheral expansion of Vγ9/Vδ2 T-lymphocytes (see Chapter 2.5), but also by the abundant presence of circular excision products of the δREC-ψJα rearrangement in peripheral T-lymphocytes. So far, the whole model of "true" TcR-$\gamma\delta^+$ T-cells and Vδ1 TcR-$\gamma\delta^+$ T-cells is speculation, and without any real evidence it will not become anything more than just an interesting point of discussion.

REFERENCES

1. Hedrick SM, Cohen DI, Nielsen EA, Davis MM. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. Nature 1984;308:149-153.
2. Saito H, Kranz DM, Takagaki Y, Hayday AC, Eisen HN, Tonegawa S. Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequences. Nature 1984; 309:757-762.
3. Allison JP, Lanier LL. The structure, function and serology of the T cell antigen receptor complex. Annu Rev Immunol 1987;5:503-540.
4. Chien Y-H, Becker DM, Lindsten T, Okamura M, Cohen DI, Davis MM. A third type of murine T-cell receptor gene. Nature 1984;312:31-35.
5. Saito H, Kranz DM, Takagaki Y, Hayday AC, Eisen HN, Tonegawa S. A third rearranged and expressed gene in a clone of cytotoxic T lymphocytes. Nature 1984; 312:36-40.
6. Brenner MB, McLean J, Dialynas D, Strominger J, Smith JA, Owen FL, Seidman J, Ip S, Rosen F, Krangel M. Identification of a putative second T cell receptor. Nature 1986;322:145-149.
7. Bank I, DePinho RA, Brenner MB, Cassimeris J, Alt FW, Chess L. A functional T3 molecule associated with a novel heterodimer on the surface of immature human thymocytes. Nature 1986;322:179-181.
8. Moingeon P, Ythier A, Goubin G, Faure F, Nowill A, Delmon L, Rainaud M, Forestier F, Daffos F, Bohuon C, Hercend T. A unique T-cell receptor complex expressed on human fetal lymphocytes displaying nature-killer-like activity. Nature 1986;323:638-640.
9. Lew AM, Pardoll DM, Maloy WL, Fowlkes BJ, Kruisbeek A, Cheng S-F, Germain RN, Bleustone JA, Schwartz RH, Coligan JE. Characterization of T-cell receptor gamma chain expression in a subset of murine thymocytes. Science 1986;234:1401-1405.
10. Moingeon P, Jitsukawa S, Faure F, Troalen F, Triebel F, Graziani M, Forestier F, Bellet D, Bohuon C, Hercend T. A γ-chain complex forms a functional receptor on cloned human lymphocytes with natural killer-like activity. Nature 1987;325:723-726.
11. Littman DR, Newton M, Crommie D, Ang S-L, Seidman JG, Gettner SN, Weiss A. Characterization of an expressed CD3-associated Ti γ-chain reveals Cγ domain polymorphism. Nature 1987;326:85-88.
12. Nakanishi N, Maeda K, Ito KI, Heller M, Tonegawa S. Tγ protein is expressed on murine fetal thymocytes as a disulfide-linked heterodimer. Nature 1987;325:720-723.
13. Pardoll DM, Fowlkes BJ, Bleustone JA, Kruisbeek A, Maloy WL, Coligan JE, Schwartz RH. Differential expression of two distinct T-cell receptors during thymocyte development. Nature 1987;326:79-81.
14. Hata S, Brenner MB, Krangel MS. Identification of putative human T cell receptor δ complementary DNA clones. Science 1987;238:678-682.
15. Lindsten T, Fowlkes BJ, Samelson LE, Davis MM, Chien Y-H. Transient rearrangements of the T cell antigen receptor α locus in early thymocytes. J Exp Med 1987;166:761-775.
16. Chien Y, Iwashima M, Kaplan KB, Elliott JF, Davis MM. A new T-cell receptor gene located within the alpha locus and expressed early in T-cell differentiation. Nature 1987;327:677-682.
17. Raulet DH. The structure, function, and molecular genetics of the γ/δ T cell receptor. Annu Rev Immunol 1989;7:175-207.
18. Porcelli S, Brenner MB, Band H. Biology of the human $\gamma\delta$ T-cell receptor. Immunol Rev 1991;120:137-183.
19. Breit TM, Van Dongen JJM. Unravelling human T-cell receptor junctional sequences. Thymus 1994;22:177-199.
20. Davodeau F, Peyrat M-A, Hallet M-M, Vié H, Bonneville M. Characterization of a new functional TCR Jδ segment in humans. J Immunol 1994;153:137-142.
21. Lewis SM. The mechanism of V(D)J joining: lessons from molecular, immunological, and comparative analyses. Adv Immunol 1994;56:27-150.
22. Redondo JM, Hata S, Brocklehurst C, Krangel MS. A T cell-specific trancriptional enhancer within the human T cell receptor delta locus. Science 1990;247:1225-1229.
23. Leiden JM. Trancriptional regulation during T-cell development: the α TCR gene as a molecular model. Immunol Today 1992;13:22-30.
24. Leiden JM. Trancriptional regulation of T cell receptor genes. Annu Rev Immunol 1993;11:539-570.
25. Hettmann T, Cohen A. Indentification of a T cell-specific transcriptional enhancer 3' of the human T cell receptor gamma locus. Mol Immunol 1994;31:315-322.
26. Lauzurica P, Krangel MS. Enhancer-dependent and -independent steps in the rearrangement of a human T cell receptor δ transgene. J Exp Med 1994;179:43-55.
27. Lauzurica P, Krangel MS. Temporal and lineage-specific control of T cell receptor α/δ gene rearrangements by T cell receptor α and δ enhancers. J Exp Med 1994;179:1913-1921.

28. Breit TM, Wolvers-Tettero ILM, Beishuizen A, Verhoeven M-AJ, Van Wering ER, Van Dongen JJM. Southern blot patterns, frequencies, and junctional diversity of T-cell receptor δ gene rearrangements in acute lymphoblastic leukemia. Blood 1993;10:3063-3074.
29. Van Dongen JJM, Comans-Bitter WM, Wolvers-Tettero ILM, Borst J. Development of human T-lymphocytes and their thymus-dependency. Thymus 1990;16:207-234.
30. De Villartay JP, Hockett RD, Coran D, Korsmeyer SJ, Cohen DI. Deletion of the human T-cell receptor delta gen by a site-specific recombination. Nature 1988;335:170-174.
31. Hockett RD Jr, Nuñez G, Korsmeyer SJ. Evolutionary comparison of murine and human δ T-cell receptor deleting elements. New Biologist 1989;1:266-274.
32. Breit TM, Wolvers-Tettero ILM, Bogers AJJC, De Krijger RR, Wladimiroff JW, Van Dongen JJM. Rearrangements of the human TcRD-deleting elements. Immunogenetics 1994;40:70-75.
33. Hockett RD, De Villartay J-P, Pollock K, Poplack DG, Cohen DI, Korsmeyer SJ. Human T-cell antigen receptor (TCR) δ-chain locus and elements responsible for its deletion are within the TCR α-chain locus. Proc Natl Acad Sci USA 1988;85:9694-9698.
34. De Villartay JP, Lewis D, Hockett RD, Waldmann TA, Korsmeyer SJ, Cohen DI. Deletional rearrangement in the human T-cell receptor α-chain locus. Proc Natl Acad Sci USA 1987;84:8608-8612.
35. De Chasseval R, De Villartay J-P. Functional characterization of the promoter for the germline T-cell receptor Jα (TEA) transcript. Eur J Immunol 1993;23:1294-1298.
36. Schatz DG, Oettinger MA, Schlissel MS. V(D)J recombination: moleculair biology and regulation. Annu Rev Immunol 1992;10:359-383.
37. Ramsden DA, Baetz K, Wu GE. Conservation of sequence in recombination signal sequence spacers. Nucleic Acid Res 1994;22:1785-1796.
38. Graninger WB, Goldman PL, Morton CC, O'Brien SJ, Korsmeyer SJ. The κ-deleting element: germline and rearranged, duplicated and dispersed forms. J Exp Med 1988;167:488-501.
39. Hieter PA, Korsmeyer SJ, Waldmann TA, Leder P. Human immunoglobulin κ light-chain genes are deleted or rearranged in λ-producing B cells. Nature 1981;290:368-372.
40. Korsmeyer SJ, Hieter PA, Sharrow SO, Goldman CK, Leder P, Waldmann TA. Normal human B cells display ordered light chain gene rearrangements and deletions. J Exp Med 1982;156:975-985.
41. Siminovitch KA, Bakhshi A, Goldman P, Korsmeyer SJ. A uniform deleting element mediates the loss of κ genes in human B cells. Nature 1985;316:260-262.
42. Klobeck HG, Zachau HG. The human Cκ gene segment and the kappa deleting element are closely linked. Nucleic Acid Res 1986;14:4591-4603.
43. Siminovitch KA, Moore MW, Durdik J, Selsing E. The human kappa deleting element and the mouse recombining segment share DNA sequence homology. Nucleic Acid Res 1987;15:2699-2705.
44. Beishuizen A, Verhoeven M-AJ, Mol EJ, Van Dongen JJM. Detection of immunoglobulin kappa light-chain gene rearrangement patterns by southern blot analysis. Leukemia 1994;in press.
45. Koop BF, Rowen L, Wang K, Lam Kuo C, Seto D, Lenstra JA, Howard S, Shan W, Deshpande P, Hood L. The human T-cell receptor TCRAC/TCRDC (Cα/Cδ) region: organization, sequence, and evolution of 97.6 kb of DNA. Genomics 1994;19:478-493.
46. Hochstenbach F, Brenner MB. Newly identified γδ and βδ T-cell receptors. J Clin Immunol 1990;10:1-18.

CHAPTER 3.2

SOUTHERN BLOT PATTERNS, FREQUENCIES AND JUNCTIONAL DIVERSITY OF T-CELL RECEPTOR δ GENE REARRANGEMENTS IN ACUTE LYMPHOBLASTIC LEUKEMIA*

Timo M. Breit[1], Ingrid L.M. Wolvers-Tettero[1], Auke Beishuizen[1], Marie-Anne J. Verhoeven[1], Elisabeth R. van Wering[2] and Jacques J.M. van Dongen[1]

1. Department of Immunology, Erasmus University/University Hospital Dijkzigt, Rotterdam; 2. Dutch Childhood Leukemia Study Group, The Hague, The Netherlands.

ABSTRACT

Southern blot analysis of TcR-δ gene rearrangements is useful for diagnostic studies on clonality of lymphoproliferative diseases. We have developed 18 new TcR-δ gene probes by use of the PCR techniques. Application of these probes for detailed analysis of the TcR-δ genes in normal control samples, 138 T-ALL, and 91 precursor B-ALL allowed us to determine the TcR-δ gene restriction map for five restriction enzymes, as well as the Southern blot restriction enzyme patterns of all theoretically possible TcR-δ gene rearrangements. Based on this information, it appeared that 97% of all 213 detected TcR-δ gene rearrangements in our series of ALL could be detected by use of the TCRDJ1 probe and that the majority (76%) of the 213 rearrangements could be identified precisely. In T-ALL, we found a strong preference for the complete rearrangements Vδ1-Jδ1 (33%), Vδ2-Jδ1 (10%), and Vδ3-Jδ1 (7%) and the incomplete rearrangement Dδ2-Jδ1 (11%). In precursor B-ALL, the majority of rearrangements consisted of Vδ2-Dδ3 (72%) and Dδ2-Dδ3 (10%). The junctional diversity of these six preferential TcR-δ rearrangements was analyzed and showed an extensive junctional insertion (~30 nucleotides) for complete Vδ-Jδ rearrangements, whereas incomplete rearrangements had correspondingly smaller junctional regions. The detailed TcR-δ gene restriction map and probes presented here, in combination with the Southern blot patterns of TcR-δ gene rearrangements, are important for TcR-δ gene studies in ALL: all TcR-δ gene rearrangements can be detected and the majority can be identified precisely. Identification of rearrangements is a prerequisite for subsequent PCR analysis of TcR-δ gene junctional regions, e.g. for detection of minimal residual disease during follow-up of ALL patients.

INTRODUCTION

The two types of antigen-specific TcR molecules are the heterodimers, TcR-αβ and TcR-γδ, in which each protein chain consists of a variable antigen-recognizing domain and

* Published in: Blood 1993;82:3063-3074.

a C domain (1,2). The variable domain is encoded by a V gene segment, (D gene segments), a J gene segment, and a junctional region linking these segments together (1,2). The V,(D), and J gene segments are joined together by rearrangement processes that are mediated via RSS (1,3-6) and regulated by RAG1 and RAG2 (7,8). During the rearrangement processes, deletion of nucleotides by trimming the recombining gene segments frequently occurs (9), as does insertion of so-called P-region nucleotides (10) and N-region nucleotides (9). P-region nucleotides represent junctional region nucleotides which are derived from the adjacent, untrimmed gene segment (10), whereas the N-region nucleotides are randomly inserted at the junctions by the enzyme TdT (9). The possible different combinations of V, (D), and J gene segments of each TcR gene determine the potential combinatorial diversity of TcR molecules (1). The junctional diversity, determined by the junctional region, is made up by D-gene-derived nucleotides (in case of TcR-β and TcR-δ genes) (11-13), P-region nucleotides (10), N-region nucleotides (9,10,12-16), and deletion of nucleotides by trimming of the involved gene segments (9,10,12-16).

The human TcR-δ gene locus is located within the TcR-α gene locus between the long stretch of Vα and the Jα gene segments on chromosome 14p11 (13,17-21). The TcR-δ gene consists of at least six Vδ gene segments, three Dδ gene segments, three Jδ gene segments and one Cδ region (13,17-21) and is flanked by TcR-δ deleting elements (22,23) (Figure 1). In humans, these deleting elements are ψJα, located 3′ of the TcR-δ gene locus, and δREC, located 5′ of the major part of the TcR-δ gene locus but 3′ of most Vδ gene segments (22,23). Rearrangement of the deleting elements to each other, or rearrangement of Vα to Jα gene segments, causes deletion of the intermediate TcR-δ gene locus (22-25).

The potential combinatorial diversity of the TcR-δ chain is limited due to the low number of V, D, and J gene segments (1). The actual combinatorial diversity is even more restricted due to preferential usage of particular Vδ and Jδ gene segments. For instance, approximately 85% of the TcR-$\gamma\delta^+$ T-lymphocytes in PB of most individuals express receptors containing a Vδ2-Jδ1-Cδ chain (26-30) and approximately 60% of TcR-$\gamma\delta^+$ thymocytes express a Vδ1-Jδ1-Cδ chain (27,29,30). The far majority of TcR-$\gamma\delta^+$ T-ALL contain at least one Vδ1-Jδ1 rearrangement (31,32) and even cross-lineage TcR-δ gene rearrangements in precursor B-ALL show preferential rearrangement patterns, i.e. Vδ2-Dδ3 and Dδ2-Dδ3 (33-35). The limited TcR-δ combinatorial diversity is compensated by extensive junctional diversity (13,16,36-39), which is especially caused by the fact that up to three Dδ gene segments, and therefore up to four N-regions, can occur in the junctional region of a complete Vδ-Jδ gene rearrangement (13,36-38).

Because the potential combinatorial diversity of the human TcR-δ gene is limited, it should be possible to determine the Southern blotting restriction pattern for each theoretically possible TcR-δ gene rearrangement. This would allow rapid identification of the various TcR-δ gene rearrangements by Southern blotting, which is important for diagnostic studies on clonality of lymphoproliferative diseases at diagnosis and during follow-up (40). For this purpose, we designed a large set of 18 new Vδ, Dδ and Jδ DNA probes, which were used to identify the various types of TcR-δ gene rearrangements and to inventory their frequencies in a large series of 91 precursor B-ALL and 138 T-ALL. The latter were divided in three phenotypic subgroups: $CD3^-$ T-ALL, TcR-$\gamma\delta^+$ T-ALL, and TcR-$\alpha\beta^+$ T-ALL. Identification of the TcR-δ gene rearrangements in these T-ALL and precursor B-ALL showed a strong preference for six types of rearrangement. The TcR-δ junctional diversity

in the ALL was studied by PCR mediated amplification and subsequent direct sequencing of the junctional regions of the six preferential rearrangements. The observed extensive junctional diversity in the majority of TcR-δ junctional regions can be applied as unique clonal markers of leukemic cells in the detection of MRD by use of PCR techniques (41-44).

METHODS

Cell samples

MNC were obtained from 138 different T-ALL patients and 91 precursor B-ALL patients and PB granulocytes obtained from 50 healthy individuals. MNC of the ALL patients were isolated from PB or BM by Ficoll-Paque (density: 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. All MNC samples were frozen and stored in liquid nitrogen. TcR-δ gene configurations of 13 TcR-$\gamma\delta^{+}$ T-ALL have been described previously (32,38). The PB granulocytes from healthy individuals were obtained by NH_4Cl lysis of the cell pellets after ficoll density centrifugation. These cells were directly used for control DNA isolation.

Immunologic marker analysis

The MNC of the T-ALL patients were analyzed for nuclear expression of TdT, for cytoplasmic expression of CD3 (UCHT1); for membrane expression of T-cell markers CD1 (66IIC7), CD2 (Leu-5b), CD3 (Leu-4), CD4 (Leu-3a), CD5 (Leu-1), CD6 (OKT17), CD7 (3A1) and CD8 (Leu-2a); for the HLA-DR antigen; and for reactivity with McAbs, BMA031 (anti-TcR-αβ), 11F2 (anti-TcR-γδ), TCRδ1 (anti-TcR-δ), Ti-γA (anti-TcR-Vγ9), δTCS1 (anti-TcR-Vδ1), and BB3 (anti-TcR-Vδ2). The MNC of the precursor B-ALL patients were analyzed for nuclear expression of TdT; for cytoplasmic expression of Ig heavy chain μ (Cyμ); for membrane expression of the B-cell markers CD9 (BA-2), CD10 (VIL-A1), CD19 (B4), CD20 (B1), CD22 (Leu-14), and CD37 (Y29/55); for membrane expression of the precursor marker CD34 (BI-3C5); and for HLA-DR antigen. A leukemia was considered to be a precursor B-ALL if the malignant cells were positive for TdT, CD19, and HLA-DR (null ALL); or for TdT, CD10, CD19, and HLA-DR (common ALL); or for TdT, CD10, CD19, HLA-DR, and Cyμ (pre-B-ALL). The rabbit anti-TdT antiserum was purchased from Supertechs (Bethesda, MD); the McAb of the Leu series, anti-HLA-DR, and 11F2 were obtained from Becton Dickinson (San Jose, CA); the CD1 antibody was obtained from Monosan/Sanbio (Nistelrode, the Netherlands); the OKT17 from Ortho Diagnostic System (Raritan, NJ); the 3A1 hybridoma from the American Type Culture Collection (Rockville, MD); TCRδ1 and δTCS1 were obtained from T-Cell Diagnostics (Cambridge, MA); anti-Ig heavy chain μ from Kallestad Laboratories (Austin, TX); BA-2 from Hybritech (San Diego, CA); B4 and B1 were obtained from Coulter Clone (Hialeah, FL); BI-3C5 was obtained from Seralab (Crawley Down, UK). The McAb BMA031, Ti-γA, and BB3 were kindly provided by Dr. R. Kurrle (Behring, Marburg, Germany), Dr. T. Hercend (Villejuif, France), and Dr. L. Moretta (Genove, Italy), respectively; VIL-A1 was kindly provided by Dr. W. Knapp (Vienna, Austria) and Y29/55 was kindly provided by Dr. H.K. Forster (Hoffman-La Roche, Basel, Switzerland). The immunofluorescence stainings were performed as described (45) and evaluated with fluorescence microscopes (Carl Zeiss, Oberkochen, Germany) and/or a FACScan flowcytometer (Becton Dickinson).

Isolation of TcR-δ gene DNA probes

TcR-δ gene DNA probes were obtained by cloning the purified PCR amplification products of granulocyte DNA from a healthy donor using specific oligonucleotide primer sets. pUC19 was used as cloning vector (46). The oligonucleotide primer sets for the TCRDV1 (Vδ1), TCRDV2 (Vδ2), TCRDV3 (Vδ3), TCRDV4 (Vδ4), TCRDV5 (Vδ5), TCRDV6 (Vδ6), TCRDD1U (Dδ1 upstream), TCRDD1 (Dδ1), TCRDD2 (Dδ2), TCRDD3U (Dδ3 upstream), TCRDD3 (Dδ3), TCRDJ1 (Jδ1), TCRDJ2 (Jδ2), TCRDJ3 (Jδ3), TCRDC1 (Cδexon1), TCRDC4 (Cδexon4), TCRDRE (δREC), and TCRAPJ (ψJα) probes are given in Table 1. All oligonucleotide primers were synthesized according to our own or published TcR-δ gene sequence data (13,18,19,24,46-51) on a 392 DNA synthesizer (Applied Biosystems, Forster City, CA) with the solid-phase phosphotriester method and used without further purification. All TcR-δ gene DNA probes will be submitted to the American Type Culture Collection and can be used for noncommercial purposes.

TABLE 1. Oligonucleotide primers used in PCR and sequencing analysis of TcR-δ gene rearrangements or construction of TcR-δ gene DNA probes.

Name	Size	Cloning sites	Code	Position[a]	Sequence[b]	Reference[c]
TcR-δ gene rearrangements[d]					5′ 3′	
		*Bgl*II/*Xba*I	Vδ4-5′XBg	−240 bp	gaagatctaGAATGTTCGTGCAGGAAAAGGAGG	48
			Vδ4-3′	−50 bp	ATCCGCCAACCTTGTCATCTCCG	48
		*Bgl*II/*Xba*I	Vδ5-5′XBg	∼−138 bp	gaagatctagATTCACCATCCCTGAGCGTCCAG	50
			Vδ6-3′	∼−29 bp	TATCATGGATTCCCAGCCTGGAG	47
		*Xba*I	Jδ2-5′X	+35 bp	ctagtctagaTTACCTGGTTCCACGATGAGTTGT	19
		*Sal*I	Jδ2-3′S	+64 bp	cgcgtcgaCCCCCTGAAGCTGTAGTAAAATGC	19
		*Sal*I	Jδ3-3′S	+88 bp	cgcgtcGACTCAAATTATCCCAGAAATATAGG	19
TcR-δ gene DNA probes						
TCRDV1	562 bp	*Hin*dIII–*Eco*RI	Vδ1p5′	−542 bp	cataagcTTCTCCAGCCTGCTGTGTGTATTT	18
			Vδ1p3′	−17 bp	CACTGTGAaTTCCCCAAGAGCAC	18
TCRDV2	491 bp	*Hin*dIII–*Sma*I	Vδ2p5′	−472 bp	gtgaagctTGCAGAGGATCTCCTCCCTCATC	51
			Vδ2p3′	−27 bp	GTCACAGGCACAGTAGTAAGACC	51
TCRDV3	335 bp	*Eco*RI–*Hin*dIII	Vδ3p5′	−321 bp	GGGTGCAGAATTCACTATTTCCTC	49
			Vδ3p3′	−26 bp	cataagctTAAAGGCACAGTAGTAAGTGGCAC	49
TCRDV4	496 bp	*Eco*RI–*Bgl*II	Vδ4p5′	−487 bp	ctggaaTTCTAGCCTGCTGAAGGTGGTC	48
			Vδ4p3′	−37 bp	gaagatctagaGCACAGAAATACATTGCTGAGTCC	48
TCRDV5	∼466 bp	*Eco*RI–*Bgl*II	Vδ5p5′	∼−46 bp	tgcgaATTCTGTGGCTTCAGCCAGACTG	50
			Vδ5p3′	∼−470 bp	gaagatctaGAGTCTCCAGGCTGGGAGGG	50
TCRDV6	∼500 bp	*Bgl*II–*Eco*RI	Vδ6p5′	∼−521 bp	ATGGACAAGATCTTAGGAGCATCAT	47
			Vδ6p3′	∼−53 bp	tggcgaatTCTCCAGGCTGGGAATCCATGATA	47
TCRDD1U	1100 bp	*Bam*HI–*Bgl*II	Dδ1Up5′	∼−1500 bp	GCTATGTTAATATTGTATCTAGAGCTAC	This report
			Dδ1-3′XBg	−30 bp	gtagatctaGAAGCCATTTGGTTAATGTCAAAAG	13
TCRDD1	584 bp	*Sal*I–*Sal*I	Dδ1-5′S	−34 bp	cgcgtcgACTCCATGTTCAAATAGATATAGTATT	13
			Dδ2-3′S	+48 bp	cgcgtcgACATAGCGGGTCACGGCTGGG	13
TCRDD2	∼450 bp	*Bgl*II–*Hin*dIII	Dδ2-5′XBg	−11 bp	gtagatctAGAAGAGGGTTTTTATACTGATGTG	13
			Dδ2p3′	∼+450 bp	aacaaagCTTTGCACTGGACATTAAGTTCTTTA	This report
TCRDD3U	659 bp	*Xba*I–*Xba*I	Dδ3Up5′	+552 bp	GCTATCTCTAgATTCACCCAGCAGG	M22197
			Dδ3-3′XBg	−46 bp	gtagatctaGAAATGGCACTTTTGCCCCTGCAG	18
TCRDD3	774 bp	*Pst*I–*Pst*I	Dδ3p5′	−43 bp	cgcgtcgaCCATATAGTGTGAAACCGAGGGG	18
			Dδ3p3′	+1010 bp	cgcgtcgACTTGGTTCCACAGTCACACGG	18
TCRDJ1	873 bp	*Hin*dIII–*Eco*RI	Jδ1p5′	+22 bp	tcaaaagcttTGACACCGATAAACTCATCTTTG	18
			Jδ1p3′	+863 bp	CAAGACACGGTCGAATTCAAATGTC	M22197
TCRDJ2	∼1300 bp	*Hin*dIII–*Eco*RI	Jδ2p5′	+24 bp	gtaaaGCTTTGACAGCACAACTCTTCTTTG	19
			Jδ2p3′	∼+1300 bp	CTGCAGAgAATTCCAAATTTCAAGTGG	This report
TCRDJ3	∼700 bp	*Eco*RI–*Hin*dIII	Jδ3p5′	+22 bp	tgtgaatTCCTGGGACACCCGACAGATG	19
			Jδ3p3′	∼+700 bp	TTCTTAATTTATAAgCTTAGAAGTCACC	M94081
TCRDC1	677 bp	*Hin*dIII–*Eco*RI	Cδex1p5′	+184 bp[e]	GAAGTACAATGCTGTCAAGCTTGG	19
			Cδex2p3′	+834 bp[e]	tgtgaattCTTTGGGTTTATGGCAGCTCTTTG	19
TCRDC4	1028 bp	*Hin*dIII–*Eco*RI	Cδex4p5′	+23 bp[f]	tgcaagcttACTGGCATGAGGAAGCTAC	19
			Cδex4p3′	+1013 bp[f]	GCACAgAATtCAGTTTAATAAATGCAATAG	19
TCRDRE	471 bp	*Eco*RI–*Hin*dIII	δRECp5′	−453 bp	gtggaattCAGCTGAAGACTGTATCATGGAAG	24
			δRECp3′	−22 bp	CCGTaaGCTtCTCACACGAGAGGATGG	24
TCRAPJ	819 bp	*Hin*dIII–*Eco*RI	ψJαp5′	+35 bp	tacaagcTTAATAGGAAACTGACATTTGGAGCC	M94081
			ψJαp3′	+815 bp	AGCCTGGaATTCAGGCTGTGAGG	M94081

a. The position of the oligonucleotide primer is indicated upstream (−) or downstream (+) relative to the heptamer RSS. The position of the DNA probes are indicated in Figure 1.
b. The sequences in lower case characters represent the aspecific nucleotides, which generate restriction sites.
c. Sequence information used to design the oligonucleotide primers was derived from the indicated literature references, EMBL databank accession numbers or from our own sequence data.
d. All other TcR-δ gene rearrangement primers are published in Ref. 38.
e. The position of the oligonucleotide primer is indicated downstream relative to the 5′ splice site of Cδ exon 1.
f. The position of the oligonucleotide primer is indicated downstream relative to the 5′ splice site of Cδ exon 4.

Southern blot analysis

DNA was isolated from fresh or frozen MNC as described previously (46,52). Fifteen μg DNA samples were digested with the restriction enzymes: *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, and/or *Kpn*I (Pharmacia), size fractionated in 0.7% agarose gels, and transferred to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) as described (46,52). TcR-δ gene rearrangements were studied using ^{32}P random oligonucleotide-labeled TcR-δ gene DNA probes (Table 1 and Figure 1).

PCR amplification analysis

PCR was essentially performed as described previously (38,46). A 0.1 μg sample of DNA, 12 pmol of the 5′ and the 3′ oligonucleotide primers, and 1 unit of *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) were used in each PCR of 100 μl. The oligonucleotide primers are listed in Table 1 or Breit *et al.* 38. The PCR reaction mixture was incubated at 94°C for 3 minutes, at 55°C for 2 minutes and at 72°C for 3 minutes in a thermal cycler (Perkin-Elmer Cetus). Following this initial cycle, denaturing, annealing and extension steps were performed for another 29 to 34 cycles at 94°C for 1 minute, at 55°C for 1 minute and at 72°C for 3 minutes, respectively. After the last cycle, an additional extension step of 72°C for 7 minutes was executed.

Direct sequencing analysis

One μl of the original PCR product, 12 pmol of the limiting primer, 600 pmol of the opposite primer, and 5 units of *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus) were used in each asymmetric PCR of 500 μl. The reaction mixture was incubated for a total of 25 to 30 cycles with the above-described regular temperature cycles. After asymmetric amplification, the PCR products were precipitated twice in 50% ethanol plus 0.1 volume of 2 M NaAc, pH 5.6 (38). The dried pellet was resolved in 22 μl H_2O, half of which was used in the sequence reaction. Twenty to fifty pmol sequence primer was used in each reaction (sequence primers are indicated in Table 1 or Breit *et al.* 38). All sequence reactions were performed with the T7-sequencing kit (Pharmacia) following the manufacturer's instructions using ^{35}S radiolabeling, and run in normal, denaturing 8% polyacrylamide sequence gels.

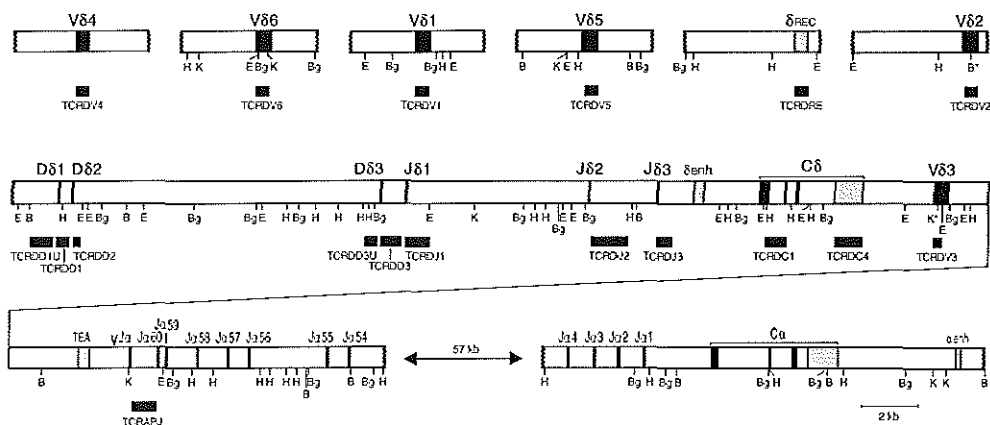


Figure 1. Restriction map of the complete human TcR-δ gene and a part of the human TcR-α gene. Restriction map of the human TcR-δ gene down to the TcR-Cα region. Protein-coding exons are indicated as solid boxes in the bars; dotted boxes in the bars represent non-coding gene segments. The relevant restriction sites are indicated: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I. *, polymorphic restriction site. Solid boxes below the restriction map represent the probes used for Southern blot hybridization. The restriction map from Cδ to the TcR-α enhancer (αenh) was based on the sequence data from Koop *et al.* (EMBL accession no. M94081).

RESULTS

TcR-δ gene probes and restriction map

Because the sequences of most human TcR-δ gene segments are published, it was possible to design oligonucleotide primer sets for the six Vδ, three Dδ and three Jδ gene segments (Table 1). In this way, we obtained DNA probes for Southern blot analysis for every known Vδ, Dδ, and Jδ gene segment. In addition to the V, D, and J probes, we designed Cδ, δREC, and ψJα probes for restriction analysis of the entire TcR-δ locus. All TcR-δ DNA probes were designed in such a way that they hybridize to DNA close to the RSS of the involved gene segment, but avoiding sequences that by sequence homology could give rise to cross-hybridization. Furthermore, all TcR-δ probes were checked for aspecific cross-hybridization by Southern blot analysis on germline granulocyte DNA. The positions and sizes of the obtained DNA probes are indicated in Table 1 and Figure 1.

Except for Vδ4, every known Vδ, Dδ, or Jδ gene segment was at least involved once in a rearrangement in our large series of 229 ALL. This allowed us to deduce a detailed restriction map of the TcR-δ gene locus by use of extensive Southern blot analysis. These Southern blot analyses included single, double and/or partial digests of the five restriction enzymes *Eco*RI, *Hin*dIII, *Bgl*II, *Bam*HI, and/or *Kpn*I and were performed on DNA from ALL patients and/or granulocyte DNA from healthy controls. The restriction map from Cδ to Cα was based on the sequence data from Koop *et al.* (EMBL accession no. M94081). Our complete human TcR-δ gene restriction map for the five restriction enzymes is given in Figure 1.

Southern blot restriction enzyme patterns

Although several attempts have been made in the literature to identify TcR-δ gene rearrangements based on Southern blot rearrangement patterns, most reports only described restriction patterns without precise identification of the rearrangements (19,32,53). Here, successive hybridization of the Southern blot filters with 3′ DNA probes (e.g. TCRDD and TCRDJ DNA probes) and 5′ DNA probes (e.g. TCRDV DNA probes) allowed identification of 76% of all detected 213 TcR-δ gene rearrangements in the 229 ALL patients. Based on the extensive Southern blot data, it was possible to identify the restriction enzyme patterns belonging to each particular TcR-δ gene rearrangements for the five selected restriction enzymes (Figure 2). However, not all theoretically possible TcR-δ gene rearrangements were present in the studied ALL. The Southern blot restriction enzyme patterns of these remaining rearrangements were deduced from the restriction map and from other Southern blot rearrangement patterns (Table 2). For instance, not all Vδ gene segments were found in a rearrangement with the Dδ3 gene segment. Nevertheless, it is obvious that the rearranged band of a Vδ-Dδ3 rearrangement will be 0.95 kb larger than the band of the same Vδ gene segment rearranged to the Jδ1 gene segment, because the Dδ3 gene segment is located 0.95 kb upstream of the Jδ1 segment without any intermediate restriction site of the applied restriction enzymes (Figures 1 and 2). For other rearrangements, the restriction enzyme pattern was deduced by calculating of the distances from the restriction sites to the RSS of a TcR-δ gene segment and subsequent adding up the 5′ restriction site→RSS distance (Vδ or Dδ) to the 3′ restriction site→RSS distance (Dδ or Jδ) of a rearrangement. This was made easier by the occurrence of rearrangements

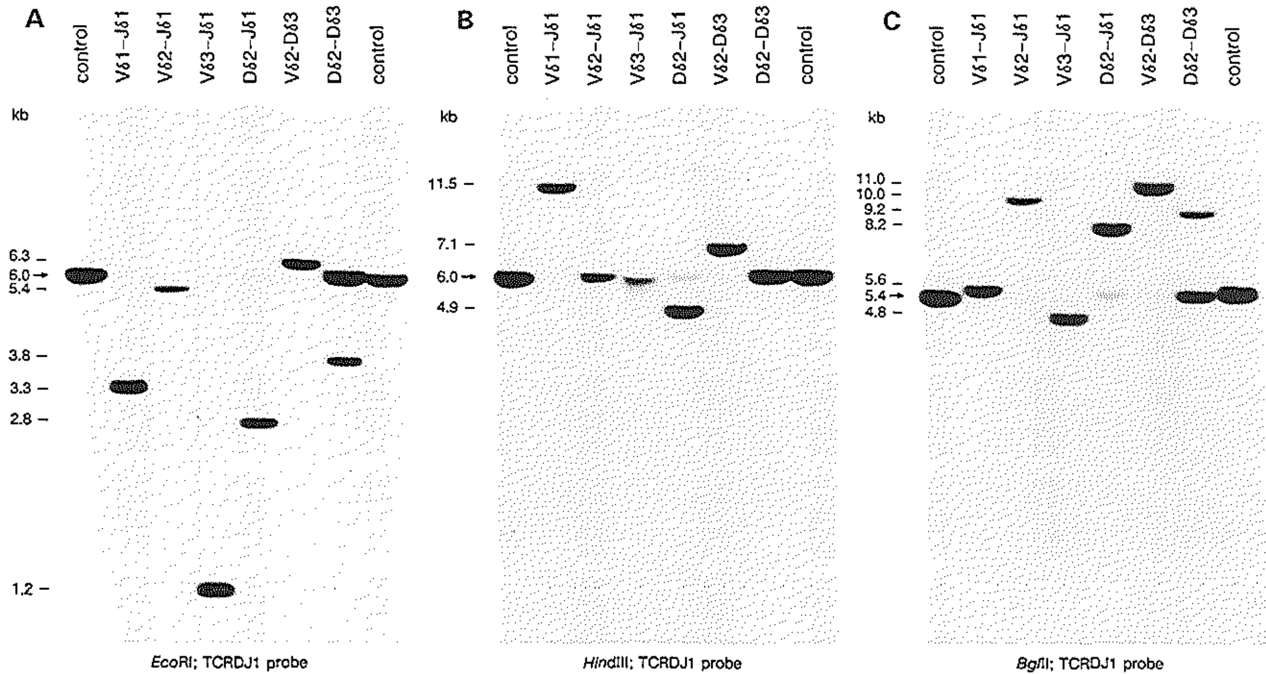


Figure 2. Southern blot analysis of the various preferential TcR-δ gene rearrangements. **(A)** *Eco*RI digests, **(B)** *Hin*dIII digests, **(C)** *Bgl*II digests of DNA from six selected patients each containing only one specific TcR-δ gene rearrangements combined with a TcR-δ gene deletion or a TcR-δ gene in germline configuration. Lane 1, germline control; lane 2, Vδ1-Jδ1 patient T046; lane 3, Vδ2-Jδ1 patient T069; lane 4, Vδ3-Jδ1 patient T071; lane 5, Dδ2-Jδ1 patient T049; lane 6, Vδ2-Dδ3, patient Bc28; lane 7, Dδ2-Dδ3 patient Bc44; lane 8, germline control. The Southern blot filters were hybridized with the TCRDJ1 probe. The sizes of the germline bands (→) and rearranged bands (–) are indicated. The junctional regions sequences of the TcR-δ gene rearrangements presented here are given in Figures 3 or 4.

TABLE 2. Sizes of Southern blot bands representing TcR-δ gene rearrangements[a].

3′ rearranged TcR-δ gene segment		5′ rearranged TcR-δ gene segment									
		Vδ1	Vδ2	Vδ3	Vδ5	Vδ6	δREC	Dδ1	Dδ2	Dδ3	
Germline		3.1	13.5	1.3	6.4	3.0	5.4	2.3	2.3	6.0	*Eco*RI
		7.1	4.2	5.7	6.8	6.1	4.5	3.6	8.0	6.0	*Hin*dIII
		1.6	7.9	4.5	6.7	2.0	8.0	5.1	5.1	5.4	*Bgl*II
		>25	13.5[b]	14.0	3.8	>25	22	3.5	3.5	17.5	*Bam*HI
		>25	10.0	16.5[c]	6.6	3.4	18.0	17.5	17.5	17.5	*Kpn*I
Dδ1	2.3	3.2[d]	5.3	1.1	1.9	1.3	5.9				*Eco*RI
	3.6	6.8	1.6	1.5	0.83	3.1	1.4				*Hin*dIII
	5.1	2.9	7.4	2.1	6.7[d]	2.0[d]	5.9				*Bgl*II
	3.5	>24	13.0[b]	5.5	5.1	>27	20				*Bam*HI
	17.5	22	20	15.0[c]	16.0	15.0	30				*Kpn*I
Dδ2	2.3	2.8	4.9	0.70	1.5	0.87	5.5[d]	1.9			*Eco*RI
	8.0	14.4	9.1	9.0	8.4	10.5	9.0	11.0			*Hin*dIII
	5.1	2.5	6.9	1.7	6.3	1.6	5.5	4.7			*Bgl*II
	3.5	>24	12.5[b]	5.1	4.7	>27	19.5	3.1			*Bam*HI
	17.5	21	20	14.5[c]	15.5	14.5	30	17.0			*Kpn*I
Dδ3	6.0	4.2	6.3	2.2	2.9	2.3	6.9	3.3	3.8		*Eco*RI
	6.0	12.5	7.1	7.0	6.3	8.6	6.9	9.1	5.9[e]		*Hin*dIII
	5.4	6.6	11.0	5.8	10.5	5.7	9.6	8.8	9.2		*Bgl*II
	17.5	>30	19.5[b]	12.5	12.0	>34	27	10.5	11.0		*Bam*HI
	17.5	10.5	9.0	3.7[c]	4.6	3.7	19.0	6.1	6.5		*Kpn*I
Jδ1	6.0	3.3	5.4	1.2[d]	2.0	1.4	6.0[e]	2.4[d]	2.8	5.1	*Eco*RI
	6.0	11.5	6.1[e]	6.0[e]	5.3	7.6	5.9[e]	8.1	4.9	5.0	*Hin*dIII
	5.4	5.6	10.0	4.8	9.4	4.7	8.6	7.8	8.2	4.4	*Bgl*II
	17.5	>30	18.5[b]	11.5	11.0	>33	26	9.4	9.8	16.5	*Bam*HI
	17.5	9.3	8.0	2.7[c]	3.6	2.7	18.0[d]	5.1	5.5	16.5	*Kpn*I
Jδ2	5.3	7.1	9.2	5.0	5.8	5.2[e]	9.8	6.2	6.6	8.9	*Eco*RI
	3.1	8.3	3.1[e]	3.0[e]	2.3	4.6	2.9	5.1	1.9	2.0	*Hin*dIII
	5.4	6.7	11.0	5.9	10.5	5.8	9.7	8.9	9.3	5.5[de]	*Bgl*II
	17.5	>24	12.0[b]	4.8	4.4	>27	19.0	2.8	3.2	9.9	*Bam*HI
	16.5[c]	19.0[c]	18.0[c]	12.5[c]	13.5[c]	12.5[c]	28[c]	15.0[c]	15.5[c]	26[c]	*Kpn*I
Jδ3	5.3	4.9	7.0	2.8	3.6	3.0[d]	7.6	4.0	4.4	6.7	*Eco*RI
	3.5	9.5	4.2[d]	4.1	3.5[e]	5.8	4.1	6.3	3.1	3.1	*Hin*dIII
	5.4	4.5	8.9	3.7	8.3	3.6	7.5	6.7	7.1	3.3	*Bgl*II
	14.0	>35	24[b]	16.5	16.0	>38	31	14.5	14.5	21	*Bam*HI
	16.5[c]	17.0[c]	15.5[c]	10.5[c]	11.5[c]	10.5[c]	26[c]	13.0[c]	13.0[c]	24[c]	*Kpn*I
ψJα	6.2	3.5	5.6	1.5	2.2	1.6	6.2[e]	2.6	3.1	5.3	*Eco*RI
	7.2	9.0	3.7	3.6	3.0	5.3	3.6	5.8	2.6	2.7	*Hin*dIII
	7.3	3.0	7.5	2.3	6.8[d]	2.1[d]	6.0	5.2[d]	5.7	1.8	*Bgl*II
	9.5	>25	17.0[b]	9.5[e]	9.1	>31	24	7.5	7.9	14.5	*Bam*HI
	6.1	6.8	5.5	0.18[c]	1.1	0.17	15.5	2.6	3.0	14.0	*Kpn*I

a. The sizes of the Southern blot bands are given with an accuracy of 1 kb above 20 kb, 0.5 kb in between 20 and 10 kb, 0.1 kb in between 10 and 1 kb, and 0.01 kb below 1 kb.
b. These Southern blot bands in *Bam*HI digests may be different due to the presence of a frequently occurring polymorphism (7%), in which a *Bam*HI site is present 10 kb downstream of the original 5′ *Bam*HI site (see Figure 1).
c. These Southern blot bands in *Kpn*I digests may be different due to the presence of a frequently occurring polymorphism (21%), in which the 3′ *Kpn*I site is absent and all bands will be enlarged by 6.1 kb (see Figure 1).
d. These rearranged bands cannot be detected with the DNA probe recognizing the 5′ TcR-δ gene segment due to comigration with the germline band.
e. These rearranged bands cannot be detected with the DNA probe recognizing the 3′ TcR-δ gene segment due to comigration with the germline band.

with a restriction site generated in the junctional region (Figures 3 and 4), which provided the exact sizes of the 3′ and 5′ restriction site→RSS distances of the involved gene segments (38). The observed and predicted sizes of rearranged Southern blot bands representing the different types of TcR-δ gene rearrangements are given in Table 2.

Because none of the used restriction enzymes cuts between the Dδ3 and Jδ1 gene segments, all Dδ3 and Jδ1 gene rearrangement patterns only differ in size (0.95 kb). To further discriminate between the two types of rearrangements, it is possible to use the TCRDD3 probe in addition to the TCRDJ1 probe (Figure 1) or to apply the restriction enzyme *Xba*I, which cuts between the Dδ3 and Jδ1 gene segments (19,53). In the latter case, a rearrangement to the Dδ3 gene segment results in a 1.6 kb *Xba*I germline band after hybridization with the TCRDJ1 probe, whereas a rearrangement to the Jδ1 gene segment will result in a rearranged band (53).

Polymorphic restriction sites

To investigate the occurrence of polymorphic restriction sites, granulocyte DNA from 50 healthy individuals were analyzed by Southern blotting with the TCRDV2, TCRDJ1 and TCRDC4 probes. For the five selected restriction enzymes, no polymorphisms were found in the restriction sites flanking the Jδ1 gene segments. This is an important observation, since virtually all TcR-δ gene rearrangements in our series of ALL (97%) were detectable with the TCRDJ1 probe. Also, in the restriction sites *Bgl*II and *Bam*HI aside the Cδ gene segment no polymorphisms occurred. However, the *Kpn*I restriction site within the Vδ3 gene segment (Figure 1) is absent in 21% (21/100) of the alleles resulting in a 23 kb instead of a 16.5 kb germline band caused by the 6.1 kb downstream located *Kpn*I site (Table 2 and Figure 1). Another remarkable polymorphism was found within the Vδ2 gene segment, which contains a polymorphic *Bam*HI restriction site in 7% (7/100) of the alleles (Figure 1). The size of the polymorphic *Bam*HI germline band of the Vδ2 gene segment is 3.4 kb instead of the normal 13.5 kb germline band (Table 2). No other gene segments were analyzed for polymorphic sites, because in the 229 ALL no indication for polymorphic restriction sites were found other than the two mentioned above.

Frequencies of TcR-δ gene rearrangements and deletions

The TcR-δ gene can occur in three consecutive configurations: germline (G), rearranged (R), and deleted (D), in which our definition of TcR-δ gene deletion is the absence of the Cδ exons. In total, 7% (20/276) of the TcR-δ alleles in T-ALL and only 23% (41/182) of the TcR-δ alleles in precursor B-ALL were in germline configuration. Rearrangements occurred on 55% (153/276) of alleles in the T-ALL and 33% (60/182) of alleles in the precursor B-ALL. The TcR-δ genes were deleted on the remaining alleles in T-ALL (37%) and precursor B-ALL (45%).

As each genome contains two TcR-δ alleles, theoretically, six different combinations of TcR-δ gene configurations can occur: G/G, R/G, R/R, D/G, D/R, and D/D. The results of the relative frequencies of TcR-δ gene configurations in the different subgroups of ALL are shown in Table 3. Whereas the T-ALL subgroups displayed preference for different TcR-δ gene configurations, the precursor B-ALL subgroups (i.e. null ALL, common ALL, and pre-B-ALL) showed no such preference and are therefore presented as one group. The G/G and R/G configurations were never found in the $CD3^+$ T-ALL, but occurred only in $CD3^-$ T-ALL

Patients	Fenotype	Vδ1 TCTTGGGGAACT	Junctional region	Jδ1 ACACCGATAAAC
T008	CD3⁻ T-ALL	TCTTGGGGAA	AAACGGGAATTCCACTGGGGGATACGcgCCC	GATAAAC
T019	CD3⁻ T-ALL	TCTTGGGGAAC	GTCCTAGACCGGGCCTACTGGGGGATCCCGCCAAAgt	ACACCGATAAAC
T046	CD3⁻ T-ALL	TCTTGGGGA	CCCCCGGAAATAGTaGGACGGA	ACCGATAAAC
T067	CD3⁻ T-ALL	TCTTGGGGAA	ATAGAAACTGGGGACACATTTGGACACCCAG	ACACCGATAAAC
T082	CD3⁻ T-ALL	TCTTGGGGAAC	GcGAACTGGGGGGGACTCCGCACTAATGGGGGATACGcCTGAGT	ACCGATAAAC
T084	TcR-γδ⁺ T-ALL	TCTTGGGGAAC	CAGGCCTCCTACgACTCTTGGGACTGGGGATCGGGC	CACCGATAAAC
T086	TcR-γδ⁺ T-ALL	TCTTGGGG	GCAACTTCCTACgATCGGGGATAGAAGCGGA	CGATAAAC
T091	TcR-γδ⁺ T-ALL	TCTTGGGG	TCTCTTTCCCCCTTC	CCGATAAAC
T106	TcR-γδ⁺ T-ALL	TCTTGGG	AAGTGGGGTAGTGCGTGCCAGAGAATGGGGAGCGATTCCCAGGGTGAG	ACCGATAAAC
T108	TcR-γδ⁺ T-ALL	TCTTGGGGAA	AGCACTTCCTCCCTCGGATGGCGGGGGAAGCCACTAACA	ACCGATAAAC
T016	TcR-αβ⁺ T-ALL	TCTTGG	CTTTCTCTCCGGgtACTGGGGGATTTgt	ACACCGATAAAC
T109	TcR-αβ⁺ T-ALL	TCTTGGGGA	CACGCATTCCTACCTGAGACCtACTGGGGGATACATAATCTTAGTt	ACACCGATAAAC
		Vδ2 GCCTGTGACACC		
T032	CD3⁻ T-ALL	GCCTGTGACACC	TCTGGGGGATGTAGG	−20
T037	CD3⁻ T-ALL	GCCTGTGA	TCGACCTACgtA	CGATAAAC
T082	CD3⁻ T-ALL	GCCTGTGAC	TCCGATGGGGGATTGGGTAGGGTGTGG	AC
T004	TcR-γδ⁺ T-ALL	GCCTGTG	TTACTACTTCTTCTTCTGGGGGATTGAG	CCGATAAAC
T005	TcR-γδ⁺ T-ALL	GCCTGTG	TGTgt	ACACCGATAAAC
T091	TcR-γδ⁺ T-ALL	GCCTGTGACACC	TTGGGTGGGGGATACTCTt	ACACCGATAAAC
T047	TcR-αβ⁺ T-ALL	GCCTGTGAC	CCCCCTCGCCAGTGTgt	ACACCGATAAAC
T069	TcR-αβ⁺ T-ALL	GCCTGT	CCGGGGGCCATACGCCGGTAGAGACGTgt	ACACCGATAAAC
T103	TcR-αβ⁺ T-ALL	+18	CGCGACCTCTAAAt	ACACCGATAAAC
		Vδ3 CTGTGCCTTTAG		
T032	CD3⁻ T-ALL	CTGTGCCTTT	TGGCGCCCTTCCTTACTGCCTTTGGGGGATACAt	ACACCGATAAAC
T033	CD3⁻ T-ALL	CTGTGCCT	AGgtACTGGGGGATAAGGTCGCG	CACCGATAAAC
T071	CD3⁻ T-ALL	CTGTGCCTTTA	ACCCTTCCTACCTTCTACCGAGA	ACACCGATAAAC
T070	TcR-γδ⁺ T-ALL	CTGTGCCT	ATAAACCTTATTTGTTCCTACTGGGGGACCAT	CGATAAAC
T106	TcR-γδ⁺ T-ALL	CTGTGCCTTT	CTCGCTGGGCAGGGGGACGGGTgt	ACACCGATAAAC
T133	TcR-γδ⁺ T-ALL	CTGTGCCTT	GCTCGGGGAGGGTATTAgCCTTATGGGGGAT	ACCGATAAAC
T050	TcR-αβ⁺ T-ALL	+14	AAACGGGCCTACCTTTGGGGGATAAAACT	CACCGATAAAC
			GAAATAGT (Dδ1) CCTTCCTAC (Dδ2) ACTGGGGGATACG (Dδ3)	

Figure 3. Representative junctional regions of complete human TcR-δ gene rearrangements. Junctional regions of the preferential complete TcR-δ gene rearrangements: Vδ1-Jδ1, Vδ2-Jδ1, and Vδ3-Jδ1. Sequences of the junctional regions are aligned with the known (double-underlined) Vδ, Dδ, and Jδ1 germline sequences. The junctional regions consist of Dδ-gene-derived nucleotides (single-underlined), N-region nucleotides, and P-region nucleotides (small characters). Generated restriction sites, *Eco*RI (**GAATTC**; patient T008) and *Bam*HI (**GGATCC**; patient T019), are indicated in bold characters. Numbers at the end of the junctional region indicate extensive deletion of nucleotides by trimming of the 5′ gene segment (+) or 3′ gene segment (−).

Patients	Fenotype	Dδ2	Junctional region	Jδ1
		TTGTGCCTTCCTAC		ACACCGATAAACTC
T039	CD3⁻ T-ALL	TTG	CTTTCGGCTCCGGGACCGCCtACTGGGGGATACGTCAGGT	CCGATAAACTC
T043	CD3⁻ T-ALL	TTGTGCCTTCCTAC	AAACGGTAGCGGAAACCAATCACCTTgt	ACACCGATAAACTC
T049	CD3⁻ T-ALL	T	GGAGGTGGGAATAGGGCgt	ACACCGATAAACTC
T107	TcR-γδ⁺ T-ALL	TT	CCAAgt	ACACCGATAAACTC
T108	TcR-γδ⁺ T-ALL	TTGTGCCTTCCTAC	CTTGGGGGATACTCC	CACCGATAAACTC
T132	TcR-γδ⁺ T-ALL	TT	AACGGGTATGGGGGGATCCTCATC	CACCGATAAACTC
T112	TcR-αβ⁺ T-ALL	TT	TAAAGGAGGGATCCAAACCCAAGTGG	ACTC
		Vδ2		**Dδ3**
		GTGCCTGTGACACC		ACTGGGGGATACGC
T039	CD3⁻ T-ALL	GTGCCTGTGACACC	GAAA	GGGATACGC
T119	CD3⁻ T-ALL	GTGCCTGTGAC	CCCTGGT	GGGATACGC
T131	CD3⁻ T-ALL	GTGCC	CGT	-14
T052	TcR-γδ⁺ T-ALL	GTGCCTGTGAC	CAGTCCTCCTt	ACTGGGGGATACGC
T134	TcR-γδ⁺ T-ALL	GTGCCTGTGACACC	GAA	GGGGGATACGC
Bn01	null-ALL	GTGCCTGTGACA	TCCTCCCt	ACTGGGGGATACGC
Bn02	null-ALL	GTGCCTGTGACACC	gTA	GATACGC
Bc02	common ALL	GTGCCTGTGACACC	CGA	ACTGGGGGATACGC
Bc22	common ALL	GTGCCTGTG	TCGGGAGC	GGGGGATACGC
Bc28	common ALL	GTGCCTGTGAC	TCCCCGGCT	GATACGC
Bc30	common ALL	GTGCCTGT	CTTt	ACTGGGGGATACGC
Bc53	common ALL	GTGCC	GGCG**GGTAC**	**C**
Bp02	pre B-ALL	GTGCCTGTG	CCT	CGC
Bp05	pre B-ALL	GTGCCTGTGAC		TGGGGGATACGC
Bp18	pre B-ALL	GTGCCTGTGACACC	gCTCTTT	CTGGGGGATACGC
Bp20	pre B-ALL	GTGCCTG	CC	CTGGGGGATACGC
Bp29	pre B-ALL	GTGCCTG	AGCG	GGGGGATACGC
		Dδ2		
		TTGTGCCTTCCTAC		
T043	CD3⁻ T-ALL	TTGTGCCTTCCTA	G	CTGGGGGATACGC
T053	CD3⁻ T-ALL	TTGTGCCTTCCTA	AAACCCTACT	GGATACGC
T122	CD3⁻ T-ALL	TT	CGGG	-34
Bc02	common ALL	TTGTGCCT	CCCTt	ACTGGGGGATACGC
Bc12.a	common ALL	TTGTGCCTTC	TTCTGTCC	GGGATACGC
Bc12.b		TTGTGCCTTCCT	CTTCCCTt	ACTGGGGGATACGC
Bc39	common ALL	TTGTGCCTTCCTA	GA	GGGGATACGC
Bc44	common ALL	TTGTGCCTTCCTAC		TGGGGGATACGC
Bc53	common ALL	TTGTGCCTTCC	CTGGG	GGGGGATACGC
Bc77	common ALL	TTGTGCCTTC	GAGG	CTGGGGGATACGC

Figure 4. Representative junctional regions of incomplete human TcR-δ gene rearrangements. Junctional regions of the preferential incomplete TcR-δ gene rearrangements: Dδ2-Jδ1, Vδ2-Dδ3, and Dδ2-Dδ3. Sequences of the junctional regions are aligned with the known (double underlined) Vδ2, Dδ, and Jδ1 germline sequences. The junctional regions consist of Dδ-gene-derived nucleotides (single-underlined), N-region nucleotides, and P-region nucleotides (small characters). Generated restriction sites, *Bam*HI (**GGATCC**; patient T112 and T132) and *Kpn*I (**GGTACC**; patient Bc53), are indicated in bold characters. Numbers at the end of the junctional region indicate extensive deletion of nucleotides by trimming of the 3′ gene segment (–).

TABLE 3. Frequencies of TcR-δ gene configuration combination in different subgroups of ALL.

	T-ALL			precursor B-ALL
	CD3⁻	TcR-γδ⁺	TcR-αβ⁺	
(Number of patients)	(73)	(25)	(40)	(91)
TcR-δ gene configuration[a]				
G/G	9.6% (7)	0% (0)	0% (0)	13.2% (12)
R/G	8.2% (6)	0% (0)	0% (0)	15.4% (14)
R/R	45.2% (33)	92.0% (23)	0% (0)	15.4% (14)
D/G	0% (0)	0% (0)	0% (0)	3.3% (3)
D/R	24.7% (18)	8.0% (2)	37.5% (15)	19.8% (18)
D/D	12.3% (9)	0% (0)	62.5% (25)	33.0% (30)

a. Abbreviations used; G, TcR-δ allele in germline configuration; R, allele contains a TcR-δ gene rearrangement; D, allele contains a deletion of Cδ gene segment.

and precursor B-ALL. The D/G configuration solely occurred in precursor B-ALL. Because the TcR-δ gene is deleted upon TcR-α rearrangement, no R/R configuration was found in TcR-αβ$^+$ T-ALL. Analogously, TcR-γδ$^+$ T-ALL need at least one rearranged TcR-δ allele and therefore never contained the D/D configuration. A noticeable high percentage (56%) of the precursor B-ALL contained one or two deleted TcR-δ alleles. In T-ALL, this was found most frequently in TcR-αβ$^+$ (100%) and CD3$^-$ T-ALL (37%), but was rare in TcR-γδ$^+$ T-ALL (8%).

Relative allelic frequencies of particular TcR-δ gene rearrangements

Southern blot analysis of the TcR-δ genes in the 229 ALL revealed that in ~70% of the T-ALL and ~51% of the precursor B-ALL one or two alleles were rearranged (Table 3). The relative allelic frequencies of the most frequent complete V-(D)-J and incomplete D-J, V-D, or D-D TcR-δ gene rearrangements in the various ALL subgroups are summarized in Table 4. A remarkably high allelic frequency of Vδ1-Jδ1 rearrangements was seen in TcR-γδ$^+$ (56%) and CD3$^-$ T-ALL (24%). In TcR-αβ$^+$ T-ALL a low frequency of rearranged TcR-δ alleles was found, due to the high frequency of TcR-δ gene deletions. These remaining rearrangements of the TcR-αβ$^+$ T-ALL did not shown an obvious preference for a specific rearrangement, although it is noticeable that only one of the incomplete rearrangements (Dδ2-Jδ1) was present in this ALL subgroup. Dδ2-Jδ1 rearrangements were observed most frequently in TcR-γδ$^+$ (15%) and CD3$^-$ T-ALL (10%), as were the Vδ2-Dδ3 rearrangements (4% and 6%, respectively). Vδ2-Dδ3 rearrangements were even more frequently present in precursor B-ALL (72%). The most 'immature' rearrangement, Dδ2-Dδ3, was observed only in precursor B-ALL (10%) and CD3$^-$ T-ALL (4%). Except for the Vδ2-Dδ3 and Dδ2-Dδ3 rearrangements, the other TcR-δ gene rearrangements in

TABLE 4. Relative allelic frequencies of six preferential TcR-δ gene rearrangements in different subgroups of ALL.

	T-ALL			precursor B-ALL
	CD3⁻	TcR-γδ⁺	TcR-αβ⁺	
(Number of rearrangements)	(90)	(48)	(15)	(60)
TcR-δ gene rearrangement				
Vδ1-Jδ1	24.4% (22)	56.3% (27)	13.3% (2)	0% (0)
Vδ2-Jδ1	10.0% (9)	6.3% (3)	20.0% (3)	0% (0)
Vδ3-Jδ1	5.6% (5)	8.3% (4)	6.7% (1)	0% (0)
Dδ2-Jδ1	10.0% (9)	14.6% (7)	6.7% (1)	0% (0)
Vδ2-Dδ3	5.6% (5)	4.2% (2)	0% (0)	71.7% (43)
Dδ2-Dδ3	4.4% (4)	0% (0)	0% (0)	10.0% (6)
all other	40.0% (36)	10.4% (5)	53.3% (8)	18.3% (11)

precursor B-ALL (18% of the rearranged alleles) could not be identified. In T-ALL, 68% of the rearranged alleles belonged to one of the six frequently occurring TcR-δ gene rearrangements (Table 4), whereas the other rearrangements either occurred at a low frequency or could not be identified.

Junctional diversity of TcR-δ gene rearrangements

To investigate the junctional diversity of the TcR-δ gene rearrangements, we analyzed a total of 100 junctional regions of the six most frequently occurring TcR-δ rearrangements by PCR-mediated amplification and subsequent direct sequencing. A representative sample of the sequenced junctional regions of complete V-D-J rearrangements and incomplete rearrangements are illustrated in Figures 3 and 4, respectively. The characteristics of the junctional regions analyzed in the total ALL group are summarized in Table 5. The complete

TABLE 5. Junctional diversity of six preferential TcR-δ gene rearrangements in ALL.

Rearrangement	Number	Junctional nucleotides	Dδ gene nucleotides	P-region[a] nucleotides	N-region[b] nucleotides	Deleted[c] nucleotides	In frame
Vδ1-Jδ1	30	31.2	11.7	1.2	18.3	4.6	57%
Vδ2-Jδ1	13	23.3	8.5	1.5	13.2	7.1	36%
Vδ3-Jδ1	10	31.0	10.8	0.6	19.6	6.8	30%
Dδ2-Jδ1	14	20.3	6.6	1.1	12.6	12.4	–
Vδ2-Dδ3	22	5.0	1.5	0.5	3.0	7.2	–
Dδ2-Dδ3	11	4.8	–	0.2	4.6	8.3	–

a. P-region nucleotides are nucleotides recognized as fulfilling the conditions for P-regions described Lafaille *et al.* (10).
b. N-region nucleotides are randomly inserted nucleotides of all N-regions.
c. Deleted nucleotides are total loss of nucleotides per junctional region as caused by trimming of the rearranged gene segments.

Vδ-Jδ rearrangements contained large junctional regions with an average size of 30 nucleotides and 5.5 deleted nucleotides. The compilation of the Vδ-Jδ junctional regions by Dδ-gene-derived nucleotides, P-region nucleotides, and N-region nucleotides was comparable between the studied Vδ-Jδ rearrangements (Table 5). The incomplete Dδ2-Jδ1 rearrangement contained larger junctional regions (mean: 20 nucleotides) and showed more deletion (mean: 12 nucleotides) than the other incomplete Vδ2-Dδ3 and Dδ2-Dδ3 rearrangements, in which average insertion and deletion was 5 and 8 nucleotides, respectively.

DISCUSSION

To detect TcR-δ gene rearrangements, it is necessary to have disposal of a set of well-located DNA probes. Due to the availability of the PCR technique, probe isolation is no longer dependent on genomic clones. Designing PCR-DNA probes only requires knowledge of the sequences flanking the sides of the probe, which implies that the location and size of the new PCR-DNA probes is independent of the restriction sites present in the human genome. By applying the PCR technique, we were able to design and clone a set of eighteen optimal DNA probes, which are located as close as possible to the RSS of the various TcR-δ gene segments, but avoiding sequences that by sequence homology could give rise to cross-hybridization in Southern blot analysis. Using these probes we were able to determine the precise human TcR-δ gene restriction map for the restriction enzymes *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, and *Kpn*I. In addition, we have determined the Southern blot restriction enzyme pattern for every theoretically possible TcR-δ gene rearrangement except those involving the Vδ4 gene segment. Although five restriction enzymes are presented, a combination of only two restriction enzymes is generally sufficient to identify most of the TcR-δ gene rearrangements by Southern blot analysis (Table 2). Moreover, virtually all the observed TcR-δ rearrangements (97%) were detectable by use of the TCRDJ1 probe.

A remarkable observation was the presence of two highly polymorphic restriction sites: *Bam*HI restriction site in the Vδ2 gene segment (54) and *Kpn*I restriction site in the Vδ3 gene segment (20). In both cases the polymorphic restriction site was caused by a non-silent point mutation within the Vδ gene segment (48,50,55,56). Both point mutations are located outside the complementary determining regions of the TcR-δ chain (1). No other polymorphic restriction sites were identified.

TcR-δ gene rearrangements occurred on 55% of the alleles in T-ALL and 33% of the alleles in precursor B-ALL. In T-ALL, this was mainly caused by high percentages of rearrangements in $CD3^-$ T-ALL (62%) and TcR-$\gamma\delta^+$ T-ALL (96%). This resulted in 70% of the T-ALL and 51% of the precursor B-ALL with at least one rearranged TcR-δ allele. TcR-δ gene deletions were frequently found in both T-ALL (37% of the alleles) and precursor B-ALL (45% of the alleles). In T-ALL, TcR-δ gene deletion most frequently occurred in TcR-$\alpha\beta^+$ T-ALL (81% of the alleles) and $CD3^-$ T-ALL (25% of the alleles). The frequent TcR-δ gene deletions in precursor B-ALL are most probably for a major part caused by Vδ2-Dδ2-Dδ3-Jα gene rearrangements (57-59)

Except for Vδ4, all other five known Vδ gene segments were at least once involved in a rearrangement in the total group of ALL. Although the Vδ4 gene segment is able to

rearrange to the Jδ1 gene segment (48,50), it frequently rearranges to Jα gene segments (14). This Vδ4 gene segment contains all conserved amino acid residues characteristic of human Vα gene segments and is almost identical (96% homology) to the Vα6 gene segments (14,48,50). Therefore, Vδ4 most probably represents a Vα gene segment. Vδ5 and Vδ6 gene segments also occur in rearrangements with both Jα and Jδ gene segments (14,50). Moreover Vδ5 and Vδ6 display a high sequence homology with Vα 17.1 and Vα 13.1, respectively (18,47,50,60). So far, there is no absolute characteristic to define Vα and Vδ gene segments, other than their preference to rearrange to either Jα or Jδ gene segments.

Of the other Vδ gene segments, the Vδ1 gene segment was most frequently used in combination with the Jδ1 gene segment, which can be explained by the assumption that T-ALL cells arise from normal thymocytes and the fact that 60% of the TcR-$\gamma\delta^+$ thymocytes express a Vδ1-Jδ1-Cδ chain (27,29,30). The predominantly expressed Vδ2-Jδ1-Cδ chain on PB T-lymphocytes is less frequently found on TcR-$\gamma\delta^+$ thymocytes (27-30), which is in line with the observation that Vδ2-Jδ1 rearrangements occur at a low frequency in T-ALL. Interestingly, almost all complete Vδ-Jδ gene rearrangements involved the Jδ1 gene segment, whereas rearrangements to the Jδ2 or Jδ3 gene segments were only sporadically observed. Also, in the incomplete TcR-δ gene rearrangements, there was a preference for particular combinations of gene segments, i.e. Dδ2-Jδ1, Vδ2-Dδ3, and Dδ2-Dδ3. Whereas these incomplete rearrangements occurred in relative low frequencies in T-ALL, they represented 82% of the rearrangements found in precursor B-ALL, which is in line with data from the literature (33-35). The preferential complete (Vδ1-Jδ1, Vδ2-Jδ1, and Vδ3-Jδ1) and incomplete (Dδ2-Jδ1, Vδ2-Dδ3, and Dδ2-Dδ3) rearrangements result in a limited actual combinatorial diversity of the TcR-δ genes in ALL.

All other rearrangements in T-ALL (32%) and precursor B-ALL (18%) could either be identified as less frequent rearrangements to other TcR-δ gene segments (e.g. Vδ3-Jδ2, Vδ5-Jδ1, δREC-Jδ1, and Vδ6-Jδ2) or could not be identified at all. The inability to identify certain TcR-δ gene rearrangements is caused by frequently occurring Vα-Jδ1 rearrangements and by translocations or other chromosomal aberrations involving the TcR-δ gene (61-63).

Sequence analysis of a total of 100 junctional regions of the above-mentioned six preferential rearrangements showed an enormous diversity caused by extensive insertion of nucleotides as well as by moderate deletion of nucleotides. This extensive junctional diversity compensates for the preferential usage of the limited combinatorial repertoire. The sizes and compilation of the junctional regions were, on average, comparable, depending on the potential number of N-regions. Only the Dδ2-Jδ1 rearrangement with two potential N-regions showed an increased number of deleted nucleotides. This biased phenomenon is caused by the fact that the relatively small size of the Dδ2 gene segment (9 nucleotides) and the extensive nucleotide deletion by trimming of the Dδ2 gene segment during the Dδ2-Dδ3 rearrangement processes can easily damage the 5′ heptamer RSS of the involved Dδ2 gene segment, thereby preventing further rearrangements to a Dδ1 or Vδ gene segment. Damage of the 5′ RSS of the Dδ2 gene segment was observed in 57% (8/14) of the analyzed Dδ2-Jδ1 gene rearrangements. For example, the single Dδ2-Jδ1 gene rearrangement observed in TcR-$\alpha\beta^+$ T-ALL contained a damaged 5′ RSS (Figure 4). Extensive trimming only damaged 9% (1/11) of the 5′ RSS in Dδ2-Dδ3 rearrangements and

6% (2/33) of the 3′ RSS in Vδ2-Dδ3 and Dδ2-Dδ3 rearrangements, indicating that the majority of these rearrangements, unlike most Dδ2-Jδ1 rearrangements, can still be involved in continuing rearrangement processes, as found in precursor-B-ALL (57-59).

Both limited combinatorial diversity and extensive junctional diversity favor the TcR-δ gene as target for the detection of MRD in ALL utilizing PCR-mediated amplification techniques (41-44). The limited combinatorial diversity allows simple identification of the rearranged TcR-δ gene segments by Southern blot analysis and usage of only a limited set of Vδ-, Dδ-, and Jδ-specific PCR oligonucleotide primers. The extensive junctional diversity allows construction of highly specific junctional region oligonucleotides probes. In ~70% of the T-ALL and ~51% of the precursor B-ALL, TcR-δ gene rearrangements were found on one or both alleles (Table 3). Approximately 80% of these T-ALL (~56% of all T-ALL) and ~91% of these precursor B-ALL (~46% of all precursor B-ALL) contain an identifiable TcR-δ gene rearrangement on at least one allele and therefore also an identifiable junctional region, which allows detection of MRD by PCR techniques in these ALL.

The restriction map and Southern blot rearrangement patterns presented here, in combination with the new TcR-δ gene probes, allow the identification of most TcR-δ gene rearrangements in T-ALL and precursor B-ALL, which can be used for diagnostic purposes at diagnosis and during follow-up. Further studies have to unravel to what extend the remaining TcR-δ gene rearrangements represent chromosome aberrations or Vα-Jδ rearrangements.

ACKNOWLEDGMENTS. The authors gratefully acknowledge Prof. Dr. R. Benner, Dr. H. Hooijkaas and Dr. H.J. Adriaansen for their continuous support; Dr. R. Kurrle (Behring, Marburg, Germany), Dr. T. Hercend (Villejuif, France), and Dr. L. Moretta (Genova, Italy) for kindly providing the BMA031, Ti-γA and BB3 antibodies; Dr. T.H. Rabbitts for kindly providing the R21XH genomic clone and Dr. T. Mak for kindly providing the E2.6 genomic clone, which were used for sequence information; Mr. T.M. van Os for excellent assistance in the preparation of the figures; Ms. A.D. Korpershoek for her secretarial support; and Dr. D. Campana, Dr. R.J. van de Griend, Dr. K. Hählen, Dr. J.C. Kluin-Nelemans, Dr. W-D. Ludwig, and Dr. C.E. van der Schoot for kindly providing ALL cell samples. The Dutch Childhood Leukemia Study Group (DCLSG) kindly provided 48 of the 229 leukemia cell samples. Board members of the DCLSG are J.P.M. Bökkerink, H. van de Berg, M.V.A. Bruin, P.J. van Dijken, K. Hählen, W.A. Kamps, F.A.E. Nabben, A. Postma, J.A. Rammeloo, I.M. Risseeuw-Appel, G.A.M. de Vaan, E.Th. van 't Veer-Korthof, A.J.P. Veerman, F.C. de Waal, M. van Weel-Sipman, and R.S. Weening.

REFERENCES

1. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988;334:395-402.
2. Raulet DH. The structure, function, and molecular genetics of the γ/δ T cell receptor. Annu Rev Immunol 1989;7:175-207.
3. Akira S, Okazaki K, Sakano H. Two pairs of recombination signals are sufficient to cause immunoglobulin V-(D)-J joining. Science 1987;238:1134-1138.
4. Blackwell TK, Alt FW. Molecular characterization of the lymphoid V(D)J recombination activity. J Biol Chem 1989;264:10327-10330.
5. Hesse JE, Lieber MR, Mizuuchi K, Gellert M. V(D)J recombination: a functional definition of the joining signals. Genes Develop 1989;3:1053-1061
6. Schatz DG, Oettinger MA, Schlissel MS. V(D)J recombination: molecular biology and regulation. Annu Rev Immunol 1992;10:359-383.
7. Schatz DG, Oettinger MA, Baltimore D. The V(D)J recombination activating gene, RAG-1. Cell 1989;59:1035-1048.
8. Oettinger MA, Schatz DG, Gorka C, Baltimore D. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. Science 1990;248:1517-1523.

9. Alt FW, Baltimore D. Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-JH fusions. Proc Natl Acad Sci USA 1982;79:4118-4122.
10. Lafaille JJ, DeCloux A, Bonneville M, Takagaki Y, Tonegawa S. Junctional Sequences of T cell receptor γδ genes: implications for γδ T cell lineages and for a novel intermediate of V-(D)-J joining. Cell 1989;59:859-870.
11. Toyonaga B, Yoshikai Y, Vadasz V, Chin B, Mak TW. Organization and sequences of the diversity, joining, and constant region genes of the human T-cell receptor β chain. Proc Natl Acad Sci USA 1985;82:8624-8628.
12. Concannon P, Pickering LA, Kung P, Hood L. Diversity and structure of human T-cell receptor β-chain variable region genes. Proc Natl Acad Sci USA 1986;83:6598-6602.
13. Loh EY, Cwirla S, Serafini AT, Phillips JH, Lanier LL. Human T-cell-receptor δ chain: genomic organization, diversity, and expression in populations of cells. Proc Natl Acad Sci USA 1988;85:9714-9718.
14. Yoshikai Y, Kimura N, Toyonaga B, Mak TW. Sequences and repertoire of human T cell receptor α chain variable region genes in mature T lymphocytes. J Exp Med 1986;164:90-103.
15. Quertermous T, Strauss W, Murre C, Dialynas DP, Strominger JL, Seidman JG. Human T-cell γ genes contain *N* segments and have marked junctional variability. Nature 1986;322:184-187.
16. Loh EY, Elliott JF, Cwirla S, Lanier LL, Davis MM. Polymerase chain reaction with single-sided specificity: analysis of T cell receptor δ chain. Science 1989;243:217-220.
17. Isobe M, Russo G, Haluska FG, Croce CM. Cloning of the gene encoding the δ subunit of the human T-cell receptor reveals its physical organization within the α-subunit locus and its involvement in chromosome translocations in T-cell malignancy. Proc Natl Acad Sci USA 1988;85:3933-3937.
18. Satyanarayana K, Hata S, Devlin P, Grazia Roncarolo M, De Vries JE, Spits H, Strominger JL, Krangel MS. Genomic organization of the human T-cell antigen-receptor α/δ locus. Proc Natl Acad Sci USA 1988;85:8166-8170.
19. Takihara Y, Tkachuk D, Michalopoulos E, Champagne E, Reimann J, Minden M, Mak TW. Sequence and organization of the diversity, joining, and constant region genes of the human T-cell δ-chain locus. Proc Natl Acad Sci USA 1988;85:6097-6101.
20. Van Dongen JJM, Wolvers-Tettero ILM, Wassenaar F, Borst J, Van den Elsen P. Rearrangement and expression of T-cell receptor delta genes in T-cell acute lymphoblastic leukemias. Blood 1989;74:334-342.
21. Hara J, Takihara Y, Yumura-Yagi K, Ishihara S, Tawa A, Mak TW, Gelfland EW, Okada S, Kawa-Ha K. Differential usage of δ recombining element and Vδ genes during T-cell ontogeny. Blood 1991;78:2075-2081.
22. De Villartay J-P, Hockett RD, Coran D, Korsmeyer SJ, Cohen DI. Deletion of the human T-cell receptor δ-gene by a site-specific recombination. Nature 1988;335:170-174.
23. Hockett RD, De Villartay J-P, Pollock K, Poplack DG, Cohen DI, Korsmeyer SJ. Human T-cell antigen receptor (TCR) δ-chain locus and elements responsible for its deletion are within the TCR α-chain locus. Proc Natl Acad Sci USA 1988;85:9694-9698.
24. Hockett RD Jr, Nuñez G, Korsmeyer SJ. Evolutionary comparison of murine and human δ T-cell receptor deleting elements. New Biologist 1989;1:266-274.
25. Van Dongen JJM, Comans-Bitter WM, Wolvers-Tettero ILM, Borst J. Development of human T lymphocytes and their thymus-dependency. Thymus 1990;16:207-234.
26. Triebel F, Faure F, Mami-Chouaib F, Jitsukawa S, Griscelli A, Genevée C, Roman-Roman S, Hercend T. A novel human Vδ gene expressed predominantly in the TiγA fraction of γ/δ$^+$ peripheral lymphocytes. Eur J Immunol 1988;18:2021-2027.
27. Bottino C, Tambussi G, Ferrini S, Ciccone E, Varese P, Mingari MC, Moretta L, Moretta A. Two subsets of human T lymphocytes expressing γ/δ antigen receptor are identifiable by monoclonal antibodies directed to two distinct molecular forms of the receptor. J Exp Med 1988;168:491-505.
28. Borst J, Wicherink A, Van Dongen JJM, De Vries E, Comans-Bitter WM, Wassenaar F, Van den Elsen P. Non-random expression of T cell receptor γ and δ variable gene segments in functional T lymphocyte clones from human peripheral blood. Eur J Immunol 1989;19:1559-1568.
29. Triebel F, Hercend T. Subpopulations of human peripheral T gamma delta lymphocytes. Immunol Today 1989;10:186-188.
30. Casorati G, De Libero G, Lanzavecchia A, Migone N. Molecular analysis of human γ/δ$^+$ clones from thymus and peripheral blood. J Exp Med 1989;170:1521-1538.
31. Gouttefangeas C, Bensussan A, Boumsell L. Study of the CD3-associated T-cell receptors reveals further differences between T-cell acute lymphoblastic lymphoma and leukemia. Blood 1990;75:931-934.
32. Breit TM, Wolvers-Tettero ILM, Hählen K, Van Wering ER, Van Dongen JJM. Limited combinatorial repertoire of γδ T-cell receptors expressed by T-cell acute lymphoblastic leukemias. Leukemia 1991;5:116-124.
33. Loiseau P, Guglielmi P, Le Paslier D, Macintyre E, Gessain A, Bories J-C, Flandrin G, Chen Z, Sigaux F. Rearrangements of the T cell receptor δ gene in T acute lymphoblastic leukemia cells are distinct from those occurring in B lineage acute lymphoblastic leukemia and preferentially involve one Vδ gene segment. J Immunol 1989;142:3305-3311.
34. Biondi A, Di Celle PF, Rossi V, Casorati G, Matullo G, Giudici G, Foa R, Migone N. High prevalence of T-cell receptor Vδ2-(D)-Dδ3 or Dδ1/2-Dδ3 rearrangements in B-precursor acute lymphoblastic leukemias. Blood 1990;75:1834-1840.
35. Yano T, Pullman A, Andrade R, Uppenkamp M, De Villartay JP, Reaman G, Crush-Stanton S, Cohen DI, Raffeld M, Cossman J. A common Vδ2-Dδ2-Dδ3 T cell receptor gene rearrangement in precursor B acute lymphoblastic leukaemia. Br J Haematol 1991;79:44-49.
36. Hata S, Satyanarayana K, Devlin P, Band H, McLean J, Strominger JL, Brenner MB, Krangel MS. Extensive junctional diversity of rearranged human T cell receptor δ genes. Science 1988;240:1541-1544.

37. Macintyre E, D'Auriol L, Amesland F, Loiseau P, Chen Z, Boumsell L, Galibert F, Sigaux F. Analysis of junctional diversity in the preferential Vδ1-Jδ1 rearrangement of fresh T-acute lymphoblastic leukemia cells by in vitro gene amplification and direct sequencing. Blood 1989;74:2053-2061.
38. Breit TM, Wolvers-Tettero ILM, Hählen K, Van Wering ER, Van Dongen JJM. Extensive junctional diversity of γδ T-cell receptors expressed by T-cell acute lymphoblastic leukemias: implications for the detection of minimal residual disease. Leukemia 5:1076-1086 and erratum 1991;6:169-170.
39. Griesinger F, Grümayer ER, Van Ness B, Kersey JH. Rearrangement and diversification of T-cell receptor delta genes in acute lymphoblastic leukemia. Leukemia 1992;6:1054-1062.
40. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of immunoglobulin and T cell receptor genes. Part II: possibilities and limitations in the diagnosis and management of lymphoproliferative diseases and related disorders. Clin Chim Acta 1991;198:93-174.
41. Hansen-Hagge TE, Yokota S, Bartram CR. Detection of minimal residual disease in acute lymphoblastic leukemia by in vitro amplification of rearranged T-cell receptor δ chain sequences. Blood 1989;74:1762-1767.
42. Macintyre EA, D'Auriol L, Duparc N, Leverger G, Galibert F, Sigaux F. Use of oligonucleotide probes directed against T cell antigen receptor gamma delta variable-(diversity)-joining junctional sequences as a general method for detecting minimal residual disease in acute lymphoblastic leukemias. J Clin Invest 1990;86:2125-2135.
43. Yokoto S, Hansen-Hagge TE, Ludwig W-D, Reiter A, Raghavachar A, Kleihauer E, Bartram CR. Use of polymerase chain reactions to monitor minimal residual disease in acute lymphoblastic leukemia patients. Blood 1991;77:331-339.
44. Van Dongen JJM, Breit TM, Adriaansen HJ, Beishuizen A, Hooijkaas H. Detection of minimal residual disease in acute leukemia by immunological marker analysis and polymerase chain reaction. Leukemia 1992;6S1:47-59.
45. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunological marker analysis of cells in the various hematopoietic differentiation stages and their malignant counterparts. In D. J. Ruiter, G. J. Fleuren, and S. O. Warnaar, editors. Application of monoclonal antibodies in tumor pathology. Dordrecht: Martinus Nijhoff 1987:87-116.
46. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning, a laboratory manual, 2nd edn. Cold Spring Harbor: Cold Spring Harbor Laboratory, 1989.
47. Klein MH, Concannon P, Everett M, Kim LDH, Hunkapiller T, Hood L. Diversity and structure of human T-cell receptor α-chain variable region genes. Proc Natl Acad Sci USA 1987;84:6884-6888.
48. Guglielmi P, Davi F, D'Auriol L, Bories J-C, Dausset J, Bensussan A. Use of a variable α region to create a functional T-cell receptor δ chain. Proc Natl Acad Sci USA 1988;85:5634-5638.
49. Hata S, Clabby M, Devlin P, Spits H, De Vries JE, Krangel MS. Diversity and organization of human T cell receptor δ variable gene segments. J Exp Med 1989;169:41-57.
50. Takihara Y, Reimann J, Michalopoulos E, Ciccone E, Moretta L, Mak TW. Diversity and structure of human T cell receptor δ chain genes in peripheral blood γ/δ-bearing T lymphocytes. J Exp Med 1989;169:393-405.
51. Dariavach P, Lefranc M-P. First genomic sequence of the human T-cell receptor δ2 gene (TRDV2). Nucleic Acids Res 1989;17:4880.
52. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of Immunoglobulin and T cell receptor genes. Part I: basic and technical aspects. Clin Chim Acta 1991;198:1-92.
53. Griessinger F, Greenberg JM, Kersey JH. T cell receptor gamma and delta rearrangements in hematologic malignancies: Relationship to lymphoid differentiation. J Clin Invest 1989;84:506-516.
54. Chuchana P, Soua Z, Brockly F, Zhang XG, Ghanem N, Lefranc G, Lefranc M-P. BamHI restriction fragment alleles of the human T-cell receptor delta (TRD) variable region V2. Nucleic Acids Res 1989;17:3622.
55. Takihara Y, Champagne E, Ciccone E, Moretta L, Minden M, Mak TW. Organization and orientation of a human T cell receptor δ chain V gene segment that suggests an inversion mechanism is utilized in its rearrangement. Eur J Immunol 1989;19:571-574.
56. Villartay J-P, Pullman AB, Andrade R, Tschachler E, Colamenici O, Neckers L, Cohen DI, Cossman J. γ/δ lineage relationship within a consecutive series of human precursor T-cell neoplasms. Blood 1989;74:2508-2518.
57. Hara J, Benedict SH, Champagne E, Takihara Y, Mak TW, Minden M, Gelfand EW. T cell receptor δ gene rearrangements in acute lymphoblastic leukemia. J Clin Invest 1988;82:1974-1982.
58. Yokota S, Hansen-Hagge TE, Bartram CR. T-cell receptor δ gene recombination in common acute lymphoblastic leukemia: preferential usage of Vδ2 and frequent involvement of the Jα cluster. Blood 1991;77:141-148.
59. Hansen-Hagge TE, Yokota S, Reuter HJ, Schwarz K, Bartram CR. Human common acute lymphoblastic leukemia-derived cell lines are competent to recombine their T-cell receptor δ/α regions along a hierarchically ordered pathway. Blood 1992;80:2353-2362.
60. Krangel MS, Yssel H, Brocklehurst C, Spits H. A distinct wave of human T cell receptor γ/δ lymphocytes in the early fetal thymus: evidence for controlled gene rearrangement and cytokine production. J Exp Med 1990;172:847-859.
61. Boehm T, Rabbitts TH. The human T cell receptor genes are targets for chromosomal abnormalities in T cell tumors. FASEB J 1989;3:2344-2359.
62. Chen Q, Cheng J-T, Tsai L-H, Schneider N, Buchanan G, Carroll A, Christ W, Ozanne B, Siciliano MJ, Baer R. The *tal* gene undergoes chromosome translocation in T cell leukemia and potentially encodes a helix-loop-helix protein. EMBO J 1990;9:415-424.
63. Rabbitts TH. Translocations, master genes, and differences between the origins of acute and chronic leukemias. Cell 1991;67:641-644.

CHAPTER 3.3

TWO PATHWAYS OF SEQUENTIAL TcR-δ GENE REARRANGEMENTS IN HUMAN THYMOCYTES*

Timo M. Breit[1], Ingrid L.M. Wolvers-Tettero[1], Ellen J. van Gastel-Mol[1], Bianca Blom[2], Ad J.J.C. Bogers[3], Ronald R. de Krijger[4], Hergen Spits[2], and Jacques J.M. van Dongen[1]

1. Department of Immunology, University Hospital Dijkzigt/Erasmus University, Rotterdam;
2. Division of Immunology, The Netherlands Cancer Institute, Amsterdam;
3. Department of Thoracic Surgery, University Hospital Dijkzigt/Erasmus University, Rotterdam;
4. Department of Pediatrics, Sophia Children's Hospital/Erasmus University, Rotterdam, The Netherlands.

ABSTRACT

Human early T-cell differentiation is characterized by rearrangements of TcR genes. The TcR-δ gene is a particular TcR gene as it is embedded in the TcR-α gene. For this reason it is assumed that the TcR-δ gene plays a key role in the commitment of early thymocytes to either the TcR-$\alpha\beta^+$ or the TcR-$\gamma\delta^+$ lineage. The order of rearrangements in the TcR-α/δ locus is: TcR-δ gene rearrangement; TcR-δ gene deletion by rearrangement of the deleting elements δREC and ψJα; TcR-α gene rearrangement. Besides these consecutive rearrangements in the TcR-α/δ locus, it is generally recognized that preferential TcR-δ gene rearrangements occur with different frequencies related to stage of ontogeny or body compartment. Fetal thymocytes predominantly contain Vδ2-Jδ3 rearrangements, whereas Vδ1-Jδ1 rearrangements dominate in post-natal thymocytes, and the majority of peripheral blood T-lymphocytes contains at least one Vδ2-Jδ1 rearrangement. In this study we have analyzed the order of TcR-δ gene rearrangements. For this purpose, fetal thymi, neonatal thymi, infant thymi, and an immature infant thymocyte subpopulation (CD34$^+$/CD4$^-$/CD8$^-$/CD3$^-$) were analyzed with Southern blot and PCR analysis directed to both the coding joints and signal joints of TcR-δ gene rearrangements. From our data we were able to deduce two major pathways of sequential TcR-δ gene rearrangements in post-natal thymocytes: the fetal-like pathway and the post-natal pathway. The fetal-like pathway rearranges Vδ2→Dδ3 and subsequently Vδ2-Dδ3→Jδ1. Homologous sequential rearrangements represent the major pathway in fetal thymocytes: Vδ2-Dδ3→Jδ3 (or Jδ2). The sequential rearrangements of the post-natal pathway are: Dδ2→Dδ3, Dδ2-Dδ3→Jδ1, Vδ1→Dδ2-Dδ3-Jδ1. The strict order in which the TcR-δ gene segments are used in sequential rearrangements may implicate genomic sites in the TcR-δ gene that are involved in the regulation of the sequential TcR-δ gene rearrangement pathways.

---

* Submitted for publication.

INTRODUCTION

The TcR-δ gene is a special gene because of its location within the TcR-α gene (1-3). The majority of the Vα gene segments are located upstream of the TcR-δ gene, whereas all Jα gene segments are located downstream (Figure 1) (1-3). This unique configuration prevents the coexistence of a TcR-δ and a TcR-α gene rearrangement on the same allele. This is one of the reasons why the TcR-α/δ locus is believed to play an important role in divergence of the TcR-αβ and TcR-γδ differentiation pathways (4).

The TcR-δ gene complex is composed of several Vδ, three Dδ, and four Jδ gene segments (Figure 1) (5-10). The exact number of Vδ gene segments is difficult to estimate because the Vδ and some Vα gene segments are located in the same region. Moreover, it appears that many Vα gene segments are capable of rearranging to the Jδ gene segments and that in return Vδ gene segments can rearrange to Jα gene segments (10-14). However, only three Vδ gene segments (Vδ1, Vδ2, and Vδ3) are frequently used in TcR-δ gene rearrangements (6,7). Another characteristic feature of the TcR-δ gene is the frequent usage of more than one Dδ gene segment in a TcR-δ gene rearrangement (5,7).

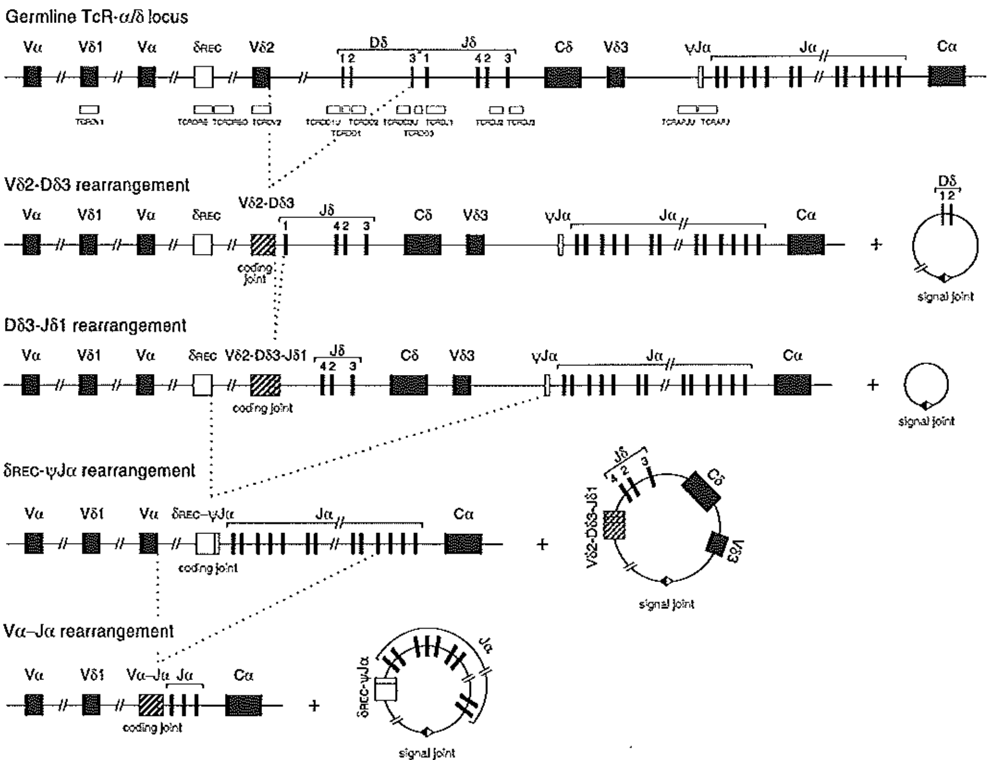


Figure 1. Sequential TcR-δ gene rearrangements. The TcR-α/δ gene is indicated with the probes for Southern blot analysis as open boxes below the gene segments they recognize (7). Indicated are the consecutive TcR gene rearrangements: Vδ2→Dδ3, Vδ2-Dδ3→Jδ1, δREC→ψJα, and Vα→Jα with the associated circular excision products.

Except for the Vδ1 gene segment, all TcR-δ gene segments are located in a region which is flanked by the so-called TcR-δ deleting elements, δREC and ψJα (Figure 1) (15-17). Rearrangement of these elements to each other will therefore delete all TcR-δ gene segments. The δREC-ψJα rearrangement is a non-functional rearrangement that is not transcribed, and it appears that this rearrangement only occurs to prepare the allele for further TcR-α gene rearrangements (Figure 1) (15,16). Thus, the δREC-ψJα rearrangement is believed to play an important role in the αβ/γδ lineage bifurcation (4,15-17).

V(D)J recombination of TcR gene segments involves the conserved heptamer-nonamer RSS, which are present at each rearranging side of a gene segment (18-20). The RSS are classified according to the size of the spacer which is located in between the heptamer and nonamer of the RSS (20). The spacer can be 23bp long (V gene segments) or 12bp long (J gene segments). The 12/23 rule defines that gene segments can in principle only rearrange to each other when the involved RSS have different spacer sizes (20). In TcR-Dδ gene segments the 5' RSS contains a J-like spacer (12bp), whereas their 3' RSS contains a V-like spacer (23bp) (5). This enables the Dδ gene segments to rearrange to Vδ, Jδ, or other Dδ gene segments.

The DNA between two rearranging gene segments is deleted by a looping-out mechanism and will form a so-called circular excision product (Figure 1) (20). The circle is closed via the so-called signal joint of the rearrangement and usually contains a perfect head-to-head fusion of the complete RSS of the rearranged gene segments without nucleotide insertion or deletion (20). Analysis of signal joints shows exactly which gene segments were rearranged to each other, whereas coding joints only show the final rearrangement in which the junctional region with nucleotide insertion and deletion might obscure the involvement of D gene segments.

Rearrangements of the TcR-δ gene occur early in thymocyte differentiation (4). And because the actual combinatorial repertoire is restricted (5-10), Southern blot analysis with TcR-δ probes will show preferential rearrangements in a polyclonal thymocyte sample (15-17). Krangel et al. (21) and McVay et al. (22) showed that the preferential rearrangements which occurred in early fetal thymus (Vδ2-Dδ3, Vδ2-Dδ3-Jδ3) were different from those present in post-natal thymus (Vδ1-Dδ2-Dδ3-Jδ1). The same preference has been observed in T-cells from human fetal liver (23). In PB of healthy individuals the far majority of TcR-$\gamma\delta^+$ T-lymphocytes (>80%) express a Vδ2-Dδ3-Jδ1 TcR-δ chain (24-27). This is in contrast with the observed preference in post-natal thymus for the Vδ1-Dδ2-Dδ3-Jδ1 rearrangement (5,21,22). Because the Vδ2 chain is almost exclusively expressed in combination with a Vγ9-Jγ1.2 chain, an extremely rare thymic rearrangement, it is assumed that the Vδ2/Vγ9 T-lymphocytes are selected by a mechanism of peripheral expansion (24-27). Because we could prove that these peripheral T-lymphocytes were selected for their expressed TcR, the observed rearrangements therefore do not reflect a preferential recombination process on the DNA level (28).

Hence, TcR-δ gene rearrangements occur with a particular preference in different stages of ontogeny or different compartments of the body. Recently, an interesting study by Lauzurica and Krangel (29) on a human Vδ1-Vδ2-Dδ3-Jδ1-Jδ2-Cδ transgenic mouse provided interesting information on early TcR-δ gene rearrangements. They observed that Vδ→Dδ rearrangement is TcR-δ enhancer independent, whereas Vδ-Dδ→Jδ is enhancer dependent and T-cell specific (29). Also, since rearrangements of the transgene occurred

in both TcR-$\gamma\delta^+$ and TcR-$\alpha\beta^+$ T-cells, it appears that no $\alpha\beta/\gamma\delta$ lineage commitment takes place before TcR-δ gene rearrangement (29).

Almost all other studies on TcR-δ gene rearrangements focussed on the final stage of these rearrangements (6,7,17,21-23). We tried to establish the order in which the TcR-δ gene rearrangements occur. For this purpose we used a large series of TcR-δ gene probes that cover the whole gene, and allow identification of the various types of TcR-δ gene rearrangements by their restriction enzyme pattern in Southern blot analysis (7). Furthermore, we performed PCR analysis of the signal joints of TcR-δ gene rearrangements to obtain information as to which gene segments rearranged to each other. And finally, we isolated a subpopulation of immature thymocytes ($CD34^+/CD4^-/CD8^-/CD3^-$) to identify the earliest rearrangements of the TcR-δ gene (30,31).

MATERIALS AND METHODS

Cell samples

MNC were isolated from PB of four healthy volunteers by Ficoll-Paque (density 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. Thymocytes were obtained from fetal thymi of legal abortions and from post-natal thymus samples of children undergoing cardiac surgery. The five fetal thymi were from: 15 weeks (2x), 16 weeks, 17 weeks, and 18 weeks of gestation. The 15 post-natal thymus samples were from neonates and children at the age of: 3 days, 8 days, 9 days, 2.5 months (2x), 5 months, 8 months, 10 months, 1 year (2x), 1.8 year, 2.4 years, 5 years, 8 years, and 15 years. The thymus samples were minced with scissors in RPMI 1640 medium containing 10% fetal calf serum and were flushed through a nylon gauze filter with 100 μm openings. All human tissue and cell samples were obtained with the approval of the Medical Ethics Committee of the Erasmus University/University Hospital Dijkzigt, Rotterdam, The Netherlands. Cell line Hela was used as a germline control in the rearrangement studies.

Southern blot analysis

DNA was isolated from fresh or frozen MNC as described previously (32). Fifteen μg DNA samples were digested with the restriction enzymes: *Bgl*II, *Eco*RI, and/or *Hin*dIII (Pharmacia), size fractionated in 0.7% agarose gels and transferred to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) as described (32). TcR-δ gene rearrangements were studied using ^{32}P random oligonucleotide-labeled TcR-δ gene DNA probes; TCRDV1 (Vδ1), TCRDV2 (Vδ2), TCRDV3 (Vδ3), TCRDD1U (Dδ1 upstream), TCRDD1 (Dδ1), TCRDD2 (Dδ2), TCRDD3U (Dδ3 upstream), TCRDD3 (Dδ3), TCRDJ1 (Jδ1), TCRDJ2 (Jδ2), TCRDJ3 (Jδ3), TCRDRE (δREC), TCRDRED (δREC downstream), TCRAPJU (ψJα upstream), and TCRAPJ (ψJα) (Figure 1) (7).

PCR amplification analysis

PCR was essentially performed as described previously (7). A 0.25-1.0 μg sample of DNA, 12 pmol of the 5' and the 3' oligonucleotide primers and 1 unit of *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) were used in each PCR of 100 μl. The oligonucleotide primers are listed in Table 1 or References 7 and 17. The PCR reaction mixture was incubated at 94°C for 3 min, at 60°C for 2 min and at 72°C for 3 min in a thermal cycler (Perkin-Elmer Cetus). Following this initial cycle, denaturing, annealing and extension steps were performed for another 35-40 cycles at 94°C for 1 min, at 60°C for 1 min and at 72°C for 3 min, respectively. After the last cycle an additional extension step of 72°C for 7 min was executed. The PCR products were analyzed after electrophoresis in a ethidium-bromide stained 1% agarose gel.

Preparation of thymocyte suspensions and thymocyte subpopulations

Thymic tissue was obtained from a 2.4 years old child undergoing corrective cardiovascular surgery. Suspensions were made by mincing tissue and pressing through a stainless steel mesh. A Lymphoprep (density 1.077 g/ml; Nycomed Pharma, Oslo, Norway) density centrifugation was performed to remove the

TABLE 1. Oligonucleotide primers used in the PCR analysis of TcR-δ genes[a].

Code[b]	Position (bp)[c]	Sequence[d]	Reference[e]
Dδ1-3'XBg	+30	gtagatctaGAAGCCATTTGGTTAATGTCAAAAG	5
Dδ2-5'XBg	−20	gtagatctAGAAGAGGGTTTTTATACTGATGTG	5
Dδ2-3's	+49	cgcgtcgACATAGCGGGTCACGGCTGGG	5
Dδ3-5's	−43	cgcgtcgaCCATATAGTGTGAAACCGAGGGG	2
Vδ1sj3'	+84	GTGTGCATTTCAGATTGGCTCCTG	5
Vδ2sj3'	+25	CTGGTCAGTGGTTTTTGAGCTGCT	47
Vδ3sj3'	+198	CTACCAGGAGTCATTCAGGTGGC	33
δRECsj3'	+213	AGGCaGATCTTGTCTGACATTTGCTCCG	16
Jδ1sj5'	−139	GTCCCTACCTGCAGATGATTAACC	5
Jδ2sj5'	−9	GCAAGGTTTTTCGTAATGATGCCTG	6
Jδ3sj5'	−130	CCCTTGGTCTCATCAAGAGCAGC	48
ψJαsj5'	−127	TaaGCTtTGAAAGGCAGAAAGAGGGCA	16

a. All primers used other than those presented in the table are described in Reference 7, 17, and 46.
b. The extensions of the codes represent restriction sites present in the oligonucleotide primers. XBg, *XbaI* and *BglII*; S, *SalI* or sj, signal joint.
c. The position of the 3' side of the oligonucleotide primer is indicated upstream (−) or downstream (+) relative to the RSS of the involved gene segment
d. The sequence in lower case characters represent the aspecific nucleotides that generate restriction sites (underlined).
e. Sequence information used to design the oligonucleotide primers was derived from the indicated references.

majority of erythrocytes and part of the $CD4^+/CD8^+$ immature thymocytes. Enrichment of $CD34^+/CD4^-/CD8^-/CD3^-/CD19^-$ cells was first performed by incubation with specific McAbs followed by magnetic bead depletion. In the first step $CD8^+$ thymocytes were removed after incubation of the thymocytes with the RPA-T8 (CD8) antibody and depletion with Biomag magnetic beads coated with goat anti-mouse antibody (Advanced Magnetics Inc., Cambridge, MA). The remaining cells were depleted of $CD4^+$, $CD27^+$, and $CD69^+$ cells by incubation with the RPA-T4 (CD4), CLB-3A12 (CD27), and L78 (CD69) antibodies and depletion with Dynabeads magnetic beads coated with sheep anti-mouse antibody (Dynal Inc., Oslo, Norway). The remaining thymocytes were incubated with PE labeled anti CD34 (HPAC-2) and with FITC labeled CD4 (Leu-3a), CD8 (Leu-2a), CD3 (Leu-4), CD19 (Leu-12) (all Leu antibodies obtained from Becton Dickinson, San Jose, CA). Leu-2a and Leu-3a are directed against different epitopes on respectively CD4 and CD8 than the RPA-T4 and RPA-T8 antibodies used for the magnetic bead depletion. CD19 was used to remove the resident thymic B-cells. $CD34^+/CD4^-/CD8^-/CD3^-/CD19^-$ cells were then sorted with the FACStar plus (Becton Dickinson Immunocytometry System). The total amount of $CD34^+/CD4^-/CD8^-/CD3^-/CD19^-$ immature thymocytes was 6×10^6 cells starting from a 1.5×10^{10} total thymocyte cell sample.

RESULTS

Preferential TcR-δ gene rearrangements in fetal and post-natal thymus

DNA from a large series of fetal and post-natal thymi was analyzed by Southern blot for TcR-δ gene rearrangements. Our series of TcR-δ probes, that cover all known Vδ, Dδ, and Jδ gene segments was used to detect and identify the various types of TcR-δ gene rearrangements (7). The most evident observation in the Southern blot analysis was the presence of a number of bands that represented preferential rearrangements in both fetal and post-natal thymus samples (Figure 2). It was remarkable how comparable the density of the rearranged bands was between the thymus samples of the same group. On the other hand, these rearranged bands varied significantly in density when compared to each other,

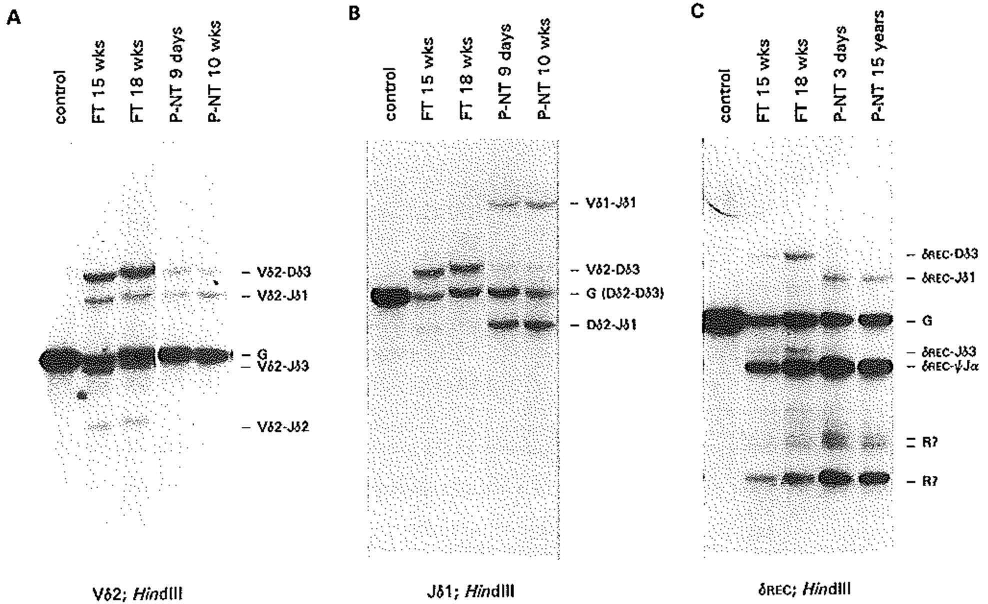


Figure 2. Preferential TcR-δ gene rearrangements in fetal (FT) and post-natal (P-NT) thymus samples. **A)** DNA digested with *Hin*dIII and hybridized with the TCRDV2 probe (Vδ2). Preferential fetal thymus rearrangements: Vδ2-Jδ3 and Vδ2-Jδ2. Other preferential rearrangements: Dδ2-Jδ1, Vδ2-Jδ1. **B)** DNA digested with *Hin*dIII and hybridized with the TCRDJ1 probe (Jδ1). Preferential post-natal thymus rearrangements: Dδ2-Jδ1, Vδ1-Jδ1, and Dδ2-Dδ3 (co-migrated with the germline band). Other preferential rearrangement: Vδ2-Dδ3. **C)** DNA digested with *Hin*dIII and hybridized with the TRCDRE probe (δREC). Preferential fetal thymus rearrangements: δREC-Dδ3 and δREC-Jδ3. Preferential post-natal thymus rearrangement δREC-Jδ1. The δREC-ψJα rearrangement is present throughout the ontogeny.

and also between the fetal versus the post-natal thymus samples (Figure 2). In fetal thymus, the most prominent bands were identified as δREC-ψJα, Vδ2-Dδ3, and Vδ2-Jδ3 rearrangements. In post-natal thymus the most prominent rearrangements were δREC-ψJα, Dδ2-Jδ1, and Vδ1-Jδ1 rearrangements (Figure 2). Some preferential rearrangements occurred in both groups of samples (δREC-ψJα, Vδ2-Dδ3, and Vδ2-Jδ1), but other rearrangements occurred in either fetal thymus (δREC-Dδ3, Vδ2-Jδ2, and Vδ2-Jδ3), or post natal thymus (Dδ2-Dδ3, Dδ2-Jδ1, Vδ1-Jδ1, Vδ3-Jδ1, and δREC-Jδ1). All preferential TcR-δ gene rearrangements as well as their occurrence, are summarized in Table 2. Although we were able to identify every known TcR-δ gene rearrangement by using an extensive series of TcR-δ probes (7), still some of the observed rearranged bands remained unidentifiable, and most likely represent rearrangements of a Dδ or Jδ gene segment to a Vα gene segment.

Coding joints of TcR-δ gene rearrangements

To confirm the Southern blot data of the various thymus samples, PCR analysis was performed on the coding joints of TcR-δ gene rearrangements. PCR reactions with primers of all possible combinations of the most frequently used TcR-δ gene segments were performed on six selected thymus (two fetal and four post-natal) samples and two MNC

TABLE 2. Southern blot analysis of preferential rearrangements in thymocyte cell samples[a].

	Fetal thymus	Post-natal thymus	Immature thymocytes
Rearrangement			
Dδ2-Dδ3	–	±	↑↑
Vδ2-Dδ3	+	±	↑↑
δREC-Dδ3	±	–	–
Dδ2-Jδ1	–	+	↑
Vδ2-Jδ2	±	–	–
Vδ2-Jδ3	+	–	–
δREC-Jδ3	±	–	–
Vδ2-Jδ1	±	±	↔
Vδ3-Jδ1	–	±	↓
Vδ1-Jδ1	–	+	↓
δREC-Jδ1	–	±	–
δREC-ψJα	+ +	+ +	–
Germline Jδ1	±	±	↑

a. Symbols; + + to –, indication of the density of rearranged band. Arrows indicate the relative increase (↑) or decrease (↓) of the density of rearranged bands observed in the immature thymocytes as compared to those observed in the post-natal thymocytes.

samples from PB of healthy volunteers. The data of the PCR analyses are summarized in Table 3.

All observed preferential rearrangements in the Southern blot analysis could be confirmed by PCR analysis (Figure 3A). In addition, several other rearrangements showed a positive signal, but this is due to the high sensitivity of the PCR amplification technique. Still, some TcR-δ gene rearrangements did not show positivity in the PCR analysis, which meant that they are extremely rare or do not occur at all. These included all rearrangements involving the Dδ1 gene segment, which is also rarely observed in junctional region sequences of TcR-δ gene rearrangements (7). Also the Dδ2 gene segment is hardly found in Vδ-Dδ2 gene rearrangements, whereas Dδ2-Jδ gene rearrangements occur frequently. It is interesting to note that almost all preferential rearrangements observed in the thymus samples by Southern blot analysis (Table 2) show PCR positivity in the PBMNC samples, whereas most other rearrangements are not detectable by PCR in PBMNC samples.

TABLE 3. PCR analysis of the coding joints in TcR-δ gene rearrangement[a].

	5' gene segment (primer)																				
	Dδ1 (Dδ1-3'XBg)			Dδ2 (Dδ2-3'S)			Dδ3 (Dδ3-3'XBg)			Jδ1 (Jδ1-3'XBg)			Jδ2 (Jδ2-3'S)			Jδ3 (Jδ3-3'S)			ψJα (ψJα-3'H)		
	FT	PNT	PB	FT	PNT	PB	FT	PNT	PB	FT	PNT	PB	FT	PNT	PB	FT	PNT	PB	FT	PNT	PB
3' gene segment (primer)																					
Vδ1 (Vδ1-5'X)	–	–	–	–	–	–	–	–	–	–	–	–									
Vδ2 (Vδ2-5'S)	–	–	–	–	–	–	±	±	–	–	–	–	–	–	–						
Vδ3 (Vδ3-5'S)	±	+	–	+	+	±	±	±	–	+	+	–	–	–	–	+	+	±			
δREC (δREC-5'E)	±	+	+	+	+	±	±	+	–	–	±	–	–	–	–	±	+	±	+	+	–
Dδ1 (Dδ1-5'S)	±	+	–	±	±	–	–	–	–	±	–	–	–	–	–	±	±	–	+	±	–
Dδ2 (Dδ2-5'XBg)	±	+	–	+	+	±	+	+	–	–	±	–	–	–	–	+	+	±	+	+	±
Dδ3 (Dδ3-5'S)	+	+	–	+	+	±	±	+	–	+	+	+	–	–	–	–	±	–	+	±	–

a. Abbreviations used: FT, fetal thymus; PNT, post-natal thymus; PB, peripheral blood.

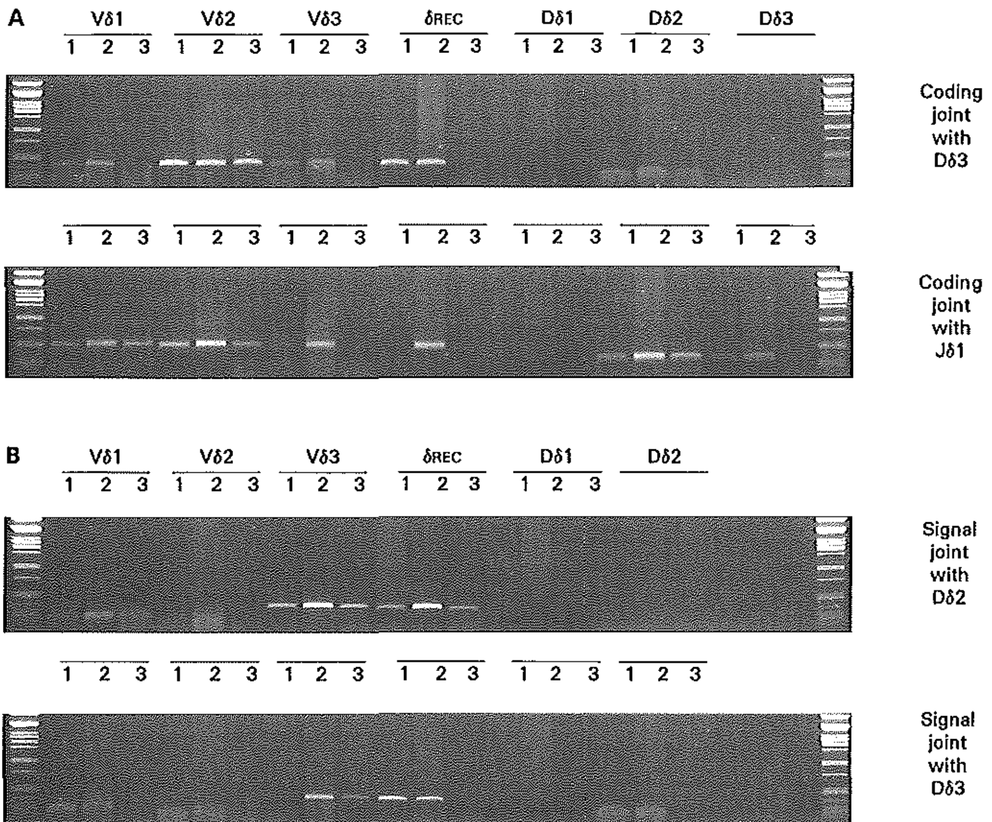


Figure 3. PCR analysis of the coding joints and signal joints in TcR-δ gene rearrangements from fetal thymocytes (lane 1), post-natal thymocytes (lane 2), and PB T-lymphocytes (lane 3). A) All combinations of 5′ coding joint primers to the Dδ3-3′ and Jδ1-3′ primers. B) All combinations of 3′ signal joint primers to the Dδ2-5′ and Dδ3-5′ primers. The PCR products are run in a ethidium-bromide-stained 1% agarose gel.

Signal joints in excision products of TcR-δ gene rearrangements

To establish the order of TcR-δ gene rearrangements, we performed Southern blot analysis on the circular excision products. These excision products were analyzed by using probes that are located downstream to the δREC or Dδ gene segments or upstream to the Dδ, Jδ or ψJα gene segments (Figure 1). Because a signal joint is also a rearrangement in itself, it can be detected as a rearranged band in a Southern blot. However, the relatively low frequency of the circular excision products prevented accurate Southern blot analysis. Only the signal joint of the δREC-ψJα rearrangement was visible as a weak band in the thymus samples and as a very faint band in the PBMNC samples.

To investigate the TcR-δ gene rearrangement order, we performed PCR analysis on the signal joints which are present in the circular excision products that arise from TcR-δ gene rearrangements. These PCR analyses were performed on DNA of six selected thymus (two fetal and four post-natal) samples and PBMNC samples of two healthy individuals. The data of the PCR analyses are summarized in Table 4. In line with the PCR analysis of the coding

TABLE 4. PCR analysis of the signal joints in circular excision products from TcR-δ gene rearrangement[a].

	5′ RSS (primer)																				
	Dδ1 (Dδ1-5′S)			Dδ2 (Dδ2-5′XBg)			Dδ3 (Dδ3-5′S)			Jδ1 (Jδ1s;5′)			Jδ2 (Jδ2s;5′)			Jδ3 (Jδ3s;5′)			ψJα (ψJαs;5′)		
	FT	PNT	PB	FT	PNT	PB	FT	PNT	PB	FT	PNT	PB	FT	PNT	PB	FT	PNT	PB	FT	PNT	PB
3′ RSS (primer)																					
Vδ1 (Vδ1s;3′)	–	–	–	–	–	–	–	–	–	–	–	–									
Vδ2 (Vδ2s;3′)	±	+	±	±	+	–	±	+	±	±	+	±	–	–	–						
Vδ3 (Vδ3s;3′)	+	±	–	+	±	–	+	±	±	+	±	–	–	–	–	±	+	±			
δREC (δRECs;3′)	–	–	–	–	–	–	–	–	–	–	±	–	–	–	–	–	–	–	–	+	–
Dδ1 (Dδ1-3′XBg)	–	–	–	–	–	–	–	±	–	–	–	–	–	–	–	–	–	–	±	±	–
Dδ2 (Dδ2-3′S)	–	–	–	–	–	–	–	–	±	–	–	–	–	–	–	–	–	–	+	+	+
Dδ3 (Dδ3-3′XBg)	+	+	±	–	–	–	±	+	±	+	+	+	–	–	–	–	–	–	±	+	+

a. Abbreviations used: FT, fetal thymus; PNT, post-natal thymus; PB, peripheral blood.

joints, the signal joint analysis did not show any involvement of the Dδ1 gene segments in TcR-δ gene rearrangement. Of more interest was the observation that the Dδ2 gene segment is frequently linked to a Vδ or δREC gene segment. This was also true for the Dδ3 gene segment, although the frequency of involvement of the two Dδ gene segments in fetal thymus was inversively proportional to that in post-natal thymus, i.e. Dδ3 gene segment involvement seems more abundant in fetal thymus, whereas involvement of Dδ2 seems more explicit in post-natal thymus, which is in line with the Southern blot data (Table 2). Also the Dδ2 gene segment is never directly rearranged to a J gene segment, which is in total concordance with the data of the coding joint PCR analyses (Table 3).

It was remarkable to see that the Jδ1 gene segment, which is the almost exclusively used Jδ gene segment in TcR-δ gene rearrangements of post-natal thymocytes, is never directly rearranged to a Vδ or Dδ2 gene segment, but predominantly rearranged to the Dδ3 gene segment (Figure 3B). The latter is also true for the Jδ2 and Jδ3 gene segments, with a minor exception of the Vδ3 gene segment. The presence of Vδ3-Jδ2 signal joints may be caused by the fact that the invested Vδ3 gene segment is located 3′ of the Cδ region and that this gene segment uses an inversional rearrangement mechanism for recombination, resulting in integration of the signal joint in the genome. The only J gene segment that does rearrange directly to Vδ gene segments is ψJα, which is to be expected because normal TcR-α rearrangements also do not include D gene segments, despite the fact that the Dδ3 gene segment is capable of rearranging to the ψJα gene segment. Still, it is interesting to find a 'difficult' inversional Vδ3-ψJα, but no 'normal' Vδ2-ψJα rearrangement.

Because it is generally assumed that the circular excision products, which arise as by-products of TcR gene rearrangement are rapidly degenerated in thymocytes and/or diluted by cell division, we were surprised by the high frequency of signal joints in PBMNC. Almost all signal joints observed in fetal and postnatal thymus were also present in PBMNC.

Incomplete TcR-δ gene rearrangements in early thymocytes

From the Southern blot and PCR data it seems as if a certain order was present in the TcR-δ gene rearrangements. To determine the sequential rearrangement pattern, we isolated an immature thymocyte subpopulation. This population was characterized by a $CD34^{+}/CD4^{-}/CD8^{-}/CD3^{-}$ phenotype and was assumed to contain the most immature thymocytes that are already in the process of rearranging their TcR-δ genes. On DNA from

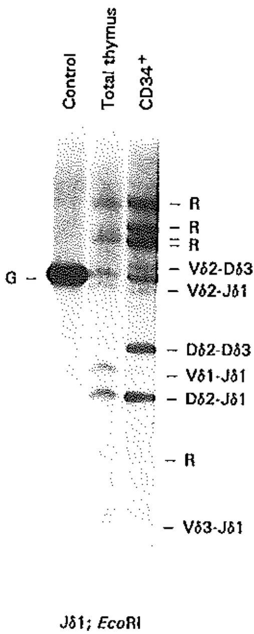


Figure 4. Preferential TcR-δ gene rearrangements in immature thymocytes. $CD34^+/CD4^-/CD8^-/CD3^-$ thymocytes were cell sorted out of a infant thymus cell sample (2.4 years). DNA of this subpopulation was digested with *Eco*RI and hybridized with the TCRDJ1 probe (Jδ1). This cell sample was compared to the total thymus sample (of the same thymus) and cell line Hela as germline control. Preferential rearrangements are indicated.

this thymocyte subpopulation we performed Southern blot analysis instead of PCR analysis, to estimate the relative frequencies of the preferential rearrangements as compared to a total thymus sample. Table 2 summarizes the data of the rearrangements observed in immature thymocytes (Figure 4). The most obvious observation was that in the early thymocyte fraction the germline band and the incomplete rearrangements (Dδ2-Dδ3, Vδ2-Dδ3, and Dδ2-Jδ1) were significantly increased in frequency, whereas the complete rearrangements Vδ1-Jδ1 and Vδ3-Jδ1, but not Vδ2-Jδ1, were evidently decreased as compared to the total thymus sample. Rearrangements involving the TcR-δ deleting elements (δREC-Jδ1 and δREC-ψJα) were totally absent. It was remarkable that in this early thymocyte subpopulation several preferential TcR-δ gene rearrangements involving either the Dδ3 or the Jδ1 gene segments were present, which did not involve any of the other known TcR-δ gene segments (Figure 4). These data together provide evidence for the existence of sequential TcR-δ gene rearrangement pathways during early thymocyte differentiation.

DISCUSSION

Previous studies found different preferential TcR-δ gene rearrangements in fetal thymus (or liver) as compared to post-natal thymus (16,17,21,22,26,33,34). The most significant preferential rearrangements in fetal thymus were: Vδ2-Dδ3 and Vδ2-Dδ3-Jδ3; in post-natal thymus: Dδ2-Dδ3-Jδ1 and Vδ1-Dδ2-Dδ3-Jδ1; and in all thymi: δREC-ψJα. The latter in fact is a TcR-δ gene deletion instead of a TcR-δ rearrangement and represents an

intermediate step towards TcR-α gene rearrangement. These studies suggest a certain order in TcR-δ gene rearrangements, but only the coding joints of the rearrangements in total thymus samples or of a (incomplete) TcR-δ transgene were analyzed so the precise sequential rearrangement steps could not be determined (16,17,21,22,29). In our present study we tried to add another dimension to these findings by analyzing not only the coding joints, but also the signal joints of TcR-δ gene rearrangements. The signal joints in circular excision products contain important information because, in contrast to the coding joints, they are not changed by ongoing rearrangements. Therefore, the signal joints tell exactly which gene segments rearranged to each other. Interesting information was also obtained from a subpopulation of immature thymocytes, because these thymocytes contained the earliest TcR-δ gene rearrangements. The combined results allowed us to define the major pathways of TcR-δ gene rearrangements in differentiating fetal and post-natal thymocytes. These pathways are schematically depicted in Figure 5.

The first pathway is the one observed in fetal thymus. This fetal pathway starts with rearrangement of the Vδ2 to the Dδ3 gene segment. In the second step, the incomplete Vδ2-Dδ3 rearrangement rearranges to a Jδ3, Jδ2 or Jδ1 gene segment. This order was deduced from the observation that Vδ2-Dδ3 appeared as a preferential fetal rearrangement in Southern blot analysis, whereas Dδ3-Jδ3 and Dδ3-Jδ2 rearrangement were only detected by a sensitive PCR analysis of the coding joints. Because the Vδ2 gene segment is located

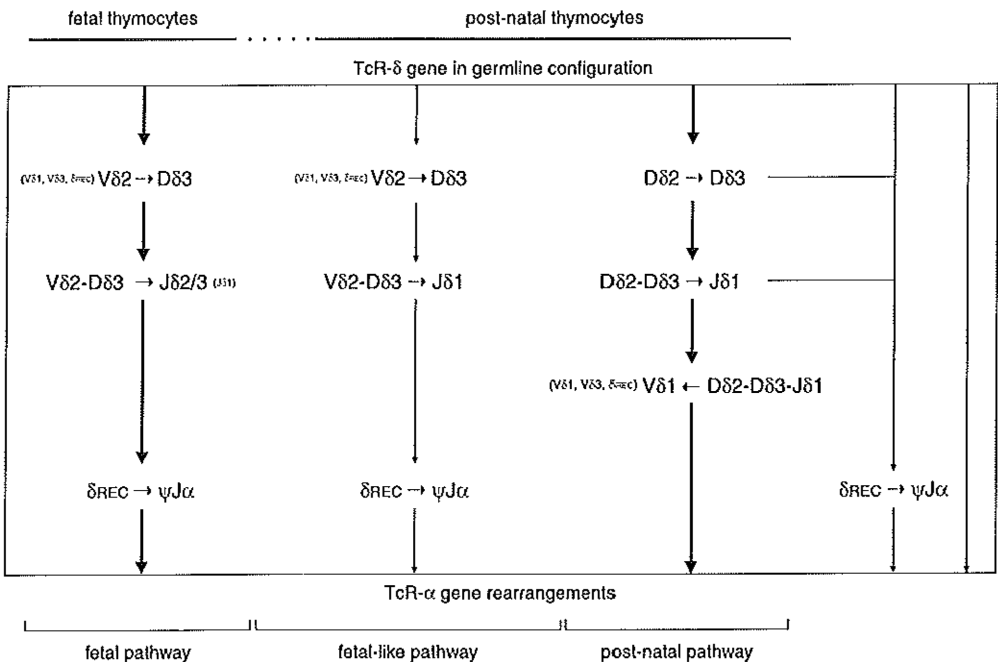


Figure 5. Schematic diagram of the pathways of human TcR-δ gene rearrangements. Indicated are the sequential TcR-δ gene rearrangements as they occur in fetal thymocytes: fetal pathway, and post-natal thymocytes: fetal-like and post-natal pathways. The gene segments between brackets are able to rearrange, but are no part of the major rearrangement pathways.

downstream of the δREC gene segment, incomplete Vδ2-Dδ3 or complete Vδ2-Dδ3-Jδ rearrangements can be deleted by the δREC-ψJα rearrangement.

This pathway could also be detected in post-natal thymus, with the exception that the incomplete Vδ2-Dδ3 rearrangement rearranges predominantly to the Jδ1 gene segment to form the complete Vδ2-Dδ3-Jδ1 rearrangement as detected in Southern blot analysis. Evidence for the sequential order of this fetal-like pathway was derived from the analysis of immature post-natal thymocytes in which the Vδ2-Dδ3 rearranged band had a significantly higher density than in the total thymus population, whereas the Vδ2-Jδ1 rearranged band showed about the same density. In this pathway all Vδ2 involved rearrangements can be deleted by the δREC-ψJα rearrangement.

The predominant pathway in post-natal thymus starts with the rearrangement of the Dδ2 to the Dδ3 gene segment, as proven in several ways. Firstly, in the PCR analysis both the coding joints and signal joints of Dδ2-Dδ3 gene rearrangements were observed. Moreover, coding joints of Dδ2-Jδ rearrangements were detected by Southern blotting and PCR, but no signal joints of these rearrangement were found by PCR analysis, which meant that they were Dδ2-Dδ3-Jδ rearrangements. The reverse situation happened with the coding joints of Vδ-Dδ2 rearrangements, which were virtually absent, but the signal joints of these rearrangement did occur, meaning that there was already a downstream gene segment linked to the Dδ2 gene segment before it rearranged to a Vδ gene segment. Secondly, the Dδ2-Dδ3 rearranged band had a significantly higher density in the immature thymocyte fraction as compared to the total thymocyte population. The second step in the post-natal pathway seems to be the rearrangement of the incomplete Dδ2-Dδ3 rearrangement to the Jδ1 gene segment. This was indicated by the fact that in the immature thymocyte fraction the Dδ2-Dδ3-Jδ1 rearrangement occurred more frequently than in the total thymus sample. However, this increase was less dramatic than that of Dδ2-Dδ3 rearrangements. Moreover, this increase was more prominent in a more mature thymocyte subpopulation ($CD4^-/CD8^-/CD27^-/CD69^-$) in which the Dδ2-Dδ3 rearrangement already decreased in frequency (results not shown). The final step in this pathway appears to be the rearrangement of a Vδ gene segment to the incomplete Dδ2-Dδ3-Jδ1 rearrangement, because in the immature thymocyte fraction most complete Vδ-Jδ1 rearrangements occurred less frequently. It is important to note that the Vδ1-Dδ2-Dδ3-Jδ1 gene rearrangement cannot be deleted by a δREC-ψJα rearrangement, because Vδ1 gene rearrangements delete the δREC gene segment.

Thus, the pathways can be divided into several different phases.

Phase 1: rearrangements of the Dδ2 or Vδ2 gene segments to the Dδ3 gene segment. It is of interest to note that the rearrangements in this phase (Dδ2-Dδ3 and Vδ2-Dδ3) exactly match with the cross-lineage TcR-δ gene rearrangements observed in precursor-B-ALL (7,35-37). Because no other dominant TcR-δ gene rearrangements occur in the precursor-B-ALL, we can assume that it is impossible for B-cells to enter the next phase of TcR-δ gene rearrangements, probably because Dδ→Jδ rearrangement is T-cell specific (29). The Vδ-Dδ rearrangement was recognized also as an initial step in the TcR-δ gene rearrangement of a human transgene. This initial step is TcR-δ enhancer independent (29).

Phase 2: rearrangements of the Jδ gene segments. The activation of the Jδ gene segments may occur in a similar way as the activation of the Jα gene segments, which is probably mediated by germline transcription from a region called T-early alpha (TEA)

(3,38,39). Recently, a region in between the Dδ3 and Jδ1 gene segments has been identified, which can be transcribed and spliced to Cδ sequences (40). This germline transcription might activate the Jδ1 gene segment. An alternative explanation might be that the promotor of the Vδ2 or Dδ2 gene segments after rearrangement to the Dδ3 gene segment activate the ≈1 kb downstream located Jδ1 gene segment by germline transcription. Anyway, rearrangements involving Jδ gene segments are TcR-δ enhancer dependent, as observed in TcR-δ transgenic mouse, and represent a distinct step in the TcR-δ gene rearrangement pathway (29). Whether the Jδ2 and Jδ3 gene segments are activated in a homologous manner as the Jδ1 gene segment, has to be elucidated. However, because of their differential usage in ontogeny one can assume that other regulating factors play a role.

Phase 3: rearrangement of the Vδ gene segments. This is the last phase of 'true' TcR-δ gene rearrangements. Little is known about the activation of TcR-Vδ gene segments and the involved regulatory sequences or proteins (20). Nevertheless, it is obvious that there is at least a significant difference in activation of the Vδ2 and the Vδ1 gene segment. In mice this is an evident stage because in SCID mice Dδ→Dδ and Dδ→Jδ rearrangements occur, but rearrangements involving a Vδ gene segment are absent (41).

Phase 4: rearrangement of the δREC to the ψJα gene segment; i.e. deletion of TcR-δ gene (rearrangements). The initiation to this phase is probably caused by the transcription of the TEA element, that results in a sterile TEA-Cα transcripts (3,38,39). It is believed that this germline transcription activates the Jα gene segments (~ 85 kb) for rearrangement. The ψJα gene segment is also activated as it is the most 5' located Jα gene segment. That other mechanisms also play an important role in recombination activation of a gene segment is evident for the δREC gene segment, which has no promotor and is therefore not transcribed (15). It is of interest that the Vδ2 and Dδ2 gene segments are the only gene segments that do not rearrange directly to ψJα (Table 4), although Vδ2-Dδ3-Jα gene rearrangement are frequently observed in B-ALL (13). Finally, after δREC-ψJα gene rearrangement, TcR-α rearrangement starts.

It is obvious from these data that the sequential rearrangement pathways are regulated by differential activation of specific TcR-δ gene segments. In Figure 6 we postulate a hypothetical scheme of the consecutive activation of the TcR-δ gene segments, in which the 5' and the 3' of the Dδ gene segments are considered to be activated independently.

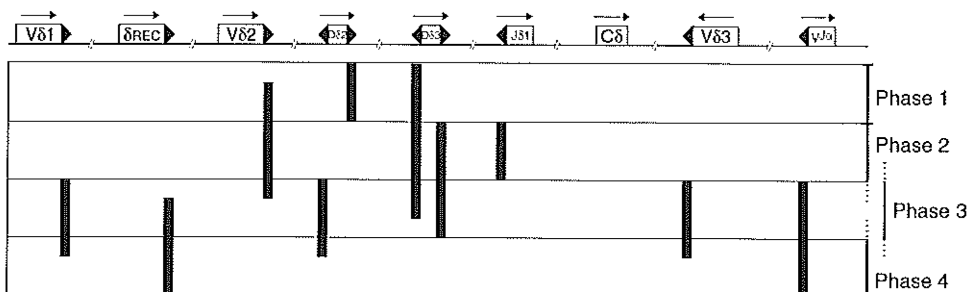


Figure 6. Hypothetical scheme of differential recombination activation of TcR-δ gene segments. The vertical bars indicate in which phase(s) a particular TcR-δ gene segment is 'accessible' for V(D)J recombination. For explanation see text.

This scheme may provide useful information as to where recombination regulating elements occur.

At this point we like to compare our data with those derived from the studies with human TcR-δ transgenic mice by Lauzurica and Krangel (29). The authors found that Vδ→Dδ rearrangements occurred frequently and in an enhancer independent way, whereas Vδ-Dδ→Jδ rearrangements were enhancer dependent. In general our data are in concordance with their observations, if we consider the fetal-like pathway. The only difference was the relative abundance of Vδ1 gene segment usage over Vδ2 gene segment usage in the Vδ-Dδ3 and Vδ-Dδ3-Jδ1 rearrangements of the transgene, whereas in our observations the reverse situation occurs. We feel that by leaving the Dδ2 gene segment out of the TcR-δ transgene, the transgenic rearrangements are forced into the fetal-like pathway (Figure 5). Because the Vδ1 gene segment is more active in post-natal thymus compared to the Vδ2 gene segment and is normally used in the post-natal pathway, the transgenic Vδ1 gene segment will be forced into the fetal-like pathway and rearranges to the Dδ3 gene segment. In their study as well as in our study the Dδ3 gene segment is recognized as the earliest activated gene segment in TcR-δ gene rearrangement and is present in all Vδ-Jδ rearrangements. From both studies it as tempting to hypothesize a cis-acting regulatory element that is located in between the Dδ2 and Dδ3 gene segments and represses gene rearrangement of the Jδ1 gene segment. Any rearrangement to the Dδ3 gene segment will delete this element and thus 'free' the Jδ1 gene segment for rearrangement.

Still some unexplainable results remain. For instance the high frequency of the δREC-ψJα rearrangement in post-natal thymus. The Vδ1-Dδ2-Dδ3-Jδ1 post-natal pathway seems to be the major pathway, but this pathway makes δREC-ψJα rearrangement impossible. Thus, the relative abundance of δREC-ψJα rearrangements are hard to explain. It might be that the TcR-δ deletion occurs at both alleles prior to TcR-α rearrangement, which in turn, may happen at one allele at the time. In frame TcR-α rearrangement and TcR-αβ expression may thus shut off the recombination processes and leave the δREC-ψJα rearrangement on the second allele. Another possibility might be that the circular excision products that derive from the δREC-ψJα gene rearrangements and contain the Cδ exons, are more stable than other types of circular excision products. An indication to support this point is found in the relative abundance of the δREC-ψJα circular excision products in PB T-lymphocytes. A final solution might be that a lot of incomplete rearrangements of the non-fetal pathway (Dδ2-Dδ3 and Dδ2-Dδ3-Jδ1) or even TcR-δ genes in germline configuration are deleted by a δREC-ψJα rearrangement (Figure 5). This might also occur in the case of TcR-α gene rearrangements. The latter possibility has been proven by cloning of circular excision products of TcR-α gene rearrangements that contained a germline TcR-δ gene (42).

We did try to enrich thymus DNA samples for circular excision products, but our efforts were greatly frustrated by the inability to find a method that does not select for the size of the circles, as has happened in previous studies (42-45). But there were also a lot of problems by the interpretation of some preliminary data because circular excision products may contain gene rearrangements themselves, or may show ongoing rearrangement, both of which will obscure the interpretation of an order in the gene rearrangements. Extensive studies on phenotypic consecutive subpopulations are therefore

probably a better tool for TcR-δ gene rearrangement studies. That TcR-δ gene rearrangement is important in early T-cell differentiation becomes clear from the presence of an increased but still small Jδ1 germline band in the immature thymocyte fraction, which means that the majority of immature thymocytes rearranges their TcR-δ genes. Because ψJα rearrangement and thus TcR-α gene rearrangement did not yet occur in this thymocyte population we can conclude that the far majority of the thymocytes will rearrange (a part of) their TcR-δ genes (before TcR-α gene rearrangement) during T-cell differentiation. This confirms the theory that αβ/γδ lineage commitment does not happen before TcR-δ gene rearrangement (29). It also suggests a role for the TcR-δ gene other than producing the TcR-δ chain in TcR-γδ$^+$ thymocytes before TcR-α activation.

The here proposed pathways of sequential TcR-δ gene rearrangements have to be considered as the *major* and not the *exclusive* pathways because PCR analysis of coding joints as well as signal joints show alternative TcR-δ gene rearrangements. However, we think that the proposed schemes are useful in determination of the critical points in the regulation of TcR-δ gene recombination. It is beyond any question that there are strict regulation mechanisms, because of the amazing homology of rearranged bands between different fetal thymi or post-natal thymi. It therefore seems that all preferential TcR-δ gene rearrangements recurrently occur in comparable frequencies in the human thymus. Unravelling the regulation mechanisms that cause these consequent frequencies of TcR-δ gene rearrangements is the next challenge in research of early T-cell differentiation.

ACKNOWLEDGEMENTS. We thank Prof. Dr. R. Benner, Dr. A. Beishuizen and Dr. H. Hooijkaas for their continuous support; Dr. G. Aversa, Dr. J.H. Phillips and Dr. R.A.W. van Lier for kindly providing the RPA-T4, RPA-T8, L78 and CLB-3A12 antibodies; Mr. T.M. van Os and Ms. A.D. Korpershoek for assistance in preparation of the manuscript.

REFERENCES

1. Isobe M, Russo G, Haluska FG, Croce CM. Cloning of the gene encoding the δ subunit of the human T-cell receptor reveals its physical organization within the α-subunit locus and its involvement in chromosome translocations in T-cell malignancy. Proc Natl Acad Sci USA 1988;85:3933-3937.
2. Satyanarayana K, Hata S, Devlin P, Grazia Roncarolo M, De Vries JE, Spits H, Strominger JL, Krangel MS. Genomic organization of the human T-cell antigen-receptor α/δ locus. Proc Natl Acad Sci USA 1988;85:8166-8170.
3. Hockett RD, De Villartay J-P, Pollock K, Poplack DG, Cohen DI, Korsmeyer SJ. Human T-cell antigen receptor (TCR) δ-chain locus and elements responsible for its deletion are within the TCR α-chain locus. Proc Natl Acad Sci USA 1988;85:9694-9698.
4. Van Dongen JJM, Comans-Bitter WM, Wolvers-Tettero ILM, Borst J. Development of human T lymphocytes and their thymus-dependency. Thymus 1990;16:207-234.
5. Loh EY, Cwirla S, Serafini AT, Phillips JH, Lanier LL. Human T-cell-receptor δ chain: genomic organization, diversity, and expression in populations of cells. Proc Natl Acad Sci USA 1988;85:9714-9718.
6. Takihara Y, Tkachuk D, Michalopoulos E, Champagne E, Reimann J, Minden M, Mak TW. Sequence and organization of the diversity, joining, and constant region genes of the human T-cell δ-chain locus. Proc Natl Acad Sci USA 1988;85:6097-6101.
7. Breit TM, Wolvers-Tettero ILM, Beishuizen A, Verhoeven M-AJ, Van Wering ER, Van Dongen JJM. Southern Blot patterns, frequencies, and junctional diversity of T-cell receptor-δ gene rearrangements in acute lymphoblastic leukemia. Blood 1993;82:3063-3074.
8. Hara J, Takihara Y, Yumura-Yagi K, Ishihara S, Tawa A, Mak TW, Gelfland EW, Okada S, Kawa-Ha K. Differential usage of δ recombining element and Vδ genes during T-cell ontogeny. Blood 1991;78:2075-2081.
9. Davodeau F, Peyrat M-A, Hallet M-M, Vié H, Bonneville M. Characterization of a new functional TcR Jδ segment in humans. J Immunol 1994;153:137-142.
10. Genevée C, Chung V, Diu A, Hercend T, Triebel F. TcR gene segments from at least one third of Vα subfamilies rearrange at the δ locus. Mol Immunol 1994;31:109-115.
11. Guglielmi P, Davi F, D'Auriol L, Bories J-C, Dausset J, Bensussan A. Use of a variable α region to create a functional T-cell receptor δ chain. Proc Natl Acad Sci USA 1988;85:5634-5638.
12. Miossec C, Faure F, Ferradini L, Roman-Roman S, Jitsukawa S, Ferrini S, Moretta A, Triebel F, Hercend T. Further analysis of the T cell receptor γ/δ$^+$ peripheral lymphocyte subset. J Exp Med 1990;171:1171-1188.

13. Yokota S, Hansen-Hagge TE, Bartram CR. T-cell receptor δ gene recombination in common acute lymphoblastic leukemia: preferential usage of Vδ2 and frequent involvement of the Jα cluster. Blood 1991;77:141-148.
14. Miossec C, Caignard A, Ferradini L, Roman-Roman S, Faure F, Michalaki H, Triebel F, Hercend T. Molecular characterization of human T cell receptor α chains including a Vδ1-encoded variable segment. Eur J Immunol 1991;21:1061-1064.
15. De Villartay J-P, Hockett RD, Coran D, Korsmeyer SJ, Cohen DI. Deletion of the human T-cell receptor δ-gene by a site-specific recombination. Nature 1988;335:170-174.
16. Hockett RD, Nuñez G, Korsmeyer SJ. Evolutionary comparison of murine and human δ T-cell receptor deleting elements. New Biol 1989;1:266-274.
17. Breit TM, Wolvers-Tettero ILM, Bogers AJJC, De Krijger RR, Wladimiroff JW, Van Dongen JJM. Rearrangements of the human TCRD-deleting elements. Immunogenet 1994;40:70-75.
18. Akira S, Okazaki K, Sakano H. Two pairs of recombination signals are sufficient to cause immunoglobulin V-(D)-J joining. Science 1987;238:1134-1138.
19. Hesse JE, Lieber MR, Mizuuchi K, Gellert M. V(D)J recombination: a functional definition of the joining signals. Genes Develop 1989;3:1053-1061.
20. Lewis SM. The mechanism of V(D)J Joining: lessons from molecular, immunological, and comparative analyses. Adv Immunol 1994;56:27-150.
21. Krangel MS, Yssel H, Brocklehurst C, Spits H. A distinct wave of human T cell receptor γ/δ lymphocytes in the early fetal thymus: evidence for controlled gene rearrangement and cytokine production. J Exp Med 1990;172:847-859.
22. McVay LD, Carding SR, Bottomly K, Hayday AC. Regulated expression and structure of T cell receptor γ/δ transcripts in human thymic ontogeny. EMBO J 1991;10:83-91.
23. Wucherpfennig KW, Liao YJ, Prendergast M, Prendergast J, Hafler DA, Strominger JL. Human fetal liver γ/δ T cells predominantly use unusual rearrangements of the T cell receptor δ and γ loci expressed on both CD4$^+$ CD8$^-$ and CD4$^-$ CD8$^-$ γ/δ T cells. J Exp Med 1993;177:425-432.
24. Triebel F, Faure F, Mami-Chouaib F, Jitsukawa S, Griscelli A, Genevée C, Roman-Roman S, Hercend T. A novel human Vδ gene expressed predominantly in the TiγA fraction of γ/δ$^+$ peripheral lymphocytes. Eur J Immunol 1988;18:2021-2027.
25. Borst J, Wicherink A, Van Dongen JJM, De Vries E, Comans-Bitter WM, Wassenaar F, Van den Elsen P. Non-random expression of T cell receptor γ and δ variable gene segments in functional T lymphocyte clones from human peripheral blood. Eur J Immunol 1989;19:1559-1568.
26. Casorati G, De Libero G, Lanzavecchia A, Migone N. Molecular analysis of human γ/δ$^+$ clones from thymus and peripheral blood. J Exp Med 1989;170:1521-1538.
27. Parker CM, Groh V, Band H, Porcelli SA, Morita C, Fabbi M, Class D, Strominger JL, Brenner MB. Evidence for extrathymic changes in the T cell receptor γ/δ repertoire. J Exp Med 1990;171:1597-1612.
28. Breit TM, Wolvers-Tettero ILM, Van Dongen JJM. Unique selection determinant in polyclonal Vδ2-Jδ1 junctional regions of human peripheral γδ T lymphocytes. J Immunol 1994;152:2860-2864.
29. Lauzurica P, Krangel MS. Enhancer-dependent and -independent steps in the rearrangement of a human T cell receptor δ transgene. J Exp Med 1994;179:43-55.
30. Spits H. Early stages in human and mouse T-cell development. Curr Opin Immunol 1994;6:212-221.
31. Galy A, Verma S, Barcena A, Spits H. Precursors of CD3$^+$ CD4$^+$ CD8$^+$ cells in the human thymus are defined by expression of CD34: delineation of early events in human thymic development. J Exp Med 1993;178:391-401.
32. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of Immunoglobulin and T cell receptor genes. Part I: basic and technical aspects. Clin Chim Acta 1991;198:1-91.
33. Hata S, Clabby M, Devlin P, Spits H, De Vries JE, Krangel MS. Diversity and organization of human T cell receptor δ variable gene segments. J Exp Med 1989;169:41-57.
34. De Villartay J-P, Pullman AB, Andrade R, Tschachler E, Colamenici O, Neckers L, Cohen DI, Cossman J. γ/δ Lineage relationship within a consecutive series of human precursor T-cell neoplasms. Blood 1989;74:2508-2518.
35. Loiseau P, Guglielmi P, Le Paslier D, Macintyre E, Gessain A, Bories J-C, Flandrin G, Chen Z, Sigaux F. Rearrangements of the T cell receptor δ gene in T acute lymphoblastic leukemia cells are distinct from those occurring in B lineage acute lymphoblastic leukemia and preferentially involve one Vδ gene segment. J Immunol 1989;142:3305-3311.
36. Biondi A, Di Celle PF, Rossi V, Casorati G, Matullo G, Giudici G, Foa R, Migone N. High prevalence of T-cell receptor Vδ2-(D)-Dδ3 or Dδ1/2-Dδ3 rearrangements in B-precursor acute lymphoblastic leukemias. Blood 1990;75:1834-1840.
37. Yano T, Pullman A, Andrade R, Uppenkamp M, De Villartay JP, Reaman G, Crush-Stanton S, Cohen DI, Raffeld M, Cossman J. A common Vδ2-Dδ2-Dδ3 T cell receptor gene rearrangement in precursor B acute lymphoblastic leukaemia. Br J Haematol 1991;79:44-49.
38. De Villartay J-P, Lewis D, Hockett R, Waldmann TA, Korsmeyer SJ, Cohen DI. Deletional rearrangement in the human T-cell receptor α-chain locus. Proc Natl Acad Sci USA 1987;84:8608-8612.
39. De Chasseval R, De Villartay J-P. Functional characterization of the promoter for the human germ-line T cell receptor J α (TEA) transcript. Eur J Immunol 1993;23:1294-1298.
40. Roman-Roman S, Ferradini L, Azogui O, Faure F, Hercend T, Triebel F. Alternatively spliced T cell receptor transcripts expressed in human T lymphocytes. Mol Immunol 1993;30:423-431.
41. Carroll AM, Bosma MJ. T-lymphocyte development in scid mice is arrested shortly after the initiation of T-cell receptor δ gene recombination. Genes Develop 1991;5:1357-1366.
42. Winoto A, Baltimore D. Separate lineages of T cells expressing the αβ and γδ receptors. Nature 1989;338:430-432.
43. Toda M, Fujimoto S, Iwasato T, Takeshita S, Tezuka K, Ohbayashi T, Yamagishi H. Structure of extrachromosomal circular DNAs excised from T-cell antigen receptor alpha and delta-chain loci. J Mol Biol 1988;202:219-231.
44. Okazaki K, Sakano H. Thymocyte circular DNA excised from T cell receptor α-δ gene complex. EMBO J 1988;7:1669-1674.
45. Takeshita S, Toda M, Yamagishi H. Excision products of the T cell receptor gene support a progressive rearrangement model of the α/δ locus. EMBO J 1989;8:3261-3270.
46. Breit TM, Wolvers-Tettero ILM, Hählen K, Van Wering ER, Van Dongen JJM. Extensive junctional diversity of γδ T-cell receptors expressed by T-cell acute lymphoblastic leukemias: implications for the detection of minimal residual disease. Leukemia 1991;5:1076-1086 and Erratum 1992;6:169-170.
47. Dariavach P, Lefranc M-P. First genomic sequence of the human T-cell receptor δ2 gene (TRDV2). Nucleic Acids Res 1989;17:4880.
48. Koop BF, Rowen L, Wang K, Lam Kuo C, Seto D, Lenstra JA, Howard S, Shan W, Deshpande P, Hood L. The human T-cell receptor TCRAC/TCRDC (Cα/Cδ) region: organization, sequence, and evolution of 97.6 kb of DNA. Genomics 1994;19:478-493.

CHAPTER 3.4

REARRANGEMENTS OF THE HUMAN TcR-δ DELETING ELEMENTS*

Timo M. Breit[1], Ingrid L.M. Wolvers-Tettero[1], Ad J.J.C. Bogers[2], Ronald R. de Krijger[3], Juriy W. Wladimiroff[4], and Jacques J.M. van Dongen[1]

1. Department of Immunology, University Hospital Dijkzigt/Erasmus University, Rotterdam;
2. Department of Thoracic Surgery, University Hospital Dijkzigt/Erasmus University, Rotterdam;
3. Department of Pediatrics, Sophia Children's Hospital/Erasmus University, Rotterdam;
4. Department of Gynaecology/Obstetrics, Division of Prenatal Diagnosis, University Hospital Dijkzigt/Erasmus University, Rotterdam, The Netherlands.

BRIEF COMMUNICATIONS

There are two types of TcR present on human PB T-lymphocytes: TcR-$\alpha\beta$ and TcR-$\gamma\delta$ (1). Although little is known about the mechanisms that commit a T-cell to the $\alpha\beta$- or $\gamma\delta$-lineage, it is generally assumed that the TcR-δ gene plays a pivotal role in the divergence of the two lineages (2-5). This assumption is based on two features of the TcR-δ gene. Firstly, the TcR-δ gene is located in the middle of the TcR-α gene and is therefore deleted during Vα-Jα rearrangement, which in principle excludes co-expression of TcR-δ and TcR-α chains (3,6). Secondly, in normal polyclonal thymocytes a predominant rearrangement is observed, which represents the rearranged δREC and ψJα gene segments (2,4,7). These two gene segments flank the major part of the TcR-δ gene and are called TcR-δ deleting elements, because their non-productive rearrangement deletes the intermediate germline and/or rearranged TcR-δ gene sequences (2). Therefore, a model is postulated in which a germline or a rearranged TcR-δ gene is deleted by the δREC-ψJα rearrangement, which in turn can be replaced by a Vα-Jα gene rearrangement (Figure 1) (5).

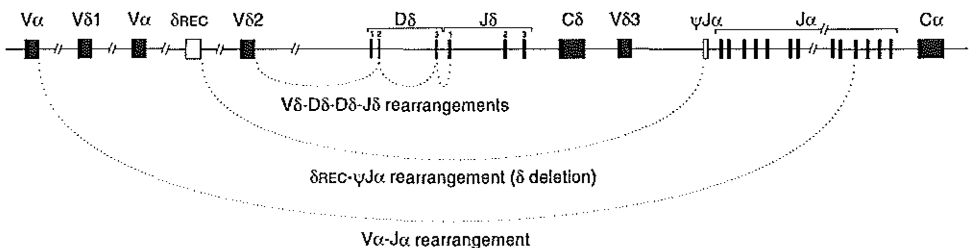


Figure 1. Schematic representation of the human TcR-α/δ locus. Indicated are the various gene segments including the TcR-δ deleting elements (δREC and ψJα). The dotted lines indicate the possible consecutive gene rearrangements: Vδ-Dδ-Dδ-Jδ, δREC-ψJα, and Vα-Jα.

* Published in: Immunogenetics 1994;40:70-75.

Although the δREC-ψJα rearrangement by its result and prominent occurrence in the thymus seems to play a distinct role in the divergence of the human TcR-αβ and TcR-γδ T-cell lineages, limited information is available in the literature concerning this rearrangement. The first δREC-ψJα rearrangements were analyzed only by identification of circular excision products with a ψJα-δREC signal joint (2). The subsequent determination of the δREC-ψJα junctional region of a T-cell line (DU.528) showed not only N-region nucleotide insertion and deletion of nucleotides by trimming of the flanking sequences comparable to a normal rearrangement, but revealed also evidence for Dδ-gene derived junctional region nucleotides (8). Other rearrangements involving one of the two TcR-δ deleting elements have also been observed, such as δREC-Jδ1, δREC-JαI, and δREC-JαII in T-ALL (9-11), as well as Dδ3-ψJα and Vα3.1-ψJα in normal thymocytes (1).

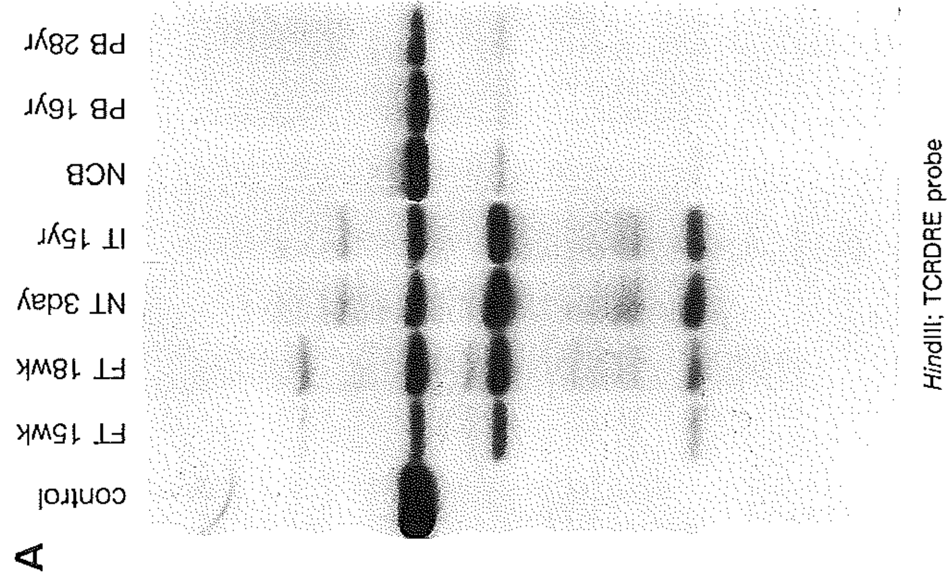
A total of 150 δREC-ψJα junctional regions were analyzed to determine their precise sequence and to investigate whether Dδ gene segments can occur in these junctional regions and if so, in what frequency they are present. To investigate at the same time potential differences in the δREC-ψJα rearrangement during human ontogeny, we studied 15 thymus and blood cell samples from fetuses, neonates, and adults.

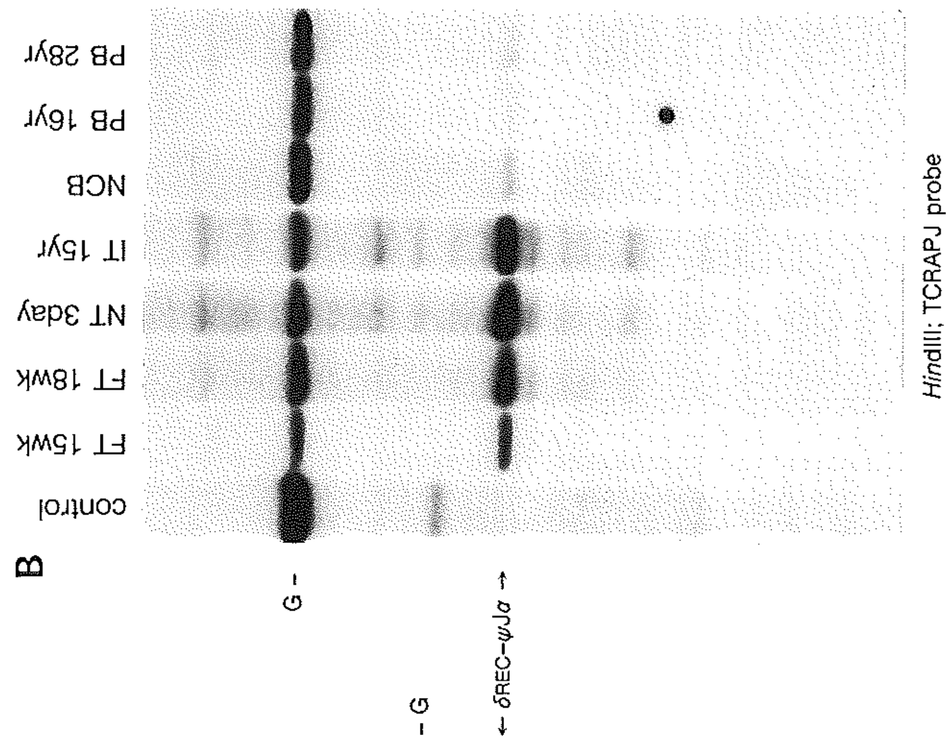
The 15 cell samples consisted of five fetal thymi (12, 15, 16, 17, and 18 weeks of gestation), five postnatal thymi (3 days, 1 month, 1 year, 5 years, and 15 years), one fetal cord blood sample (18 weeks of gestation), one neonatal cord blood sample, and three adult PB samples (16 years, 27 years, and 28 years). DNA was extracted from the obtained cell samples as described (12). Fifteen μg of each DNA sample was digested with *Eco*RI, *Hin*dIII, and/or *Bgl*II and analyzed by Southern blot analysis, using the δREC probe (TCRDRE) and ψJα probe (TCRAPJ) (13). Of each DNA sample, 0.25-0.5 μg was amplified in a normal 100 μl PCR reaction, using the oligonucleotide primers δREC-5′E (ctaagaatTCGATCCTCAA-GGGTCGAGACTGTC) and ψJα-3′H (cctgaagcTTAAGGCACATTAGAATCTCTCACTG) as described (13). The obtained polyclonal PCR products (~500 bp) were digested with *Eco*RI and *Hin*dIII and cloned in the pUC19 vector. Ten single bacteria colonies of each sample were randomly picked and sequenced with the universal pUC reverse sequencing primer as described (14).

Southern blot analysis of the various cell samples confirmed the prominent presence of the δREC-ψJα rearrangement in all thymic cell samples, but in PB mononuclear cells this rearrangement was hardly visible (5% detection limit) (Figure 2). The latter observation is probably caused by the predominant biallelic Vα-Jα gene rearrangements in peripheral TcR-$\alpha\beta^+$ T-lymphocytes, which have deleted the preexisting δREC-ψJα rearrangements.

The sequences of the δREC-ψJα junctional regions in the various cell samples are presented in Figure 3. Although there are some differences, in all cell samples N-region and P-region nucleotide insertion occurred in addition to deletion of nucleotides from the flanking sequences. The characteristics of the δREC-ψJα junctional regions in Table 1 show that the total nucleotide insertion in fetal thymocytes (1.7-3.7) is on average lower than in

Figure 2. Southern blot analysis of the δREC-ψJα rearrangement in various human cell samples. Lane 1, control DNA (cell line HELA); lane 2, Fetal Thymus 15 weeks; lane 3, Fetal Thymus 18 weeks; lane 4, Neonatal Thymus 3 days; lane 5, Infant Thymus 15 years; lane 6, Neonatal Cord Blood; lane 7, infant PB 16 years; lane 8, adult PB 28 years. A) Hybridization of *Hin*dIII digests with the TCRDRE (δREC) probe. B) Rehybridization with the TCRAPJ (ψJα) probe. The band representing the preferential δREC-ψJα rearrangement is indicated. All other rearranged bands represent other preferential rearrangements to either the δREC or ψJα gene segment. G indicates the germline band.


A
control
FT 15wk
FT 18wk
NT 3day
IT 15yr
NCB
PB 16yr
PB 28yr
– G
← δREC–ψJα →
*Hind*III; TCRDRE probe


B
control
FT 15wk
FT 18wk
NT 3day
IT 15yr
NCB
PB 16yr
PB 28yr
G –
*Hind*III; TCRAPJ probe

FETAL THYMUS

Sample	δREC	junctional region	ψJα
	TGTGAGGAGCC		GGTACCGGGTT
fetal thymus 12 weeks	-19		CCGGGTT
	TGTG		GTACCGGGTT
	TGTGAGG		TACCGGGTT
	TGTGAGGAGCC		GGGTT
	TGTGAGGAGCC		CGGGTT
	TGTGAGGA	TA	GTACCGGGTT
	TGTGAGGAGCC	TTG	-15
	TGTGAGGAGCC	gAG	CGGGTT
	TGTGAGGAGC	TATT	TACCGGGTT
	-14	CTTTT	GGGTT
fetal thymus 15 weeks	TGTGAG		-14
	TGTG		TACCGGGTT
	TGTGAGGAGCC		GGGTT
	TGTGAGGAGC		ACCGGGTT
	TGTGAGGAGCC		GGTACCGGGTT
	TGTGAGGAGC	TTT	TACCGGGTT
	TGTGAGGAGCC	ggAG	CGGGTT
	TGTGAGGA	TGCCTA	TACCGGGTT
	TGTGAGGAGC	TTGGCT	GGTACCGGGTT
	TGTGAGGAGCC	CGGTGGGA	TT
fetal thymus 16 weeks	TGTGAGGA		CCGGGTT
	TGTGAGGAG		TACCGGGTT
	TGTGAGGAGCC		ACCGGGTT
	TGTGAGGAGCC	C	CCGGGTT
	TGTGAGGA	CA	GTACCGGGTT
	TGTGAGGAGC	TT	GTACCGGGTT
	T	TTT	TACCGGGTT
	TGTGAGGAGCC	ggA	GGGTT
	TGTGAGGAGCC	CGCC	GTACCGGGTT
	TGTGAGGAGCC	ggTGA	CGGGTT
fetal thymus 17 weeks	TGTGAGGAGC		GTACCGGGTT
	TGTGAG	AC	CCGGGTT
	TGTGAGGAGCC	gg	CCGGGTT
	-12	CCT	GTACCGGGTT
	TGTGAGGAGCC	gTG	ACCGGGTT
	TGTGAGG	GCGT	TACCGGGTT
	TGTGAGGAGC	TCTA	GTACCGGGTT
	TGTGAG	TCCCA	TACCGGGTT
	TGTGAGGAG	TCCCTC	TACCGGGTT
	TGTG	CTGTCCTA	GTACCGGGT*
fetal thymus 18 weeks	TGTGAGGAGCC		CCGGGTT
	TGTGAGGAGC		GTACCGGGTT
	TGTGAGG	T	GTACCGGGTT
	TGTGA	CAT	TACCGGGTT
	TGTGAGGAGC	GGT	CCGGGTT
	TGTGAGG	TTTC	TACCGGGTT
	TGTGAGGA	TTCAG	ACCGGGTT
	TGTGAGGAGCC	ggGCA	GGTACCGGGTT
	TGTGAGGAGCC	gTGGAG	CGGGTT
	TGTGAGGAGC	TATGGTCCT	TACCGGGTT

POSTNATAL THYMUS

Sample	δREC	junctional region	ψJα
	TGTGAGGAGCC		GGTACCGGGTT
neonatal thymus 3 days	TGTGAGG		-11
	-13	T	-17
	T	CCT	TACCGGGTT
	TGTGAGG	GGTCTC	GTACCGGGTT
	TGTGAGGAGCC	AAATAGG	CGGGT*
	TGTGAGGAGCC	CCCCCGAG	-15
	TGTGAGGAGCC	ggGTTATAT	CCGGGTT
	TGTGAGGAG	TTGATAGGGC	T
	TGTGAGGAGCC	ggATTGATTC	CCGGGTT
	TGTGAGGAGCC	ggCGCCTGTAC	-15
infant thymus 1 month	TGTGAGGAGCC	gg	ACCGGGTT
	TGTGAGGAGC	TCT	TACCGGGTT
	TGTGAGGAGCC	CCCGT	GGGTT
	-12	CCCCGC	GTACCGGGTT
	TG	GGGGCC	GTACCGGGTT
	TGTGA	AATGGG	CCGGGTT
	TGTGA	GACTCA	ACCGGGTT
	TGTGAGGAGCC	GAAGGAGAG	CGGGTT
	TGTGAGGAGCC	ggGGGCGGGA	-16
	TGTGAGGAGCC	ggGTTACTAT	CCGGGTT
infant thymus 1 year	TGTGAGGAGCC	g	ACCGGGTT
	TGTGAGGAGC	G	GGTACCGGGTT
	TGTGAGGAGC	TG	CCGGGTT
	TGTGAGGAG	TC	GTACCGGGTT
	-12	TCC	-14
	-11	GGC	ACCGGGTT
	TGTGAGGAGCC	TAGGGA	CGGGTT
	TGTGAGGAGC	AGTGGA	ACCGGGTT
	TGTG	CTCACAGAG	CGGGTT
	TGTGAGGA	AACTACgTA	GTACCGGGTT
infant thymus 6 years	TGTGAGGAGCC	g	CCGGGTT
	TGTGAGGAGCC	A	ACCGGGTT
	TGTGAGGAGCC	CT	TACCGGGTT
	TGTGAGG	GCC	-11
	TGTGAGGAG	ACAA	CGGGTT
	TGTGAGGAG	TCTTC	CCGGGTT
	TGTGAGGAGCC	gAGGGA	CGGGTT
	TGTGAGGAGCC	gAGGGAG	CGGGTT
	TGTGAGG	ggCCTTAGAG	CGGGTT
	TGTGAGGAGCC	CTGATTTGTAATGc	GGTACCGGGTT
adult thymus 15 years	TGTGAGGAG		T
	TGTGAGGAGCC		CCGGGTT
	TGT	C	CCGGGTT
	TGTGAGGAGCC	A	GTACCGGGTT
	TGTGAGGAGCC	CTTC	CCGGGTT
	TGTG	CATAA	-16
	TGTGAGG	TCCGA	ACCGGGTT
	TGTGAGGA	CCTTCTAGC	TACCGGGT*
	-19	ACGACGGAGGG	-13
	TGTGAGGA	CCCCCTCGACAT	TACCGGGTT

PERIPHERAL BLOOD

Sample	δREC	junctional region	ψJα
	TGTGAGGAGCC		GGTACCGGGTT
fetal cord blood 18 weeks	T		ACCGGGTT
	TGTGAGGAGCC		CGGGTT
	-11	AA	GGTT
	TGTGAGGAGCC	gT	CCGGGTT
	TGTGAGGAGCC	gCG	CGGGTT
	TGTGAGGAG	TTC	TACCGGGTT
	TGTGAGGAGC	GGCC	-14
	TGT	AGGCG	GGGTT
	TGTGAG	AGGGGGGTG	ACCGGGT*
	TGTGAGGAGCC	ggGGTTCTGGGGATTCT	TACCGGGT*
neonatal cord blood	TGTGAGGAGCC		CGGGTT
	TGTGAGGAGC		GTACCGGGTT
	-14	C	-14
	TGTGAGGAG	T	GTACCGGGTT
	TGTGAGGAGCC	gA	ACCGGGTT
	TGTGAGGAGC	TC	GTACCGGGTT
	TGTGAGGAG	TACTC	GTACCGGGTT
	TGTGA	CCCTAC	TACCGGGT*
	TGTGAGGAGCC	AAAGGTG	-15
	TGTGAGGAG	TCCCCCG	ACCGGGTT
PBMNC 16 years	TGTGAGGAGCC	gg	ACCGGGTT
	TGTGAGGAGCC	gTCC	GGTACCGGGTT
	TGTGAGGA	CCGGT	CCGGGTT
	TGTGAGGAG	GGACTC	TACCGGGTT
	TG	CGATAAA	ACCGGGTT
	TGTGAGGAG	GTAGGGC	CCGGGTT
	TGTGAGGAGC	TACgGTAG	CGGGTT
	-22	AGGTCAGGAA	GGGTT
	TGTGAGGAGC	ATATAAATCCGAGG	-20
	TGTGAGGAGCC	gTACTAAATCAATCGAcc	GGTACCGGGTT
PBMNC 27 years	TGTGAGGA		CGGGTT
	TGTGAGGAGCC	CG	CCGGGTT
	TGTGAGG	CCA	GTACCGGGTT
	TGTGAGG	CCG	GGTACCGGTT
	-16	GGAC	CCGGGTT
	TGTGAGGAGCC	ggTT	TT
	TGTGAGG	GGAC	CCGGGTT
	TGTGAGGAGCC	gAGG	CGGGTT
	TGTGAGG	GCgCCT	GTACCGGGTT
	TGTGAGG	GAGCTGAGG	-14
PBMNC 28 years	TGTGAGGAG		TACCGGGTT
	TG	GATC	CCGGGTT
	TGTGAGGAGCC	TAAC	TACCGGGTT
	TGTGAGGA	TCTAAG	GGGTT
	TGTGAGGAGCC	ACGAGGG	CGGGTT
	TG	GTATGGGA	GTT
	TGTGAGG	gCCTCCGGG	ACCGGGTT
	TGTGAGGAGCC	GGGGGAAGGGG	CGGGT*
	-11	CGAGGCCTAAGAGA	GTT
	TGTGAGG	TCGTCTCCCCGGGC	GTT

TABLE 1. Junctional region diversity of human δREC-ψJα rearrangements.

	No. of inserted nucleotides				No. of deleted nucleotides		
	N-region	Dδ	P-region	Total	δREC	ψJα	Total
Fetal thymus							
12 weeks	1.3	0.3	0.1	1.7	4.8	4.7	9.5
15 weeks	1.7	0.8	0.2	2.7	1.8	4.3	6.1
16 weeks	1.6	0	0.4	2.0	1.9	2.9	4.8
17 weeks	1.7	1.7	0.3	3.7	3.7	2.1	4.8
18 weeks	2.3	1.0	0.3	3.6	2.0	2.4	4.4
Postnatal thymus							
3 days	3.7	2.2	0.6	6.5	3.3	8.4	11.7
1 month	3.9	1.8	0.6	6.3	3.4	4.5	7.9
1 year	2.6	1.4	0.2	4.2	3.8	3.9	7.7
5 years	2.8	1.9	0.6	5.3	1.2	4.4	5.6
15 years	3.0	1.8	0	4.8	4.6	5.9	10.5
Fetal cord blood							
18 weeks	2.5	1.7	0.4	4.6	3.7	5.1	8.8
Neonatal cord blood							
NCB5	1.6	1.4	0.1	3.1	2.8	4.6	7.4
Peripheral blood							
16 years	5.4	2.0	0.7	8.1	4.0	4.6	8.6
27 years	3.2	0.3	0.4	3.9	3.9	4.7	8.6
28 years	5.3	2.3	0.1	7.7	4.2	5.1	9.3

At least ten junctional regions were analyzed per cell sample.

postnatal cell samples (4.2-6.5). Especially in 12 weeks thymocytes, N-region nucleotide insertion was very low, probably due to low expression of the enzyme terminal deoxynucleotidyl transferase (15), which mediates the random N-region nucleotide insertion. Nucleotide deletion was also more extensive in postnatal cell samples, suggesting that rearrangements in early fetal thymocytes are performed by an "immature" recombinase complex, which is less capable of nucleotide deletion. Furthermore, in almost all cell samples, nucleotide deletion by trimming of the δREC gene segment was less extensive than trimming of the ψJα gene segment, indicating that the activity of the recombinase enzyme complex has a direction, which may be related to the size of the spacers in the recombination signal sequences (16).

Usually, Dδ nucleotides are identified in TcR-δ junctional regions based on the guideline that at least one third of the Dδ gene segment has to be present with a minimum of three consecutive nucleotides. By use of this guideline, we could identify putative Dδ-gene derived nucleotides in 36% of the δREC-ψJα junctional regions. However, there was no Dδ gene segment present in any junctional region, whereas in normal Vδ-Jδ junctional regions complete Dδ2 and Dδ3 gene segments frequently occur (10,17). Moreover, applying the

Figure 3. Junctional region sequences of δREC-ψJα rearrangements in various human cell samples. Sequences of the δREC-ψJα junctional regions are aligned with the known (double underlined) δREC and ψJα germline sequences. Single underlined sequences represent putative Dδ-gene derived nucleotides. Lower case characters represent P-region nucleotides and all other junctional region nucleotides represent N-region nucleotides. * Indicates junctional regions (n=7) with a long string of (≥5) Dδ-gene derived nucleotides.

same guideline to published $V\alpha$-$J\alpha$ or even $V\gamma$-$J\gamma$ junctional regions, revealed comparable frequencies of putative $D\delta$-gene derived nucleotides, although these junctional regions should not contain $D\delta$ gene segments (10,18). In fact, the δREC-$\psi J\alpha$ junctional regions are highly homologous to $V\alpha$-$J\alpha$ and $V\gamma$-$J\gamma$ junctional regions (with only one N-region) and are essentially smaller than $V\delta$-$J\delta$ junctional regions (with primarily two or three N-regions). We therefore conclude that most putative $D\delta$-gene derived nucleotides observed in the δREC-$\psi J\alpha$ junctional regions probably represent N-region nucleotides, which is in line with the finding that putative $D\delta$-gene derived nucleotides are virtually absent, if the number of N-region nucleotides is low (12 weeks old fetal thymocytes).

Nevertheless, in some δREC-$\psi J\alpha$ junctional regions (7/150) we discovered longer strings ($\geq$5) of putative $D\delta$ gene nucleotides (Figure 3), suggesting that in these particular instances $D\delta$-gene derived nucleotides are indeed present. All three $D\delta$ gene segments were present at least once, indicating that the δREC and $\psi J\alpha$ gene segments are able to rearrange to each $D\delta$ gene segment.

The finding that δREC-$\psi J\alpha$ and $V\alpha$-$J\alpha$ junctional regions rarely include a $D\delta$ gene segment may be due to the order of rearrangements on one allele: firstly a $D\delta$-$J\delta$ or $V\delta 2/3$-($D\delta$)-$J\delta$ rearrangement, followed by the TcR-δ gene deleting δREC-$\psi J\alpha$ rearrangement, and finally a $V\alpha$-$J\alpha$ rearrangement (Figure 1). In principle, $D\delta$ gene segments can only be involved in δREC-$\psi J\alpha$ or $V\alpha$-$J\alpha$ rearrangements, if germline $D\delta$ gene segments are available at the time of rearrangement, i.e. germline TcR-δ genes or incompletely rearranged TcR-δ genes. Apparently this does not occur frequently, or there are other (yet unknown) restrictions excluding $D\delta$ gene segments from these rearrangements. Overall, the δREC-$\psi J\alpha$ rearrangement appears to be just a $V\alpha$-$J\alpha$-like rearrangement, committing the thymocyte to the TcR-$\alpha\beta$ lineage.

It can be concluded that the predominant TcR-δ gene deleting δREC-$\psi J\alpha$ rearrangement is present in human thymocytes throughout ontogeny. The size of the δREC-$\psi J\alpha$ junctional regions increases during thymic ontogeny, but no further ontogenic differences were observed. The TcR-δ gene deleting elements can potentially rearrange to $D\delta$ gene segments, but our extensive sequencing analyses of 150 δREC-$\psi J\alpha$ junctional regions revealed that they rarely contain $D\delta$-gene derived nucleotides.

ACKNOWLEDGMENTS. The authors gratefully acknowledge Prof. Dr. R. Benner, Dr. H. Hooijkaas and Ms. E.J. Mol for their continuous support; Prof. Dr. E. Bos for kindly providing the postnatal thymus samples; Ms. W.M. Comans-Bitter for collecting the fetal thymus samples; Mr. T.M. van Os for his excellent assistance in the preparation of the figures; and Ms. A.D. Korpershoek for her secretarial support.

REFERENCES

1. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988;334:395-402.
2. De Villartay J-P, Hockett RD, Coran D, Korsmeyer SJ, Cohen DI. Deletion of the human T-cell receptor δ-gene by a site-specific recombination. Nature 1988;335:170-174.
3. Hockett RD, De Villartay J-P, Pollock K, Poplack DG, Cohen DI, Korsmeyer SJ. Human T-cell antigen receptor (TCR) δ-chain locus and elements responsible for its deletion are within the TCR α-chain locus. Proc Natl Acad Sci USA 1988;85:9694-9698.

4. Hockett RD, Nuñez G, Korsmeyer SJ. Evolutionary comparison of murine and human δ T-cell receptor deleting elements. The New Biologist 1989;1:266-274.
5. Van Dongen JJM, Comans-Bitter WM, Wolvers-Tettero ILM, Borst J. Development of human T lymphocytes and their thymus-dependency. Thymus 1990;16:207-234.
6. Isobe M, Russo G, Haluska FG, Croce CM. Cloning of the gene encoding the δ subunit of the human T-cell receptor reveals its physical organization within the α-subunit locus and its involvement in chromosome translocations in T-cell malignancy. Proc Natl Acad Sci USA 1988;85:3933-3937.
7. De Villartay J-P, Lewis D, Hockett RD, Waldmann TA, Korsmeyer SJ, Cohen DI. Deletional rearrangement in the human T-cell receptor α-chain locus. Proc Natl Acad Sci USA 1987;84:8608-8612.
8. Begley CG, Aplan PD, Davey MP, De Villartay J-P, Cohen DI, Waldmann TA, Kirsch IR. Demonstration of δREC-pseudo Jα rearrangement with deletion of the δ locus in a human stem-cell leukemia. J Exp Med 1989;170:339-342.
9. Breit TM, Wolvers-Tettero ILM, Hählen K, Van Wering ER, Van Dongen JJM. Limited combinatorial repertoire of γδ T-cell receptors expressed by T-cell acute lymphoblastic leukemias. Leukemia 1991;5:116-124.
10. Breit TM, Wolvers-Tettero ILM, Hählen K, Van Wering ER, Van Dongen JJM. Extensive junctional diversity of γδ T-cell receptors expressed by T-cell acute lymphoblastic leukemias: implications for the detection of minimal residual disease. Leukemia 1991;5:1076-1086.
11. Hara J, Takihara Y, Yumura-Yagi K, Ishihara S, Tawa A, Mak TW, Gelfand EW, Okada S, Kawa-Ha K. Differential Usage of δ recombining element and Vδ genes during T-cell ontogeny. Blood 1991;78:2075-2081.
12. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of immunoglobulin and T cell receptor genes. Part I: basic and technical aspects. Clin Chim Acta 1991;198:1-91.
13. Breit TM, Wolvers-Tettero ILM, Beishuizen A, Verhoeven M-AJ, Van Wering ER, Van Dongen JJM. Southern blot patterns, frequencies and junctional diversity of T-cell receptor δ gene rearrangements in acute lymphoblastic leukemia. Blood 1993;10:3063-3074.
14. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning, a laboratory manual, 2nd edn. Cold Spring Harbor: Cold Spring Harbor Laboratory 1989.
15. Campana D, Janossy G, Coustan-Smith E, Amlot PL, Tian W-T, Ip S, Wong L. The expression of T cell receptor-associated proteins during T cell ontogeny in man. J Immunol 1989;142:57-66.
16. Breit TM, Mol EJ, Wolvers-Tettero ILM, Ludwig W-D, Van Wering ER, Van Dongen JJM. Site-specific deletions involving the *tal*-1 and *sil* genes are restricted to cells of the T cell receptor α/β lineage: T cell receptor δ gene deletion mechanism affects multiple genes. J Exp Med 1993;177:965-977.
17. Panchamoorthy G, McLean J, Modlin RL, Morita CT, Ishikawa S, Brenner MB, Band H. A predominance of the T cell receptor Vγ2/Vδ2 subset in human mycobacteria-responsive T cells suggests germline gene encoded recognition. J Immunol 1991;147:3360-3369.
18. Porcelli S, Yockey CE, Brenner MB, Balk SP. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood $CD4^{-}8^{-}$ α/β T cells demonstrates preferential use of several Vβ genes and an invariant TCR α chain. J Exp Med 1993;178:1-16.

CHAPTER 3.5

HUMAN T-CELLS WITH AN ACTIVE V(D)J RECOMBINASE COMPLEX FOR TcR-δ GENE DELETION*

Timo M. Breit[1], Ingrid L.M. Wolvers-Tettero[1], Ellen J. van Gastel-Mol[1], Karel Hählen[2], and Jacques J.M. van Dongen[1]

1. Department of Immunology, Erasmus University/University Hospital Dijkzigt, Rotterdam; 2. Department of Pediatrics, Subdivision of Hematology-Oncology, Sophia Children's Hospital, Rotterdam, The Netherlands.

ABSTRACT

The rearrangements in the various TcR and Ig genes are mediated by the V(D)J recombinase enzyme system and occur in an hierarchical order during different phases of T-cell and B-cell differentiation. This suggests that the V(D)J recombinase system contains elements which are specific for each separate Ig/TcR gene complex. Based on the special position of the TcR-δ gene complex between the Vα and Jα gene segments, the TcR-δ gene is assumed to play an important role in the divergence of the γδ versus αβ differentiation lineages. This is supported by the presence of the so-called TcR-δ deleting elements, δREC and ψJα, which flank the major part of the TcR-δ gene complex. By rearranging to each other, the δREC and ψJα gene segments delete the TcR-δ gene and prepare the allele for TcR-α rearrangement. This intermediate rearrangement is thought to be caused by a specific V(D)J recombinase complex. In our search for a suitable monoclonal model to study the elements of this putative TcR-δ deletion recombinase complex, we identified a T-ALL with continuous activity of the δREC-ψJα gene rearrangement process. Extensive Southern blotting, PCR, and sequence analyses of the coding joints as well as the signal joints of the δREC-ψJα rearrangements in this patient, allowed us to prove that these rearrangements occurred in the leukemic cells and that these cells therefore represent a polyclonal subpopulation of the otherwise monoclonal T-ALL. In additional studies we identified several other T-ALL as well as a T-cell line which exhibit continuous activity of the δREC-ψJα rearrangement process. These T-ALL and T-cell line can serve as an experimental model for further studies on the elements of the V(D)J recombinase complex which are specific for TcR-δ gene deletion.

INTRODUCTION

T-ALL are assumed to originate from differentiating cortical thymocytes (1-3). This is

* Submitted for publication.

supported by the presence of the enzyme TdT in T-ALL cells, because cortical thymocytes are the only T-cells expressing TdT (4). During the recombination processes in early T-cell differentiation, the enzyme TdT causes the random insertion of nucleotides in the junctional regions, which connect the T-cell receptor (TcR) V, (D) and J gene segments (5,6). In principle a T-ALL is a clonal cell population in which all cells originate from a single malignantly transformed thymocyte. Therefore the leukemic cells in a T-ALL have the same monoclonal TcR gene rearrangements (2,4).

Comparable to cortical thymocytes, T-ALL cells can express TcR molecules in combination with the signal transducing CD3 complex (2-4). Because complete TcR molecules exist of $\alpha\beta$ chains or $\gamma\delta$ chains, T-ALL can express at least three possible configurations of the TcR/CD3 phenotype: TcR$^-$/CD3$^-$, TcR-$\gamma\delta^+$/CD3$^+$, and TcR-$\alpha\beta^+$/CD3$^+$. The TcR-δ gene plays an important role in the divergence of the $\alpha\beta$ lineage versus the $\gamma\delta$ lineage (4,7-9). Firstly, the major part of the TcR-δ gene is located in between the Vα and Jα gene segments, which exclude the possibility of simultaneous TcR-δ and TcR-α gene rearrangements on the same allele (8,10). Secondly, in man there are two TcR-δ gene deleting elements, δREC and ψJα, which delete the intermediate TcR-δ gene by rearrangement to each other (Figure 1). This TcR-δ gene deleting δREC-ψJα rearrangement is a dominant process in cortical thymocytes (7,9,11). As for now, the hierarchical model of gene rearrangements in the human TcR-α/δ locus may start with a TcR-δ gene

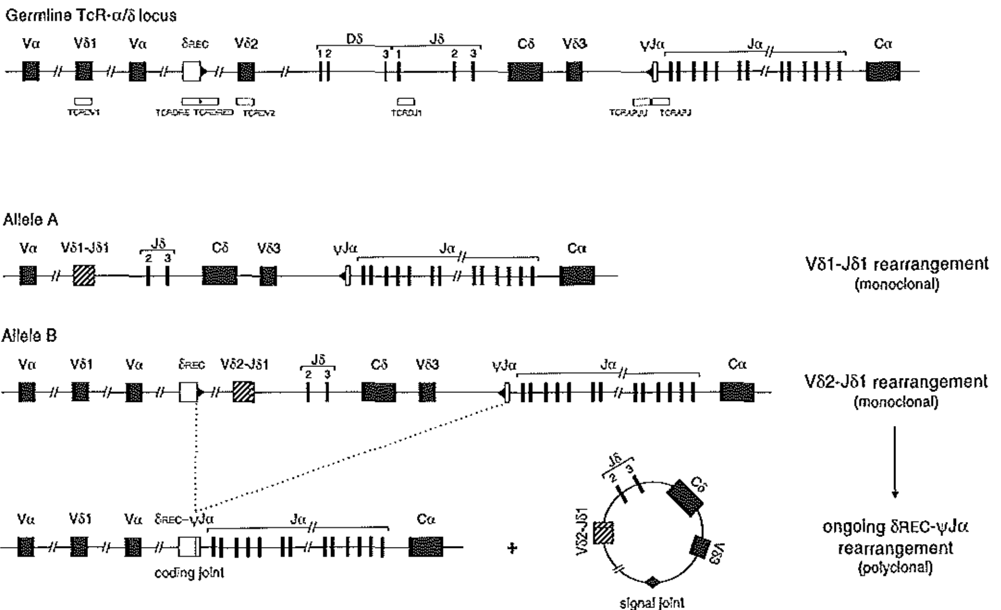


Figure 1. Schematic representation of ongoing δREC-ψJα rearrangements in leukemic cells of T019. Top: the germline TcR-α/δ locus. The open bars represent the different TcR-δ and ψJα probes. Bottom: allele A contains a monoclonal Vδ1-Jδ1 rearrangement and allele B contains a monoclonal Vδ2-Jδ1 rearrangement. The dotted lines indicate ongoing TcR-δ deletion on allele B, which results in polyclonal δREC-ψJα gene rearrangements, as well as their corresponding circular excision products. The presence of the circular products can be analyzed using Southern blot with the TCRDRED (δREC downstream) and the TCRAPJU (ψJα upstream) probes.

rearrangement, followed by deletion of the rearranged (or germline) TcR-δ gene by the δREC-ψJα rearrangement, which in turn is deleted by subsequent Vα-Jα rearrangement (4). Support for this rearrangement model was found in the junctional regions of δREC-ψJα rearrangements in thymocytes which rarely contained Dδ gene derived nucleotides, implying that the δREC gene segment directly rearranges to the ψJα gene segment (12).

Indication for the existence of a specific TcR-δ gene deletion recombinase complex was found in the skewed distribution of a specific chromosome aberration, the so-called *tal*-1 deletion (13). The *tal*-1 deletion is a site-specific, ~90 kb deletion on chromosome band 1p32 that deletes the major part of the *sil* gene and thereby juxtaposes the 5′ part of the *tal*-1 gene to the upstream located *sil* gene promotor (14,15). Because the heptamer-nonamer RSS and fusion regions of the *tal*-1 deletion breakpoints strongly resemble the RSS and junctional regions of TcR gene rearrangements, it is generally assumed that they are caused by a similar V(D)J recombinase complex (13-15). Noticeable, the *tal*-1 deletions were exclusively observed in αβ lineage T-ALL i.e. T-ALL with two non-functional TcR-δ gene rearrangements or a TcR-δ gene deletion on one or both alleles (13,16). Differential demethylation (≈ accessibility) of the breakpoint region in the *tal*-1 gene could only partly explain the restricted occurrence of *tal*-1 gene deletion in T-ALL of αβ lineage and therefore the coincidence of *tal*-1 deletion and TcR-δ gene deletion lead to the postulation of the existence of a special recombinase complex for TcR-δ gene deletion, which - as a side effect - also causes the *tal*-1 deletion (17).

In our search for a suitable monoclonal model to study the elements of a putative specific TcR-δ gene deletion recombinase complex, we here describe a $CD3^-$ T-ALL with complete out-of-frame Vδ-Jδ rearrangements on both alleles and with a minor subpopulation containing δREC-ψJα rearrangements. Extensive PCR, Southern blotting, and sequence analysis, allowed us to prove that this subpopulation was polyclonal and was caused by ongoing δREC-ψJα rearrangements in the T-ALL cells. The results of this T-ALL were compared with other T-ALL and T-ALL-derived T-cell lines and showed that some of them also contain an activity of the recombinase complex for TcR-δ gene deletion.

MATERIAL AND METHODS

Cell samples

MNC and/or granulocytes were isolated from PB or BM of 139 T-ALL patients and four healthy volunteers by Ficoll-Paque (density: 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. All MNC samples were frozen and stored in liquid nitrogen. Thymocytes were obtained from thymus samples of children undergoing cardiac surgery. The thymic samples were minced with scissors in RPMI 1640 medium containing 10% fetal calf serum and were flushed through a nylon gauze filter. All human cell samples were obtained with the approval of the Medical Ethics Committee of the Erasmus University/University Hospital Dijkzigt, Rotterdam, The Netherlands. Twelve T-cell lines, seven B-cell lines, two myeloid cell lines and one non-hematopoietic cell line were used.

Immunologic marker analysis

The MNC of the T-ALL patients were analyzed for nuclear expression of TdT; for cytoplasmic expression of CD3 (UCHT1); for membrane expression of T-cell markers CD1 (66IIC7), CD2 (Leu-5b), CD3 (Leu-4), CD4 (Leu-3a), CD5 (Leu-1), CD6 (OKT17), CD7 (3A1), and CD8 (Leu-2a); for the HLA-DR antigen; and for reactivity with McAb, BMA031 (anti-TcR-αβ), 11F2 (anti-TcR-γδ), TCRδ1 (anti-TcR-δ). The rabbit anti-TdT antiserum was purchased from Supertechs (Bethesda, MD); the McAb of the Leu series, anti-HLA-DR, and

11F2 were obtained from Becton Dickinson (San Jose, CA); the CD1 antibody was obtained from Monosan/Sanbio (Nistelrode, the Netherlands); the OKT17 from Ortho Diagnostic System (Raritan, NJ); the 3A1 hybridoma from the American Type Culture Collection (Rockville, MD); TCRδ1 from T Cell Diagnostics (Cambridge, MA); The McAb BMA031 was kindly provided by Dr. R. Kurrle (Behring, Marburg, Germany,). The immunofluorescence stainings were performed as described (18) and evaluated with fluorescence microscopes (Carl Zeiss, Oberkochen, Germany) and/or a FACScan flow cytometer (Becton Dickinson).

Isolation of DNA probes

DNA probes recognizing sequences just downstream of the δREC gene segment (TCRDRED), just upstream of the ψJα gene segment (TCRAPJU), and in the coding region of the RAG genes (RAG-1 and RAG-2) were obtained by cloning the purified PCR amplification products of granulocyte DNA from a healthy volunteer using specific oligonucleotide primer sets and pUC19 as cloning vector (19). The oligonucleotide primer sets are given in Table 1 and were synthesized according to published sequence data (9,20-22) on a 392 DNA synthesizer (Applied Biosystems, Forster City, CA) with the solid-phase phosphotriester method and used without further purification.

Southern blot analysis

DNA was isolated from fresh or frozen cells as described previously (2). Fifteen μg DNA samples were digested with the restriction enzymes: *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII and/or *Kpn*I (Pharmacia), size fractionated in 0.7% agarose gels, and transferred to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) as described (2). TcR-δ gene rearrangements were studied using ^{32}P random oligonucleotide-labeled TcR-δ gene DNA probes: TCRDV1 (Vδ1), TCRDV2 (Vδ2), TCRDV3 (Vδ3), TCRDRE (δREC), TCRDRED (δREC downstream), TCRDD1 (Dδ1), TCRDD2 (Dδ2), TCRDD3 (Dδ3), TCRDJ1 (Jδ1), TCRDC4 (Cδexon4), TCRAPJU (ψJα upstream), and TCRAPJ (ψJα) (Figure 1) (23). Other TcR genes were analyzed with the TcR-γ gene probes: Jγ1.2, Jγ1.3, and Jγ2.1 (2) and TcR-β gene DNA probes: TCRBJ1 (Jβ1), TCRBJ2 (Jβ2), and TCRBC (Cβ) (Breit et al., unpublished results).

TABLE 1. Oligonucleotide primers used in PCR and sequencing analysis of δREC-ψJα rearrangements or in the construction of DNA probes.

Name	Size	Cloning sites	Code	Position[a]	Sequence[b]	Reference[c]
DNA probes					5′ 3′	
TCRDRED	283 bp	*Hin*dIII	δRECDp5′	+52 bp	TCGAaagCtTGCCTAAACCCTGCAGCTGGCAC	9
		*Eco*RI	δRECDp3′	+286 bp	cacagaatTCGCGATCTCCACACAAAGTCCTT	9
TCRAPJU	865 bp	*Eco*RI	ψJαUp5′	−861 bp	GTTCTAgAATTCTCTCTAAAATCATAAAC	22
		*Hin*dIII	ψJαUp3′	−40 bp	TGGGaAgCTTTACAAAAACCAGAGGTGTCAGC	9
RAG1	1071 bp	*Bam*HI	RAG1p5′	+35 bp[d]	GCCTggaTCCCACCCACCTTGGGACTCAG	20
		*Hin*dIII	RAG1p3′	+1059 bp[d]	TTGCaagCTTTTGCTGGACATTTCACCATCAGG	20
RAG2	1632 bp	*Eco*RI	RAG2p5′	−25 bp[d]	AGTCATTTTATTTTAgAATTCTTTCAGACA	21
		*Hin*dIII	RAG2p3′	+1588 bp[d]	ACACCTGAATCTGAAAaGCTTTTGCA	21
δREC-ψJα rearrangement						
		*Eco*RI	δREC-5′E	−204 bp	CTAAgAATtcGATCCTCAAGGGTCGAGACTGTC	9
		*Hin*dIII	ψJα-3′H	+105 bp	CCTGaagcTTAAGGCACATTAGAATCTCTCACTG	9
		*Bgl*II	δRECsj3′	+213 bp	AGGCaGATCTTGTCTGACATTTGCTCCG	9
		*Hin*dIII	ψJαsj5′	−127 bp	TaaGCTtTGAAAGGCAGAAAGAGGGCA	9
δREC polymorphism						
		–	δRECseq	−106 bp	ATGAAATTTATGAACACATGCTGAGG	9
		*Hin*dIII	δRECp3′	−22 bp	CCGTaaGCTtCTCACACGAGAGGATGG	9
δREC-ψJα signal joint probe						
		–	δREC-ψJαsj	RSS[e]	CAC<u>TCCTGTG</u> <u>CACGGTG</u>ATGC	9

a. The position of the 3′ side of the oligonucleotide primer is indicated upstream (−) or downstream (+) relative to the RSS.
b. The sequences in lower case characters represent the aspecific nucleotides, which generate restriction sites.
c. Sequence information used to design the oligonucleotide primers was derived from the indicated literature references.
d. The position of the 3′ side of the oligonucleotide primer is indicated upstream (−) or downstream (+) relative to the ATG codon.
e. The δREC-ψJα signal joint probe contains the head to head fused heptamers (underlined) of the δREC and ψJα gene segments.

Northern blot analysis

Total RNA was isolated using the method as described by Chromzynski and Sacchi (24). Approximately 15 μg of total RNA was size-fractionated in 1.0% agarose gels and transferred to Nytran-13N nylon membranes (Schleicher and Schuell) as described (2). RNA expression was studied using the ^{32}P random oligonucleotide-labeled probes for the RAG genes (RAG-1 and RAG-2 probes), and for the GAPDH gene (GAPDH probe) (25).

PCR amplification analysis

PCR was essentially performed as described previously (13). A 1.0 μg sample of DNA, 12 pmol of the 5′ and the 3′ oligonucleotide primers and 1 unit of *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) were used in each PCR of 100 μl. The oligonucleotide primers are listed in Table 1. The PCR reaction mixture was incubated at 94°C for 3 minutes, at 60°C for 1 minute, and at 72°C for 3 minutes in a thermal cycler (Perkin-Elmer Cetus). Following this initial cycle, denaturing, annealing, and extension steps were performed for another 29-34 cycles at 94°C for 1 minute, at 60°C for 1 minute, and at 72°C for 2.5 minutes, respectively. After the last cycle an additional extension step of 72°C for 7.5 minutes was executed.

Sequencing analysis

The δREC-ψJα PCR products were cloned into a pUC 19 vector as described previously (12). For the direct sequencing analysis one μl of the original PCR product, 12 pmol of the limiting primer, 600 pmol of the opposite primer and 5 units of *AmpliTaq* DNA polymerase were used in each asymmetric PCR of 500 μl. The reaction mixture was incubated for a total of 25-30 cycles with the above-described regular temperature cycles. After asymmetric amplification, the PCR products were precipitated twice in 50% ethanol plus 0.1 volume of 2 M NaAc, pH 5.6 (13). The dried pellet was resolved in 22 μl H_2O, half of which was used in the sequence reaction. Twenty to fifty pmol sequence primer was used in each reaction; the sequence primers δREC seq and ψJα-3′H are listed in Table 1. All sequence reactions were performed with the T7-sequencing kit (Pharmacia) following the manufactor's instructions using ^{35}S radiolabeling, and run in normal, denaturing 8% polyacrylamide sequence gels.

RESULTS

δREC-ψJα coding joint analysis by PCR amplification

To identify clonal T-cell populations in which the TcR-δ gene deletion mechanism via δREC-ψJα rearrangement is active, we analyzed various cell samples for the presence of this particular rearrangement. The primary approach was a PCR reaction with the primers δREC-5′E and ψJα-3′H, which amplify the coding joint of the δREC-ψJα rearrangement (Figure 1). The reactions were performed on DNA from thymic cell samples, PBMNC, T-ALL, and cell lines (Figure 2). Hybridization of the PCR products with the δREC-3′ oligonucleotide probe confirmed their identity (Figure 2). All thymic cell samples as well as the PBMNC and the majority of 139 tested T-ALL were strongly positive (Table 2). Of all 21 cell lines tested only one T-cell line (TCL1) was strongly positive, because one allele contains a δREC-ψJα rearrangement (Table 2) (7). However, a second T-cell line (TCL2) appeared to be weakly positive (Table 2). This is remarkable, because this cell line contains a functional Vδ1-Jδ1 rearrangement on one allele and an unidentified rearrangement to the Jδ1 gene segment on the other allele (Table 2).

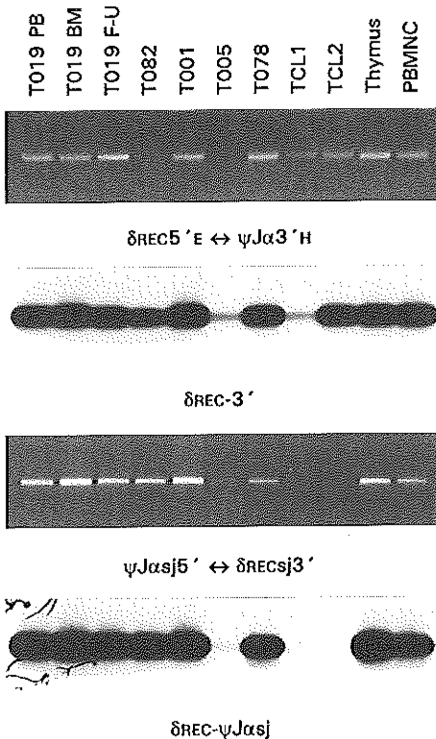


Figure 2. PCR analysis of coding joints (top) and signal joints (bottom) of δREC-ψJα rearrangements. Indicated are the PCR products analyzed by electrophoresis in ethidium-bromide-stained agarose gels and subsequent blotting and identification by oligonucleotide probe hybridization.

δREC-ψJα rearrangement analysis by Southern blot

To confirm the results of the PCR analysis, DNA of the cell samples was analyzed by Southern blotting. The advantage of the Southern blot over the PCR technique is that it does not amplify the DNA and will therefore allow a realistic estimation of the frequency of the δREC-ψJα rearrangement. However, the disadvantage is the low sensitivity, i.e. a detection limit of 5%. By using the δREC probe (TCRDRE) and the ψJα probe (TCRAPJ) (Figure 1), a clear δREC-ψJα rearranged band was detected in each thymic cell sample with δREC-ψJα as preferential, polyclonal rearrangement, and the T-ALL and the cell line with δREC-ψJα as monoclonal rearrangement on one allele (e.g. patient T078 and cell line TCL1) (Figure 3 and Table 2). Weak bands were also visible in PBMNC of all healthy volunteers, as well as in some T-ALL. All tested cell lines other than TCL1 were negative, including TCL2. Because of the presence of the δREC-ψJα rearrangements in PBMNC, it was difficult to determine whether the observed rearrangements in T-ALL were caused by "background" rearrangements in normal PBMNC or whether these rearrangements originated from the T-ALL cells.

δREC-ψJα signal joint analysis by PCR amplification

To determine the origin of the δREC-ψJα rearrangements we investigated the circular excision products that are formed as by-product of this particular rearrangement (Figure 1). PCR amplification using the δRECsj-3′ and ψJαsj-5′ primers gave δREC-ψJα signal joint PCR products, which identity was confirmed by hybridization with the δREC-ψJαsj oligonucleotide

TABLE 2. Characteristics of T-cells with and without ongoing δREC-ψJα rearrangements[a].

											δREC-ψJα rearrangement					
				Rearrangement					RNA expression		PCR		Southern blot		Direct sequencing	
	CD3 phenotype	CD4/CD8	δREC germline[b] polymorphism	TcR-α/δ	frame[c]	TcR-γ	frame[c]	TcR-β	RAG-1	RAG-2	coding joint	signal joint	rearran-gement	excision products	junctional δREC region	poly-morphism[d]
Patients																
T019	CD3^{-}	4^{+}/8^{+}	C	Vδ1-Jδ1	−	Vγ2-Jγ1.3	+	Jβ2	+	+	+	+	+	+	polyclonal	T
			T	Vδ2-Jδ1	−	Vγ2-Jγ1.3	−	Jβ2								
T001	CD3^{-}	4^{+}/8^{+}	C	Vδ1-Jδ1	−	Vγ8-Jγ2.3	ND	Jβ2	ND	ND	+	+	±	±	polyclonal	T
			T	Vδ2-Jδ1	−	G	O	G								
T082	CD3^{-}	ND	ND	Vδ1-Jδ1	−	Vγ3-Jγ2.3	ND	Jβ1	+	+	+	+	+	+	polyclonal	T
			T	Vδ2-Jδ1	−	Vγ8-Jγ2.3	ND	Jβ2								
T005	CD3^{+}	4^{+}/8^{-}	ND	Vδ1-Jδ1	+	Vγ2-Jγ1.1	+	Jβ1	±	±	±	±	−	−	ND	T
			T	Vδ2-Jδ1	−	Vγ2-Jγ1.3	+	G								
T078	CD3^{-}	4^{+}/8^{+}	ND	Vδ3-Jδ2	−	Vγ3∼Jγ1.3	ND	Jβ1	±	±	+	±	+[e]	±	monoclonal	C
			C	δREC-ψJα	O	Vγ2/4−Jγ2.3	ND	G								
T-cell line																
TCL1	CD3^{+}	4^{+}/8^{-}	ND	Vδ1-Jδ1	+	Vγ5-Jγ2.3	−	Jβ1	±	±	±	±	−	−	polyclonal	T
			T	R	ND	Vγ8-Jγ2.3	−	Jβ2								
TCL2	CD3^{-}	4^{-}/8$^{\pm}$	T	δREC-ψJα	O	Vγ8-Jγ2.3	ND	Jβ1	−	−	+	−	+[e]	−	monoclonal	T
			ND	δREC-Jα?	O	Vγ10-Jγ2.3	−	Jβ1								
controls[f]																
Thymus	CD3^{-}/CD3^{+}	O	O	Vδ-Jδ	±	Vγ-Jγ1	±	Jβ1	+	+	+	+	+	+	polyclonal	O
			O	Vα-Jα	±	Vγ-Jγ2	±	Jβ2								
PBMNC	CD3^{+}	O	O	Vδ-Jα	±	Vγ-Jγ1	±	Jβ1	−	−	+	+	±	±	polyclonal	O
			O	Vα-Jα	±	Vγ-Jγ2	±	Jβ2								
HELA	CD3^{-}	4^{-}/8^{-}	ND	G	O	G	O	G	−	−	−	−	−	−	O	O
			ND	G	O	G	O	G								

a. Abbreviations used; ND, not determined; O, not applicable.
b. This polymorphism is present in the δREC germline sequence at position − 84 relative to the RSS.
c. Frame; −, rearrangement is out of frame; +, rearrangement is in frame.
d. For these δREC polymorphisms cf Figures 5 and 6.
e. These bands are 50% of the Southern blot signal, representing a monoclonal δREC-ψJα rearrangement on one allele.
f. The rearrangements indicated in Thymus and PBMNC represent the most frequently occurring rearrangements.

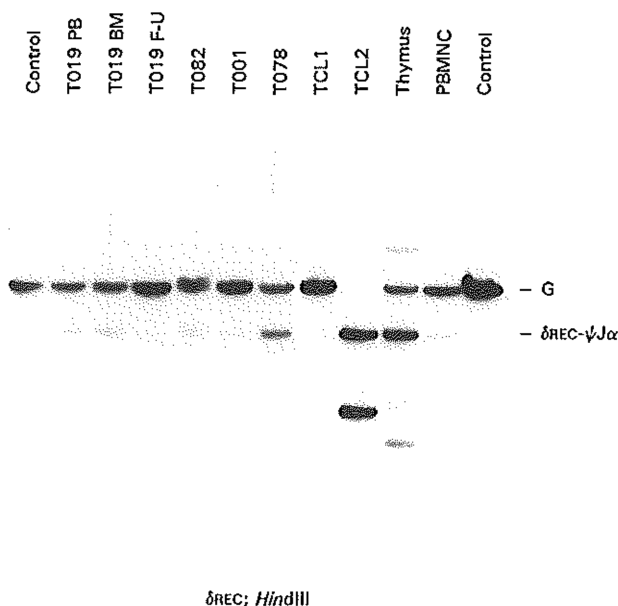


Figure 3. Southern blot analysis of δREC-ψJα rearrangements. A Southern blot filter with *Hin*dIII digests of DNA from various cell samples hybridized with the ^{32}P labelled TCRDRE (δREC) probe. The sizes of the germline (G) band and preferential δREC-ψJα rearrangement are indicated (23).

probe (Figure 2). All thymic cell samples were strongly positive, whereas the tested T-ALL varied from strongly positive to absolute negative results (Figure 2 and Table 2). Surprisingly, the control PBMNC were also positive, meaning that δREC-ψJα excision products do occur in normal PBMNC. Therefore, it was impossible to distinguish background signal joints from T-ALL cells derived signal joints, and thus still impossible to determine the origin of the δREC-ψJα rearrangement in the T-ALL samples. The tested cell lines were all negative, except for cell line TCL2, in which a weakly positive signal proved - by lack of background - that this T-cell line actively rearranges δREC to ψJα.

δREC-ψJα circular DNA analysis by Southern blot

If the δREC-ψJα excision products found by PCR in normal peripheral T-lymphocytes originate from an early thymocyte differentiation stage, we assumed that the frequency of this circular DNA will decrease dramatically due to degradation and dilution, caused by cell proliferation. To analyze the δREC-ψJα circular DNA without amplification, we developed two new probes, TCRDRED (δREC downstream) and TCRAPJU (ψJα upstream), which are able to detect δREC-ψJα excision products in Southern blot analysis (Figure 1). The thymic cell samples showed a clear band representing the δREC-ψJα excision product, whereas some of the analyzed T-ALL showed a weak band (Figure 4 and Table 2). Also, a very faint band was visible in control PBMNC, whereas no excision product could be detected in all cell lines tested. Because T-ALL cell samples generally contain $<10\%$ background PBMNC, these data showed that the analyzed T-ALL in which a (weak) band was visible, actively rearrange δREC to ψJα.

Based on positive and negative results we selected at this point five T-ALL and two T-cell lines which we analyzed further for their ability to form δREC-ψJα rearrangements (Table 2). The most important phenotypic and genotypic characteristics of these T-ALL and T-cell lines are summarized in Table 2.

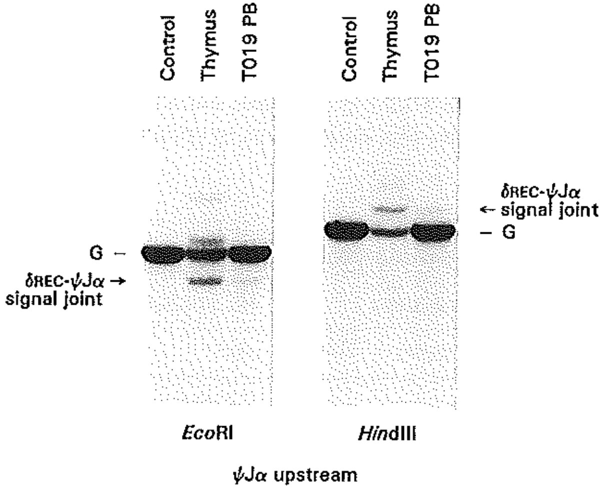


Figure 4. Southern blot analysis of δREC-ψJα circular excision products. A Southern blot filter with *Eco*RI or *Hin*dIII digests of DNA from thymus and patient TO19 hybridized with the TCRAPJU (ψJα upstream) probe. The sizes of the germline (G) band and δREC-ψJα signal joint are indicated.

δREC-ψJα junctional regions

To further prove that the δREC-ψJα rearrangements in the selected T-ALL originate from leukemic cells and thus define a polyclonal subpopulation within the clonal malignancy, we investigated the junctional regions of these rearrangements.

Direct sequencing analysis of PCR products from the δREC-ψJα coding joint can ascertain the clonality status, because in a monoclonal situation the sequence can be read from the δREC gene segment through the junctional region into the ψJα gene segment, whereas in a polyclonal situation the random junctional region will make the sequence unreadable just after the δREC gene segment. An example is presented in Figure 5. All tested samples were polyclonal except for patient TO78 and T-cell line TCL1, both of which contain a monoclonal δREC-ψJα rearrangement on one allele (Table 2).

Cloning and sequencing of the PCR products revealed the actual junctional regions of

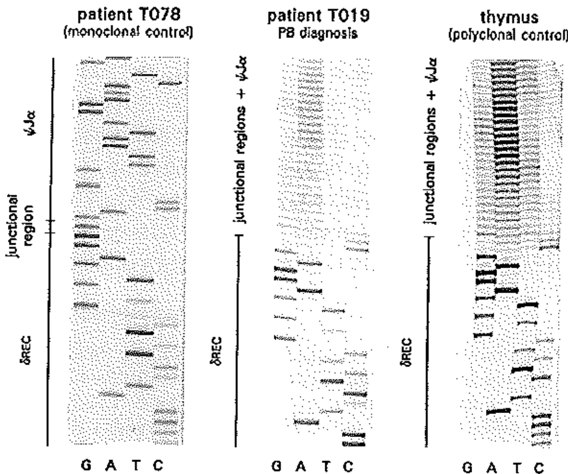


Figure 5. Direct sequencing analysis of δREC-ψJα rearrangement PCR products. The δREC-ψJα rearrangements of patient TO19 are compared with a monoclonal control (patient TO78) and a polyclonal control (thymus).

the δREC-ψJα rearrangements present in these T-ALL patients and T-cell lines (Figure 6). The sequences show no significant differences with those present in normal thymocytes or T-lymphocytes (Table 3) (12). This was most obvious in patient T019 in which the δREC-ψJα junctional regions derived from T-ALL cells of PB or BM at diagnosis were similar to those derived from normal MNC cells of BM at the time this patient was in complete remission (Figure 6 and Table 3).

TABLE 3. Junctional region diversity of human δREC-ψJα rearrangements.

		No. of inserted nucleotides				No. of deleted nucleotides		
		N-region	Dδ	P-region	Total	δREC	ψJα	Total
T019 PB/BM	(n=36)	3.3	1.4	0.4	5.1	3.1	4.5	7.6
T001 BM	(n=12)	6.0	1.4	0.3	7.7	1.4	5.5	6.9
T001 PB	(n=12)	2.9	1.5	0.2	4.6	5.8	5.1	10.9
TCL2	(n=12)	2.1	2.6	0.5	5.2	2.2	4.4	6.6
T019 BM follow-up	(n=12)	3.3	1.3	0.6	5.3	4.2	3.2	7.3
Fetal thymus	(n=50)	1.7	0.8	0.3	2.7	2.8	3.3	5.9
Post-natal thymus	(n=50)	3.2	1.8	0.4	5.4	3.3	5.4	8.7
PBMNC	(n=42)	4.5	1.5	0.3	6.4	3.3	5.3	8.6

Allele specific δREC rearrangement

If the δREC-ψJα rearrangements originate from T-ALL cells, they can only use the δREC gene segments that are still present in these cells. Four of the five tested T-ALL had Vδ1-Jδ1 and Vδ2-Jδ1 rearrangements, whereas the fifth contained a Vδ3-Jδ2 and a δREC-ψJα rearrangement. Because the Vδ1-Jδ1 rearrangement deletes the intermediate δREC gene segment, the only available δREC gene segment is present on the allele with the Vδ2-Jδ1 rearrangement. In some individuals it is possible to distinguish δREC gene segments of different alleles on basis of a polymorphism present at position −87 (relative to the heptamer) in this gene segment, which contains either a T or a C nucleotide (Figure 7 and Table 2). In patient T019 we were able to prove that only one δREC allele was used in the polyclonal δREC-ψJα rearrangements in BM and PB diagnosis, because this patient contained on one allele a C and on the other a T nucleotide at this position (Figure 7): All δREC-ψJα rearrangements in the T-ALL cell samples at diagnosis contained the δREC gene segment with the T nucleotide, whereas in the normal cells of this patient during follow-up, exactly half of the δREC-ψJα rearrangements contained a T and half a C nucleotide (Figure 6 and Table 2). Sequencing of the δREC germline DNA sequence in the leukemic cells revealed a T nucleotide, whereas in the δREC germline DNA sequence of normal cells both nucleotides

Figure 6. Junctional region sequences of δREC-ψJα rearrangements in various human cell samples. Sequences of the δREC-ψJα junctional regions are aligned with the known (double underlined) δREC and ψJα germline sequences. Single underlined sequences represent putative Dδ-gene derived nucleotides. Lower case characters represent P-region nucleotides and all other junctional region nucleotides represent N-region nucleotides. The P↓ indicates the configuration of the polymorphism in the involved δREC gene segment at position -87 bp upstream of the RSS.

patient T019 (PB diagnosis)

p	δREC	junctional region	ψJα
↓	GTGTGAGGAGCC		GGTACCGGGTTA
T	GTG	CG	-15
T	GTGTGAGGA	C	GTACCGGGTTA
T	GTGTGAGGA	CCCTCT	TACCGGGTTA
T	GTGTGAGGAGCC	TACCCGGGC	TACCGGGTTA
T	GTGTGAGGAGCC	TTGTGGCATCCCTACT	GGTACCGGGTT*
T	GTGTGAGGAGCC	TCCTTGGGAAACG	CGGGTTA
T	GTGTGAGGAGC	TCTACCGTGG	CGGGTTA
T	GTGTGAGGAG	AGGGAc	GGTACCGGGTTA
T	GTGTGAGGAGCC		GGTACCGGGTTA
T	GTGTGAGGA	CT	GTACCGGGTTA
T	GTG	CTC	GTACCGGGTTA
T	GTGTGAGGAGCC	CG	CCGGGTTA
T	GTGTGAGGAGC	GGACGGGCAAG	ACCGGGTTA
T	GTGTG	GGGC	CCGGGTTA
T	GTGTGAGGAGCC	CT	-13
T	GTGTGAGGAGCC	ggTTGGCGT	CGGGTTA
T	GTGTGAGGAGCC	gCGG	CGGGTTA
T	GTGTGAGGAGCC	CTTT	GTACCGGGTTA
T	GTGTGAGGAGCC	ggGAAA	TACCGGGTTA
T	GTGTGAGGAGCC	ggTCC	TACCGGGTTA
T	GTGTGAGGAGCC	ggG	CGGGTTA
T	GTGTGAGGA	AAATCG	CCGGGTTA
T	GTGTGAGGAGCC	ggT	CCGGGTTA
T	GTGTGAGGAGCC	CCG	ACCGGGTTA

patient T019 (BM diagnosis)

p	δREC	junctional region	ψJα
↓	GTGTGAGGAGCC		GGTACCGGGTTA
T	GTGTGAGGAGCC	gAAGAGGAGGT	-24
T	GTGTGAGGAGCC	T	GTACCGGGTTA
T	GTGTGAGGAGC	GTC	TACCGGGTTA
T	-38		-14
T	GTGTGAGGAG	GGC	TACCGGGTTA
T	GTGTGAGGAGCC	CTTCT	TACCGGGTTA
T	GTG	CCTGATGTGG	CCGGGTTA
T	GTG	CCCCT	-15
T	GTG	CC	CCGGGTTA
T	GTGTGAGGAGCC	TACA	GTACCGGGTTA
T	GTGTGAGGAGC	TAATAAG	ACCGGGTTA
T	GTGTGAGGAGCC	gAGG	CGGGTTA

patient T019 (BM follow up)

p	δREC	junctional region	ψJα
↓	GTGTGAGGAGCC		GGTACCGGGTTA
C	GTGTGAGGAGCC	g	ACCGGGTTA
C	GT	TTC	TACCGGGTTA
T	GTGTG	GGAC	ACCGGGTTA
C	GTGTGAGGAG	ACGACTCGACCT	TACCGGGTTA
T	-13	TCT	TACCGGGTTA
C	GTGTGAGGAGCC	ggGG	CCGGGTTA
C	GTGTGAGGA	ATAGAGGT	CGGGTTA
T	GTGTGAGGAGCC	ggG	CCGGGTTA
T	GTGTG	GGG	CGGGTTA
T	GTGTGAGGAGCC	TTGACGA	CGGGTTA
T	GTGTGAGGAGCC	ggGGTTC	GTACCGGGTTA
C	GTGT	AAAGGCGC	TACCGGGTTA

patient T078 and cell line TCL1 (monoclonal controls)

p	δREC	junctional region	ψJα
↓	GTGTGAGGAGCC		GGTACCGGGTTA
C	GTGTGAGG	GG	ACCGGGTTA
T	GTGTG	CCACCTAGG	CGGGTTA

control PBMNC (polyclonal control)

p	δREC	junctional region	ψJα
↓	GTGTGAGGAGCC		GGTACCGGGTTA
T	GTGTGAGGA	C	GTACCGGGTTA
T	GTGTGAGGAGC	CGGGATAGG	CCGGGTTA
T	GTGTGAGGAG	TTATTACT	GTACCGGGTTA
T	GTGTGAGGAGCC	TTTTAG	-20
T	GTGTGAGGA	CCCGTTCTCCGG	ACCGGGTTA
T	GTGTGAGGAGC	TAGA	ACCGGGTTA
T	GTGTGAGGAGCC	CCGAGcc	GGTACCGGGTTA
T	GTGTGAGGAGCC	GGCGAT	CGGGTTA
T	GTGTGAGG	AGCCGGTGGAGG	CCGGGTTA
T	GTGTGAGGAGCC		ACCGGGTTA
T	GTGTGAGGA	AGGG	-21
T	GTGTGAGGAGC	GG	-12

patient T001 (BM diagnosis)

p	δREC	junctional region	ψJα
↓	GTGTGAGGAGCC		GGTACCGGGTTA
T	GTGTGAGGAGCC	ggC	-12
T	GTGTGAGGAGCC	GGGGGATCCATTTTAA	GGTT*
T	GTGTGAG	CCCAATGATGGT	TACCGGGTTA
T	GTGTGAGGAGCC	ggCGG	CGGGTTA
T	GTGTGAGG	TCTCTCTCCCT	CGGGTTA
T	GTGTGAGGAGCC	T	TTA
T	GTGTGAGG	TACTCTGA	TACCGGGTTA
T	GTGTGAGGAGCC		CGGGTTA
T	GTGTGAGGAGCC	CGTTGAACCGTTT	TACCGGGTTA
T	GTGTGAGGAGCC	CCCAGGG	-13
T	GTGTGAGG	CCCAC	GTACCGGGTTA
T	GTGTGAGGAGCC	TCCTTTACCATC	TACCGGGTTA

patient T001 (PB diagnosis)

p	δREC	junctional region	ψJα
↓	GTGTGAGGAGCC		GGTACCGGGTTA
C	GTGTGAGGAGCC	T	TACCGGGTTA
T	GTGTGAGGAGC	TAGATT	CCGGGTTA
T	-14	CGA	TACCGGGTTA
T	GTGTG	GTACC	-17
C	GTGTGAGGAGCC	ggAGGGGGA	CGGGTTA
T	-18	AGACA	ACCGGGTTA
T	GTGTGAG	CT	GTACCGGGTTA
T	GTGTGAGGAGCC	CCC	GTACCGGGTTA
C	GT	CCCTT	GTACCGGGTTA
C	GTGTGAGGAGCC	ggAGAAATCGATCCGG	-15
T	GTGTGAGGAGCC		GGGTTA
T	-15		CCGGGTTA

patient T082 (PB diagnosis)

p	δREC	junctional region	ψJα
↓	GTGTGAGGAGCC		GGTACCGGGTTA
T	GTGTGAGGAG	TC	GTACCGGGTTA
T	GTGTGAGGAGCC	gCGGGGCTA	CGGGTTA
T	GTGTGAGGAGC		GGTACCGGGTTA
T	GTGTGAGGAG		TACCGGGTTA
T	GTGT	CACGTGGG	-19
T	GTGTGAGGAGCC	GCG	CGGGTTA
T	GTGTGAGGAGCC	TTCG	CCGGGTTA
T	GTGTGAGGAG	TC	GTACCGGGTTA
T	GTGTGAGGAGCC		GGTACCGGGTTA
T	GTGTGAGGAG	GTGAAG	CCGGGTTA
T	GTGTGAGGAGCC	CACTTTCTAAc	GGTACCGGGTTA
T	GTGTGAGGA	CCTAGGGTAGTC	CCGGGTTA

patient T005 (PB diagnosis)

p	δREC	junctional region	ψJα
↓	GTGTGAGGAGCC		GGTACCGGGTTA
T	GTGTGAGGAGCC	ACCTAGG	CGGGTTA
T	GTGTGAGG	TTACCA	ACCGGGTTA
T	GTGTGAGGAGCC	ggTGGTCCCTGTG	CGGGTTA
T	GTGTGAGGAG		GTTA
T	GTGTGAGGAGCC	ggTGGCTGAG	-22
T	GTGTGAGG	TTACCTA	TACCGGGTTA
T	GTGTGAGGAGCC	ggTGGTCCGCTGTGCGA	GTTA
T	GTGTGAGGAGCC	ggGAA	CGGGTTA
T	GTGTGAGGAGCC	CCTATTTAATTGGACG	CCGGGTTA
T	GTGTGAGGAGCC	ggTGG	CCGGGTTA
T	GTGTGAGGAGCC	TCCGCC	CCGGGTTA

cell line TCL2

p	δREC	junctional region	ψJα
↓	GTGTGAGGAGCC		GGTACCGGGTTA
T	GTGTGAGGAGCC	AAATAGG	CGGGTTA
T	GTGTGAGGAGCC	ggGTAGG	CGGGTTA
T	GTGTGAGGA	CGTTCA	GGGTTA
T	GTGTGAGGAGC	TCTA	GTACCGGGTTA
T	GTGTGAGGAGCC	CTCTTTACA	CGGGTTA
T	GTGTGAGGAGCC	GGGAT	GGGTT*
T	GTG	AAGGGAA	CGGGTTA
T	GTGTG	G	CGGGTTA
T	GTGTGAGGAGCC	ggA	ACCGGGTTA
T	GTGTGAGGAGCC	CA	GTACCGGGTTA
T	GTGTGA	CTA	ACCGGGTTA
T	GTGTGAGGAGCC	ggAAGAAA	GTTA

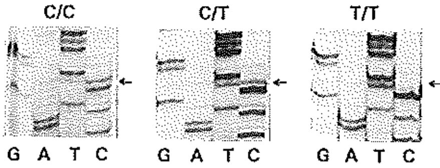


Figure 7. The polymorphism in the δREC gene segment at position -87 bp from the RSS. This nucleotide is either a T or a C and there are therefore three possible configurations: C/C, C/T, and T/T.

were present. Therefore, by using this polymorphism we were able to prove that the δREC-ψJα rearrangements observed in the polyclonal subpopulation of patient T019 all used the same δREC, present on the allele with the Vδ2-Jδ1 rearrangement. Thus, the ALL cells were actively deleting the Vδ2-Jδ1 rearrangement by δREC-ψJα recombination (Figure 1). This was also the case in the T-ALL cell samples derived from BM of patient T001 (Figure 6 and Table 2). However, in leukemic cells derived from PB of patient T001 a mixture of T and C nucleotides was observed in the δREC-ψJα rearrangements. This difference is in line with the high tumor load in the BM sample (88%) versus the low tumor load in the PB sample (45%). Taken together, it can be concluded that in the BM sample of patient T001, the δREC-ψJα rearrangements came from T-ALL cells, whereas in the PB sample a part originated from "background" PBMNC. In patient T082 no normal cells were present to determine the presence of this polymorphism, and although only T nucleotides were observed in the δREC-ψJα rearrangements (Figure 6) this does not exclude background rearrangements.

Expression of RAG genes

The products of the RAG genes are essential for V(D)J recombination and are therefore also involved in the recombinase complex that mediates TcR-δ gene deletion. To prove RAG expression in the T-ALL and T-cell line with ongoing δREC-ψJα rearrangement, we designed RAG-1 and RAG-2 probes for Northern blot analysis. Although not quantitated, it appeared that all T-ALL tested and T-cell line TCL2 express both RAG genes as is the case in thymic cell samples (Figure 8 and Table 2). Control PBMNC, cell line HELA and cell line TCL1 did not express these genes (Figure 8 and Table 2).

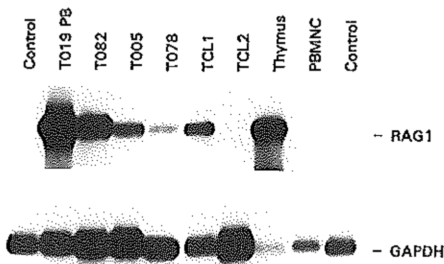


Figure 8. Northern blot analysis of the RAG-1 gene transcription in several cell samples. A Northern blot filter was successively hybridized with the RAG1 and GAPDH probes.

DISCUSSION

The rearrangement of the human TcR-δ gene deleting elements, δREC and ψJα, to each other represents a decisive step in the γδ versus the αβ lineage commitment of differentiating thymocytes (4,8,9,11), because the δREC-ψJα rearrangement is an intermediate rearrangement between TcR-δ and TcR-α gene rearrangement (4,8). The only reason for this non-functional rearrangement appears to be the deletion of the TcR-δ gene in order to prepare the allele for subsequent TcR-α gene rearrangement. The high frequency of this particular rearrangement in thymic cell samples strongly suggests that a major fraction of thymocytes will use the δREC-ψJα rearrangement to delete their TcR-δ genes during differentiation (7,9,11,26-28).

There are several indications that the δREC-ψJα rearrangement is caused by a V(D)J recombinase complex which contains at least some components that are different from those used in TcR-δ and TcR-α gene rearrangement. For instance, it is noticeable that the δREC gene segment rearranges with such a high preference to the ψJα gene segment, although the whole Jα region (61 gene segments) is assumed to be opened by transcription from the T early alpha (TEA) element (4,7,21,27,28). Also other gene segments can rearrange to the ψJα gene segment, but it most frequently rearranges to the δREC gene segment, and not to the proximally located Vδ2 gene segment. In addition, rearrangement of δREC to ψJα occurs at a later stage of thymic differentiation than TcR-δ gene rearrangement (Blom *et al.*, unpublished results), meaning that different factors play a role in (the regulation of) this unique rearrangement. Furthermore, our studies on the *tal*-1 deletions on chromosome band 1p32 in T-ALL, and especially their occurrence in combination with TcR-δ gene deletion, strongly suggest the existence of a unique TcR-δ deleting recombinase complex (13,16,17).

To identify the specific components of this particular recombinase complex, an experimental model is needed in which this recombinase complex is active. The first idea was to select thymocytes that were actively deleting their TcR-δ genes. But there are two main obstacles to do that: firstly it is unknown in which differentiation stage the thymocytes delete their TcR-δ genes and whether there exist specific markers that define this differentiation stage, which can also be applied as selection markers. Secondly, from the fact that δREC-ψJα rearrangements are rarely found in T-ALL, in contrast to TcR-δ and TcR-α gene rearrangements, we concluded that the differentiation stage in which the TcR-δ gene deleting recombination occurs is a short transient stage. Because these facts would greatly hamper our ability to obtain by cell-sorting sufficient cells for our experimental purposes, we decided to look for a monoclonal cell population with an active TcR-δ gene deleting recombinase.

By screening a large number of T-ALL and cell lines by PCR, Southern blot and direct sequencing analysis, we were able to identify several T-ALL and one T-cell line with small subpopulations containing the δREC-ψJα rearrangement. To investigate whether these cells represented just leukemic subclones or rearrangements from "background" MNC, not only the coding joints, but also the excision products of the δREC-ψJα rearrangements were analyzed. An unexpected problem we encountered was the presence of circular excision products of the δREC-ψJα rearrangements in normal PBMNC. To distinguish between normal "background" and ALL derived rearrangements, we applied several Southern blotting and

PCR-sequencing methods. Frequently occurring circular excision products in ALL cells and polyclonal δREC-ψJα junctional region sequences provided the evidence that several T-ALL and T-cell line TCL1 contain small polyclonal subclones. A polymorphism in the δREC gene segment provided the most convincing evidence that these cells were actively rearranging δREC to ψJα on one allele, thereby generating a polyclonal subpopulation. This is a remarkable observation, because subpopulations in ALL tend to be monoclonal or oligoclonal (29,30).

It is especially surprising to find such a polyclonal subpopulation in a cell line, because this means that although continuous rearrangement occurs, none of these cells was able to grow out to a substantial subclone. This is basically the same for the T-ALL. It becomes even more surprising in cell line TCL1, because the δREC-ψJα rearrangement deletes the TcR-δ allele that is not expressed. Therefore, it is hard to imagine that the δREC-ψJα rearrangement will influence the cell growth. What might occur is that this TcR-δ gene deleting rearrangement is immediately followed by a TcR-α rearrangement, which in turn deletes the δREC-ψJα rearrangement and may lead to an expression of a new TcR-$\alpha\beta^+$. These cells may have lower grow capacity and therefore never become a substantial subclone.

In fact this may also be the case for the T-ALL, because for instance in patient T019 with a $TcR^-/CD3^-$ T-ALL, double immunofluorescence stainings revealed that a minor TcR-$\alpha\beta^+/CD3^+$ subpopulation (14%) was present (unpublished results). This confirmed the continuous rearrangement capacity of the T-ALL cells. Because the rearrangements occur in T-ALL cells that are outside the thymus, or in an *in vitro* cultured cell line, this suggests that δREC-ψJα rearrangements do not explicitly require the thymic environment and all other specific extracellular signals.

It was remarkable that all T-ALL that showed the most intense ongoing δREC-ψJα rearrangement, were $CD3^-$ and contained a Vδ1-Jδ1 and a Vδ2-Jδ1 rearrangement. Moreover, two of three T-ALL were tested for their CD4/CD8 phenotype and both were double positive (Table 2), which is in line with the phenotype of a $CD4^+/CD8^+$ thymocyte population in which the TcR-δ gene deleting rearrangement is believed to occur (Blom *et al.*, unpublished results). However, the characteristics of T-cell line TCL1 were different compared to these T-ALL, because this cell line was $CD3^+$, and contains a Vδ1-Jδ1 and an unknown TcR-δ gene rearrangement.

We can conclude that there are T-ALL and cell lines that contain an active TcR-δ gene deleting recombinase complex. But although it is still a long way to the identification of regulating elements involved in the deletion of the TcR-δ gene, at least we now have the (monoclonal) experimental model to start these studies.

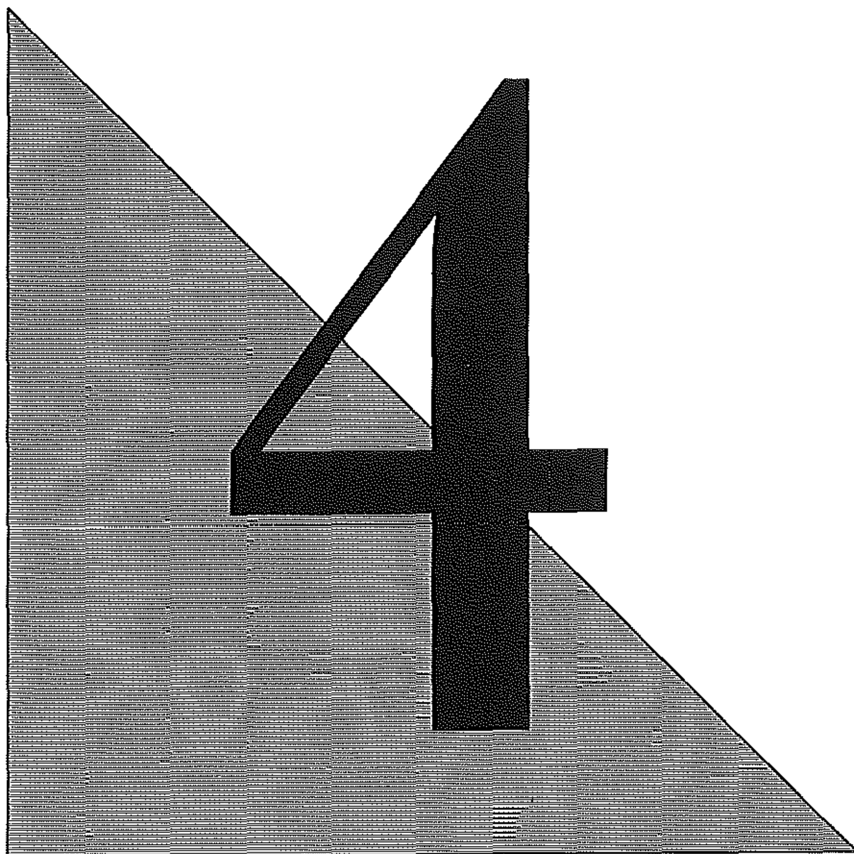
ACKNOWLEDGMENTS. The authors gratefully acknowledge Prof. Dr. R. Benner, Dr. H. Hooijkaas, and Dr. A. Beishuizen for their continuous support; Dr. R. Kurrle (Behring, Marburg, Germany), Dr. T. Hercend (Villejuif, France) and Dr. L. Moretta (Genova, Italy) for kindly providing the BMA031, Ti-γA and BB3 antibodies; Dr. T. Quertermous and Dr. M.-P. Lefranc for kindly providing the TcR-γ probes. Dr. D. Campana for kindly providing ALL cell samples; Mrs. W.M. Comans-Bitter for providing immunofluorescence data of patient TO19; Mr. T.M. van Os for excellent assistance in the preparation of the figures; and Ms. A.D. Korpershoek for her secretarial support.

REFERENCES

1. Foon KA, Todd RF III. Immunologic classification of leukemia and lymphoma. Blood 1986;68:1-31.
2. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of immunoglobulin and T cell receptor genes. Part I: basic and technical aspects. Clin Chim Acta 1991;198:1-91.
3. Breit TM, Wolvers-Tettero ILM, Van Dongen JJM. Phenotypic and genotypic characteristics of human early T-cell differentiation: the T-cell acute lymphoblastic leukaemia model. Res Immunol 1994;145:139-143.
4. Van Dongen JJM, Comans-Bitter WM, Wolvers-Tettero ILM, Borst J. Development of human T lymphocytes and their thymus-dependency. Thymus 1990;16:207-234.
5. Alt FW, Baltimore D. Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-JH fusions. Proc Natl Acad Sci USA 1982;79:4118-4122.
6. Schatz DG, Oettinger MA, Schlissel MS. V(D)J recombination: molecular biology and regulation. Annu Rev Immunol 1992;10:359-383.
7. De Villartay J-P, Hockett RD, Coran D, Korsmeyer SJ, Cohen DI. Deletional of the human T-cell receptor δ-gene by a site-specific recombination. Nature 1988;335:170-174.
8. Hockett RD, De Villartay J-P, Pollock K, Poplack DG, Cohen DI, Korsmeyer SJ. Human T-cell antigen receptor (TCR) δ-chain locus and elements responsible for its deletion are within the TCR α-chain locus. Proc Natl Acad Sci USA 1988;85:9694-9698.
9. Hockett RD, Nuñez G, Korsmeyer SJ. Evolutionary comparison of murine and human δ T-cell receptor deleting elements. The New Biologist 1989;1:266-274.
10. Isobe M, Russo G, Haluska FG, Croce CM. Cloning of the gene encoding the δ subunit of the human T-cell receptor reveals its physical organization within the α-subunit locus and its involvement in chromosome translocations in T-cell malignancy. Proc Natl Acad Sci USA 1988;85:3933-3937.
11. De Villartay J-P, Lewis D, Hockett RD, Waldmann TA, Korsmeyer SJ, Cohen DI. Deletional rearrangement in the human T-cell receptor α-chain locus. Proc Natl Acad Sci USA 1987;84:8608-8612.
12. Breit TM, Wolvers-Tettero ILM, Bogers AJJC, De Krijger RR, Wladimiroff JW, Van Dongen JJM. Rearrangements of the human TCRD-deleting elements. Immunogenet 1994;40:70-75.
13. Breit TM, Mol EJ, Wolvers-Tettero ILM, Ludwig W-D, Van Wering ER, Van Dongen JJM. Site-specific deletions involving the *tal*-1 and *sil* genes are restricted to cells of the T cell receptor αβ lineage: T cell receptor δ gene deletion mechanism affects multiple genes. J Exp Med 1993;177:965-977.
14. Brown L, Cheng J-T, Chen Q, Siciliano MJ, Crist W, Buchanan G, Baer R. Site-specific recombination of the *tal*-1 gene is a common occurrence in human T cell leukemia. EMBO J 1990;9:3343-3351.
15. Aplan PD, Lombardi DP, Ginsberg AM, Cossman J, Bertness VL, Kirsch IR. Disruption of the human SCL locus by "illegitimate" V-(D)-J recombinase activity. Science 1990;250:1426-1429.
16. Macintyre EA, Smit L, Ritz J, Kirsch IR, Strominger JL. Disruption of the SCL locus in T-lymphoid malignancies correlates with commitment to the T-cell receptor αβ lineage. Blood 1992;80:1511-1520.
17. Breit TM, Wolvers-Tettero ILM, Van Dongen JJM. Lineage specific demethylation of *tal*-1 gene breakpoint region determines the frequency of *tal*-1 deletions in αβ lineage T-cells. Oncogene 1994;9:1847-1853.
18. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunological marker analysis of cells in the various hematopoietic differentiation stages and their malignant counterparts. In Application of monoclonal antibodies in tumor pathology. Ruiter DJ, Fleuren GJ, Warnaar SO, eds. Dordrecht, The Netherlands, Martinus Nijhoff, 1987:87-116.
19. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning, a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1989.
20. Schatz DG, Oettinger MA, Baltimore D. The V(D)J recombination activating gene, RAG-1. Cell 1989;59:1035-1048.
21. Ichihara Y, Hirai M, Kurosawa Y. Sequence and chromosome assignment to 11p13-p12 of human RAG genes. Immunol Lett 1992;33:277-284.
22. Koop BF, Row L, Wang K, Kuo CL, Seto D, Lenstra JA, Howard S, Shan W, Wilke E, Hood L. The human T-cell receptor Cα/Cδ region: organization, sequence and evolution of 97.6 kb of DNA. Genomics 1994;19:478-493.
23. Breit TM, Wolvers-Tettero ILM, Beishuizen A, Verhoeven M-AJ, Van Wering ER, Van Dongen JJM. Southern blot patterns, frequencies, and junctional diversity of T-cell receptor-δ gene rearrangements in acute lymphoblastic leukemia. Blood 1993;82:3063-3074.
24. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-159.
25. Benham FJ, Hodgkinson S, Davies KE. A glyceraldehyde-3-phosphate dehydrogenase pseudogene on the short arm of the human X-chromosome defines a multigene family. EMBO J 1984;3:2635-2640.
26. De Villartay J-P, Mossalayi D, De Chasseval R, Dalloul A, Debré P. The differentiation of human pro-thymocytes along the TcR-α/β pathway *in vitro* is accompanied by the site-specific deletion of the TcR-δ locus. Int Immunol 1991;3:1301-1305.

27. De Villartay J-P, Lewis D, Hockett R, Waldmann TA, Korsmeyer SJ, Cohen DI. Deletional rearrangement in the human T-cell receptor α-chain locus. Proc Natl Acad Sci USA 1987;84:8608-8612.
28. De Villartay J-P, Pullman AB, Andrade R, Tschachler E, Colamenici O, Neckers L, Cohen DI, Cossman J. γ/δ lineage relationship within a consecutive series of human precursor T-cell neoplasms. Blood 1989;74:2508-2518.
29. Bird J, Galili N, Link M, Stites D, Sklar J. Continuing rearrangement but absence of somatic hypermutation in immunoglobulin genes of human B cell precursor leukemia. J Exp Med 1988;168:229-245.
30. Beishuizen A, Hählen K, Hagemeijer A, Verhoeven M-AJ, Hooijkaas H, Adriaansen HJ, Wolvers-Tettero ILM, Van Wering ER, Van Dongen JJM. Multiple rearranged immunoglobulin genes in childhood acute lymphoblastic leukemia of precursor B-cell origin. Leukemia 1991;5:657-667.

GENE REARRANGEMENT OF THE *tal*-1 GENE



GENE REARRANGEMENT OF THE *tal*-1 GENE

4.1 TAL1, a bHLH protein implicated in T-ALL 155

4.2 Site-specific deletions involving the *tal*-1 and *sil* genes are restricted to cells of the T-cell receptor $\alpha\beta$ lineage: T-cell receptor δ gene deletion mechanism affects multiple genes 169
Published in: J Exp Med 1993;177:965-977.

4.3 Lineage specific demethylation of *tal*-1 gene breakpoint region determines the frequency of *tal*-1 deletions in $\alpha\beta$ lineage T-cells 187
Published in: Oncogene 1994;9:1847-1853.

CHAPTER 4.1

TAL-1, A bHLH PROTEIN IMPLICATED IN T-ALL

Timo M. Breit, Ingrid L.M. Wolvers-Tettero, Ellen J. van Gastel-Mol, and Jacques J.M. van Dongen

Department of Immunology, University Hospital Dijkzigt/Erasmus University, Rotterdam, The Netherlands.

Introduction

It is generally assumed that the oncogenic events in many B- and T-cell malignancies are mediated by "illegitimate" activity of the V(D)J recombinase complex in early stages of lymphoid differentiation (1-7). This assumption is based on the findings that many oncogenic translocations in these malignancies involve one of the Ig/TcR genes (1-3,6). Another type of chromosome aberrations result in chimeric oncoproteins (4-6,8). For instance, in null ALL with t(4;11)(q21;q23) chimeric HRX-FEL proteins are formed; pre-B-ALL with t(1;19)(q23;p13.3) produces a E2A-PBX1 fusion protein; in AML the chimeric AML1-ETO oncoprotein occurs due to a t(8;21)(q22;q22) (4,8). The most frequently involved site in reciprocal translocations with fusion proteins is chromosome band 11q23 on which the gene *HRX* (also called *MLL*, *ALL*-1 or *HTRX*-1) is disrupted by translocation to several chromosomes; t(4;11), t(6;11), t(9;11) and t(11;19) (8). So far, in T-ALL no chromosome aberrations which result in fusion protein are observed.

Members of the gene family that encode DNA-binding proteins with a bHLH domain, are frequently affected by chromosome aberrations in ALL (4-6,8,9). At least five bHLH genes are suspected to play a role in the oncogenesis of B-ALL (*E2A*) or T-ALL (*lyl*-1, *myc*, *tal*-1, and *tal*-2) (4-6,8).

Here we want to summarize some data concerning the role of V(D)J recombination in the development of chromosome aberrations present in T-ALL and the information this provides about oncogenic transformation processes and normal T-cell differentiation. We will illustrate this with aberrations that involve the gene for the TAL-1 bHLH protein (10-12). The *tal*-1 gene is the target for several aberrations, which are non-randomly distributed in T-ALL (13,14). The implications of the observed restriction of these *tal*-1 aberrations to T-ALL with a particular immunophenotype and immunogenotype will be discussed.

V(D)J recombinase and chromosome aberrations in T-ALL

It is not surprising that translocations involving the Ig and TcR genes are restricted to ALL, because these genes and the V(D)J recombination activating genes, RAG-1 and RAG-2, normally are only active in lymphoid cells (7). In fact, the majority of the Ig gene translocations occur in B-cell leukemias, whereas those involving TcR genes occur in T-cell leukemias (1,4-6). Because the Ig/TcR and RAG genes are inactive in myeloid cells, other recombination processes and other translocated genes are found in AML (4,6,8).

The chromosome aberrations that occur in T-ALL are summarized in Table 1. The TcR genes that are affected are TcR-β (chromosome band 7q35) and TcR-α/δ (chromosome band 14q11) on the one hand, and genes that code for DNA binding proteins involved in gene activation, on the other hand (2-6). These translocations probably activate the involved non-TcR gene via the enhancer/promoter of the translocated TcR gene (2-6,8). It is noticeable that up to date, no translocations to the TcR-γ gene are reported in ALL, although inversions of this gene to the TcR-β locus do occur in ataxia telangiectasia patients (15). As suggested in the literature, reason for this may be the fact, that if a T-cell is committed to the $\alpha\beta$ lineage, the TcR-γ gene becomes inactivated and therefore also the translocated putative oncogene (16). Without an active TcR-γ enhancer/promoter the oncogenic transformation will stop (16).

Involvement of TcR genes in translocations may be an indication that V(D)J recombinase plays a significant role in these recombination processes, but there is other and more convincing evidence. Most of the genes that translocate to a TcR gene have at their breakpoint region, sequences that are homologous to the heptamer-nonamer RSS sequences of Ig/TcR genes (1-7). These translocation RSS often exist of a sole heptamer, but still it is a strong indication for V(D)J recombinase involvement. The RSS

TABLE 1. Characteristics of chromosome aberrations in T-ALL[a].

Aberration	Involved TcR gene	Other[b] gene	Protein domain	Effect	Recombination signal sequence
tal-1 deletion	–	*sil*	–	~90 kb deletion	heptamer
		tal-1	bHLH	overexpression	RSS
t(1;3)(p32;p21)	–	*tal*-1	bHLH	overexpression	heptamer
		?	–	?	pur/pyr
t(1;7)(p32;q35)	β	*tal*-1	bHLH	overexpression	–
t(1;14)(p32;q11)	δ	*tal*-1	bHLH	overexpression	RSS
t(1;7)(p34;q35)	β	*lck*	SRC	overexpression	–
t(8;14)(q24;q11)	α/δ	*c-myc*	bHLH	overexpression	RSS
t(8;14)(q24;q11)	δ	*pvt*-1	?	?	heptamer
t(7;9)(q35;q32)	β	*tal*-2	bHLH	overexpression	heptamer
t(7;9)(q35;q34.3)	β	*tan*-1	–	truncation/overexpr.	(heptamer)
t(7;10)(q35;q24)	β	*HOX11*	homeobox	overexpression	RSS
t(10;14)(q24;q11)	α/δ	*HOX11*	homeobox	overexpression	(heptamer)
t(7;11)(q35;p13)	β	*RBTN2*	LIM	overexpression	RSS
t(11;14)(p13;q11)	α/δ	*RBTN2*	LIM	overexpression	(heptamer)
t(11;14)(p15;q11)	δ	*RBTN1*	LIM	overexpression	heptamer
t(7;14)(q35;q32.1)	β	(*tcl*-1)	?	?	heptamer
inv(14)(q11;q32.1)	α	(*tcl*-1)	?	?	heptamer
t(14;14)(q11;q32.1)	α	(*tcl*-1)	?	?	heptamer
t(7;19)(q35;p13)	β	*lyl*-1	bHLH	overexpression	–

a. Data derived from references 1-6,12,64,65,115-120.
b. Other names for the involved genes are: *tal*-1 = *SCL* or *TCL5*; *tan*-1 = *TCL3*; *RBTN1* = *TTG1*; *RBTN2* = *TTG2*.

sequences are also found in a submicroscopic deletion in chromosome band 1p32, the so-called *tal*-1 deletion, in which two closely located genes (*sil* and *tal*-1) show site-specific recombination to each other (12,14). The unique breakpoint in the *sil* gene contains a single heptamer, whereas the several breakpoints in the *tal*-1 gene contain a complete RSS (Figure 1A) (12,14). From this site-specific deletion further evidence for "illegitimate" V(D)J recombination emerges, in the form of randomly-inserted N-region nucleotides at the fusion site of the breakpoints (7,17). In translocations to TcR genes one cannot exclude that the N-region nucleotides were already present in a preexisting rearrangement of the TcR gene, instead of that they were included in the translocation fusion region during the recombination processes. However, in the breakpoint fusion regions of *tal*-1 deletion, N-region nucleotides, P-region nucleotides, as well as deletion of nucleotides by exonucleic nibbling from the flanking sequences were found, all of which are hallmarks of V(D)J recombination (7,12,14,17). In fact these fusion regions of *tal*-1 deletion were extremely comparable to junctional regions of TcR gene rearrangements. This strongly supports the assumption that the chromosome aberrations in T-ALL are caused by "illegitimate" V(D)J recombinase activity (1-7).

Besides the RSS, there must be other sequences involved in these recombination processes. For instance, it is hard to believe that in *tal*-1 deletions a poorly conserved,

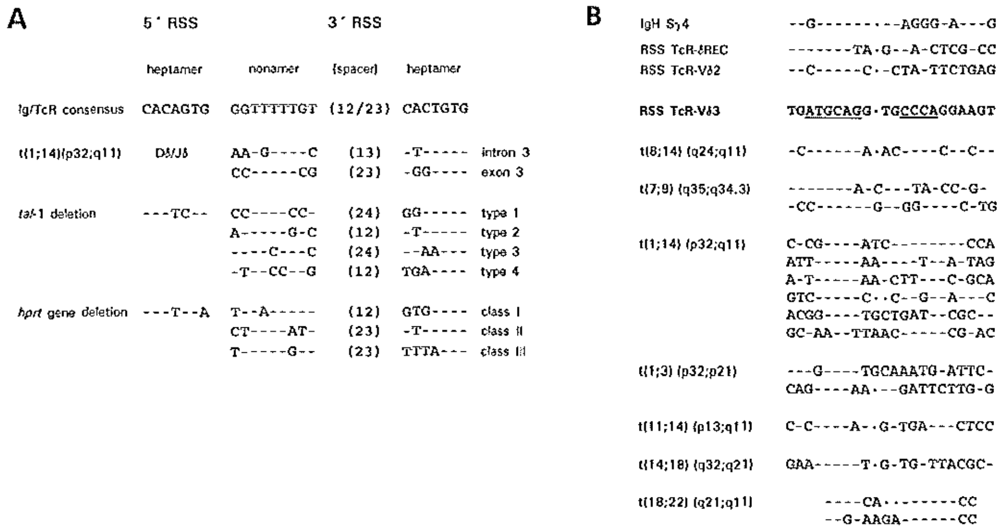


Figure 1. Signal sequences in V(D)J recombination. A) Recombination signal sequences (RSS) used in rearrangement processes mediated by V(D)J recombinase. The RSS used in the various types of translocations t(1;14)(p32;q11) (10,11,46,58,60,62), *tal*-1 deletions (12,14,45,66,68), and *hprt* deletions (101) are aligned with the consensus heptamer-nonamer RSS of TcR genes (75,76). ---, nucleotides homologous to the RSS consensus sequence. B) Sequence homology in the t(1;14)(p32;q11) and t(8;14)(q24;q11) breakpoint regions. A signal-like sequence motif (underlined) has been observed that represents a recognition sequence for the DNA binding proteins ReHF-1 and ReHF-2 (18,19). The sequences are aligned with the spacer sequence of the Vδ3 RSS of the human TcR-δ gene which also binds the ReHF proteins (19). Sequence data are derived from references 10,11,18,45,61,64,65,82,83,117,119,124, and 125. ---, nucleotides homologous to the human Vδ3 RSS; ···, spacing for sequence alignment.

single heptamer sequence in the *sil* gene is the sole sequence that causes the strict location of this aberration which occurs relatively frequently in T-ALL (Figure 1A) (12,14). If indeed other sequences are involved in the "illegitimate" V(D)J recombination processes, than it is likely that they will also be involved in the "normal" V(D)J recombination processes and could therefore lead to new insight in these extremely complex processes. In fact, this idea was persuaded in the t(1;14)(p32;q11) and t(8;14)(q24;q11), which share a signal-like sequence motif at their breakpoints in addition to the RSS (Figure 1B) (18). Recent studies showed that this sequence motif represents a recognition sequence for DNA binding proteins (ReHF-1 and ReHF-2) (19). But even more interesting was the finding that this signal-like sequence motif was also present in the spacer of the Vδ3 RSS. Together with the expression of the ReHF genes in early T-cell lines, this is a remarkable way to find new proteins involved in V(D)J recombination (Figure 1B) (19).

bHLH proteins involved in chromosome aberrations

As already mentioned, the members of the gene family coding for bHLH proteins are frequently affected by chromosome aberrations in ALL (4-6,8,9). The bHLH proteins normally play a role in the control of cell proliferation or differentiation (9,20-22).

The HLH motif is a structural domain of about 40-50 amino acids that mediates protein dimerization (Figure 2). This domain has the potential to form two amphipathic α helices separated by an intervening loop (9,20,23,24). Most HLH proteins contain a stretch of basic residues at the amino-terminus of the first helix that mediates sequence-

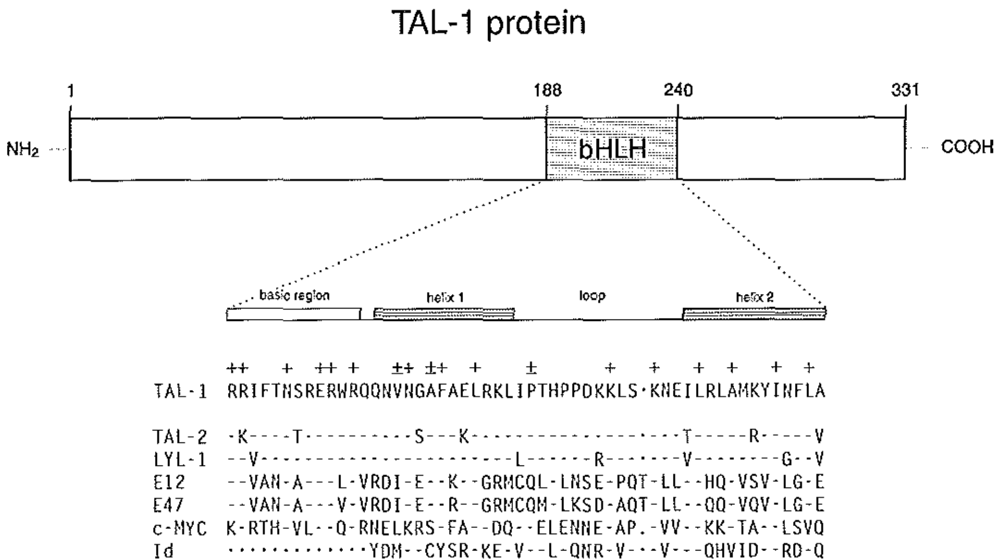


Figure 2. Schematic representation of the TAL-1 protein. Indicated are the position and amino acid sequence of the bHLH domain in the TAL-1 protein (43,44). The TAL-1 protein bHLH domain is compared with that of other bHLH proteins (9,41,115,117,118). +, strongly conserved amino acid residues; ±, less conserved amino acid residues; ---, amino acid residues homologous to the TAL-1 amino acid sequence; ···, spacing for sequence alignment.

specific DNA binding (Figure 2) (25-27). DNA recognition by the bHLH domain is dependent on protein dimerization (9,20,23,28,29). Thus, bHLH proteins may form homodimers by self-association, or may form heterodimers with other bHLH proteins. The dimeric bHLH complexes specifically recognize DNA sequences containing the so called consensus 'E-box' motif (CANNTG) (23,30-36), a *cis*-acting regulatory element found in many eukaryotic transcription enhancers (30,31). bHLH proteins contain transcription regulation domains and have been shown to activate transcription of a gene upon binding to E-box elements of an associated enhancer (21,28,34,40). It is therefore assumed that regulation of transcription is the primary function of most bHLH proteins (21).

Several bHLH proteins are expressed in a wide variety of tissues, where they can function as homodimers but are also able to form functional heterodimers with other bHLH proteins, which exhibit tissue-specific expression patterns (20). Some HLH proteins do not have a basic domain and are therefore unable to bind to DNA. These proteins act as inhibitors, because they form non-functional heterodimers with bHLH proteins (21,41,42). Since dimer formation is essential for DNA binding activity, the function of bHLH proteins is largely dependent on the pattern of dimerization (9,20,23,24). The family of (b)HLH proteins can therefore be divided in at least six classes, which are indicated in Table 2.

TABLE 2. Classification of bHLH proteins[a].

Class I	bHLH proteins, which are broadly expressed and able to form homodimers as well as heterodimers (examples: E2A, E2-2, and HEB).
Class II	bHLH proteins, which are tissue-specific and can only form heterodimers with class I proteins (examples: MyoD, Myogenin, TAL-1, TAL-2, and LYL-1).
Class III	bHLH-zip proteins, which are related to MYC and are involved in cell growth control (examples: C-MYC, N-MYC, L-MYC, and TFE3).
Class IV	bHLH-zip proteins, which are able to form heterodimers with class III proteins (examples: MAX, and MAD).
Class V	HLH proteins, which lack a basic domain, but are able to dimerize with class I proteins. In this way they inhibit DNA binding of class I proteins (examples: Id1, Id2, Id3, and Id4).
Class VI	bHLH proteins characterized by the presence of a proline residue in their basic region. (examples: Hairy, E-(spl), HES1, HES3, and HES5).

a. Classification according to Murre *et al.* (121)

bHLH protein TAL-1

Within the bHLH gene family, the *tal*-1 gene (also called *TCL5* or *SCL*) that codes for the bHLH protein TAL-1, is most recurrently affected in T-ALL. The *tal*-1 gene is a transcriptionally complex locus consisting of eight exons, in which the 5′ non-coding region has at least three distinct transcription initiation sites and a variable pattern of alternative exon utilization (Figure 3) (43-46). At least six different forms of mRNA are expressed, predominantly in early hematopoietic cells (44,45,47-49). The TAL-1 protein contains a bHLH DNA binding motif, which is highly homologous to those present in the TAL-2 protein and LYL-1 protein (Figure 2) (9,20,43,44,47,50,51). TAL-1 proteins dimerize with bHLH class I proteins and it has been shown that the TAL-1 protein is able

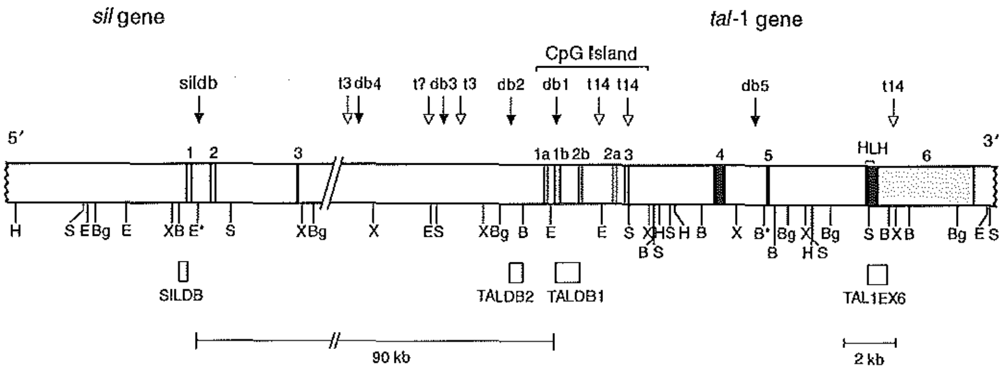


Figure 3. Restriction map of the *tal*-1 locus (right side) and the 5′ part of the *sil* gene (left side) involved in the ~90 kb *tal*-1 deletion (14). The various types of *tal*-1 deletion breakpoints are indicated with closed arrows (12,14,45,66,70): sildb, breakpoint in the *sil* gene; db1-5, the five breakpoints in the *tal*-1 gene. Translocation breakpoints are indicated with open arrows: t3, t(1;3) (65, Breit unpublished results); t14, t(1;14)(p32;q11) (10,11,46,57,58,60,62,63); t?, a yet unknown translocation. Non-coding exons are indicated as dotted boxes; solid boxes represent protein-coding exons. The CpG island in the *tal*-1 gene promotor region is indicated (44,122,123), as well as the HLH motif in *tal*-1 exon 6 (43,44). The relevant restriction sites are indicated: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; S, *Sac*I; X, *Xba*I; *, polymorphic restriction site. Open boxes below the restriction map represent the probes which can be used for Southern and Northern blot analysis (14).

to form a stable heterodimer with E2A proteins that is able to bind DNA (36,52-54).

Recently, the DNA binding site for the TAL-1/E2A complex has been identified: AACAGATGGT (36). This site contains the E-box motif and can be divided in two half-sites of recognition: AACAG, to which the E2A protein binds; and ATGGT, to which the TAL-1 protein binds. Although several TAL-1 DNA binding sites are proposed (36), still limited information is available about the normal or malignant functions of the TAL-1 protein, other than that there are indications that it might work as a transcription regulator (51).

To illustrate the complexity of the mechanisms in which this protein is involved, recent studies describe that the TAL-1 protein can repress transcription by recruiting E47 into bHLH complexes (i.e. TAL-1/E47 heterodimers) with less transcriptionally activity than E47/E47 homodimers (55). However, in other settings, the TAL-1 protein can activate transcription because TAL-1/E47 heterodimers are more resistant to negative regulation by Id proteins (i.e. E47/Id heterodimers)(55,56). Hence, the TAL-1 protein can potentially regulate transcription in either a positive or negative fashion, depending on the intracellular environment (55).

Chromosome aberrations affecting the *tal*-1 gene

The *tal*-1 gene on human chromosome 1p32 is frequently affected in T-ALL. In t(1;14)(p32;q11), as well as in the rarely occurring t(1;7)(p32;q35), and t(1;3)(p32;p21) the *tal*-1 gene is recombined to the TcR-δ gene complex (10,11,46,57-63), the TcR-β gene complex (64), and a yet unidentified transcriptional unit on chromosome 3p21 (65), respectively (Table 1 and Figure 3). In addition, 15-25% of T-ALL contain a site-specific, so-called *tal*-1 deletion (12-14,66-73), in which all coding exons of the *tal*-1

gene (43,44) are juxtaposed to the first non-coding exons of the *sil* gene (74) (Figure 3). As a result of this ~90 kb deletion, the *sil* coding exons are deleted and the expressed *sil-tal*-1 fusion mRNA in principle codes for an intact TAL-1 protein, which is transcriptionally controlled by the *sil* gene promoter (45,66). In the above mentioned translocations, the *tal*-1 gene is placed under the control of either a promoter or enhancer of the other gene involved in the translocation (10,11,46,60-63). Here also the *tal*-1 mRNA in principle codes for an intact TAL-1 protein.

The site-specificity of the breakpoints in t(1;14) and *tal*-1 deletions is most probably caused by the fact that both aberrations are mediated via RSS, which are homologous to the consensus heptamer-nonamer sequences in Ig/TcR genes (Figure 1A) (10,12,14,45,60,68,75,76). In most t(1;14) the breakpoints cluster at two RSS around *tal*-1 exon 2a and 3 (Figures 1A and 3) (10,11,60,62). However, five types of *tal*-1 deletions are described, which all use the same 5′ RSS consisting of a sole heptamer located in the first *sil* intron, but different 3′ RSS consisting of heptamer-nonamer sequences located in the 5′ region of the *tal*-1 locus (Figures 1A and 3) (12,14,45,66,68,70). The *tal*-1 deletions type 1 and type 2 occur most frequently, whereas the three other types thus far have each been observed only once (12,14,45,68,70).

Both t(1;14) and *tal*-1 deletions appear to result in 'ectopic' (over)expression of intact TAL-1 proteins, i.e. expression in T-cell differentiation stages where TAL-1 proteins are normally not observed (12,45,64-66,68). Additionally, we and others were unable to detect any *tal*-1 deletions in normal tissues, including cortical thymocytes, which represent the normal counterparts of T-ALL cells (67,73). These findings support the assumption that *tal*-1 deletions are involved in the oncogenesis of the affected T-ALL. However, we observed a few T-ALL with a *tal*-1 deletion, but without detectable *tal*-1 gene transcription, suggesting that (over)expression of TAL-1 protein might not be necessary for preserving the malignant capacity of T-ALL cells. However, the precise role of TAL-1 proteins in the oncogenic transformation of differentiating cortical thymocytes into T-ALL is still unknown (77).

tal-1 deletions correlate with T-cell differentiation stages

tal-1 deletions appear to be restricted to malignancies of the T-cell lineage, because they have not been observed in other hematopoietic malignancies (71,73,78). However, they occur in different frequencies in various T-ALL subgroups. We and others showed that *tal*-1 deletions are correlated with CD3 phenotype, because no *tal*-1 deletions were found in TcR-$\gamma\delta^+$ T-ALL (n = 29), whereas 11% of the $CD3^-$ T-ALL (n = 73) and 31% (n = 42) of the TcR-$\alpha\beta^+$ T-ALL contained such a deletion (13,14).

The normal counterparts of T-ALL (i.e. the cortical thymocytes) differentiate from $CD3^-$ stages to $CD3^+$ stages with either TcR-$\gamma\delta$ or TcR-$\alpha\beta$ expression. The TcR-α/δ gene complex plays a pivotal role in the $\gamma\delta$ versus $\alpha\beta$ lineage commitment of thymocytes (79-81). TcR gene rearrangement starts with rearrangement of the TcR-δ alleles, followed by TcR-γ gene rearrangement (86). If no TcR-$\gamma\delta$ expression occurs due to nonfunctional rearrangements of TcR-δ and/or TcR-γ genes, the rearranged TcR-δ genes will be deleted via a special deletion mechanism involving the so-called δREC and ψJα TcR-δ deleting elements (81-84). This TcR-δ gene deletion prepares the allele for

subsequent rearrangement of the TcR-α gene segments, thereby forcing the T-cell to differentiate into the αβ lineage.

Based on these consecutive recombination processes, we used the TcR-δ configuration to subdivide the T-ALL into several subgroups (14,73,85). This revealed that *tal*-1 deletions exclusively occurred in $CD3^-$ or $CD3^+$ T-ALL of the αβ lineage with a frequency of 20% in T-ALL with one deleted TcR-δ allele, and 36% in T-ALL with TcR-δ gene deletions on both alleles (14). Because *tal*-1 deletions were almost exclusively observed in T-ALL with at least one deleted TcR-δ allele, combined with the fact that biallelic TcR-δ gene deletions coincide with a doubling of the *tal*-1 deletion frequency, we initially proposed two possible mechanisms for this phenomenon:

- Differential accessibility of the *tal*-1 deletion breakpoint regions for recombination depends on differentiation-stage-related transcription and/or (de)methylation;
- The existence of a specific TcR-δ gene deletion recombinase complex (containing at least one unique component) with the capacity not only to delete TcR-δ genes by rearranging the δREC and ψJα gene segments, but also to possibly cause *tal*-1 deletions.

Effect of transcription and (de)methylation on *tal*-1 deletions

Because rearrangement of Ig and TcR genes is preceded or accompanied by germline transcription of parts of these genes, it has been proposed that transcription is essential to 'open up' the DNA for V(D)J recombination (86,87). To investigate this principle for *tal*-1 deletions, we analyzed the expression of the involved genes. The *sil* gene is ubiquitously expressed, and therefore we detected *sil* mRNA in all tested T-ALL, T-cell lines, thymic cell samples, and PBMNC cell samples. The normal expression profile of the TAL-1 protein shows predominant transcription in early hematopoietic cells (44,47,48). In postnatal thymic cell samples, *tal*-1 gene expression can not be detected (43,48), but we observed a relatively abundant expression of *tal*-1 mRNA in a fetal thymic cell sample of 17 weeks. It might be that *tal*-1 gene expression is only present during early phases of thymic differentiation, and that such cells are limited in postnatal thymus, but abundant in fetal thymus. However, only a few T-ALL and T-cell lines contained *tal*-1 mRNA. Almost all T-ALL samples with *tal*-1 gene transcripts contained an aberrant *tal*-1 gene, except for two T-cell lines with apparently normal *tal*-1 genes. On the other hand, as mentioned before, not all T-ALL with an affected *tal*-1 gene contained transcripts. These data indicate that expression of the *sil* gene cannot cause the αβ lineage restriction of *tal*-1 deletions, and suggest that the observed *tal*-1 gene expression most likely is the *result* and not the *cause* of the aberration.

Besides transcription of the involved genes, demethylation of the rearranging gene segments has been recently recognized as an even more important factor in the regulation of V(D)J recombination processes (88-98). We therefore studied the DNA (de)methylation (i.e. DNA accessibility) of the *tal*-1 deletion breakpoints to find an explanation for the differences in the *tal*-1 deletion frequencies between the various T-ALL subgroups (99). The deletion breakpoint in the *sil* gene (sildb) was demethylated in all cell samples tested. This was in contrast to the deletion breakpoint of *tal*-1 deletion type 1 (taldb1), which showed three demethylation configurations (complete, partial, and absent). As for now, it appears that in relatively immature T-ALL with still

unrearranged TcR-δ genes, all stages of taldb1 methylation can occur, whereas TcR-δ gene rearrangement is correlated with methylation in the *tal*-1 gene (which might be caused by the mechanism of so-called protective methylation (100)), and TcR-δ gene deletion is correlated with *tal*-1 gene demethylation (99).

Because the frequencies of demethylated taldb1 in the various T-ALL subgroups parallel the observed frequencies of *tal*-1 deletions, they neutralize the initially observed difference in *tal*-1 deletion frequency between $CD3^-$ and TcR-$\alpha\beta^+$ T-ALL. It can be concluded that differential taldb1 demethylation causes the difference in *tal*-1 deletion frequency in these CD3 subgroups. The same situation occurred in the T-ALL subgroups defined by the TcR-δ configuration, and we conclude that the initial doubling of *tal*-1 deletion frequency in T-ALL with both TcR-δ alleles deleted versus T-ALL with one TcR-δ allele deleted is caused by differential demethylation of taldb1 (99). However, between the completely demethylated subgroups with TcR-δ gene rearrangement and TcR-δ gene deletion, still a noticeable difference in frequency of *tal*-1 deletions remains (0% versus 39%). This finding is indirect evidence for the existence of a special recombinase complex for TcR-δ gene deletion, which is exclusively present in immature thymocytes committed to the αβ lineage (99). Whether this specific recombinase complex seizes at the TcR-δ gene deleting elements (δREC and ψJα) or represents just a specific Vα-Jα recombinase complex has to be elucidated.

tal-1 deletion versus t(1;14) in TcR-$\alpha\beta^+$ T-ALL

Despite several homologies between *tal*-1 deletions and t(1;14), the translocations do not seem to be associated directly with TcR-δ gene deletions. It appears that t(1;14) occur at an earlier stage in T-cell differentiation than *tal*-1 deletions, because all reported translocations involved either Dδ or Jδ gene segments, implying that they occurred during the stage of rearrangements in the TcR-δ gene (10,11,46,60-62). However, most T-ALL with t(1;14) express TcR-αβ (13). This suggests that the oncogenic event occurred in an earlier stage of T-cell differentiation (during TcR-δ gene rearrangement) than the differentiation stage expressed by the T-ALL (during TcR-δ gene deletion). Because this mechanism of ongoing maturation in T-ALL with t(1;14) might also occur in T-ALL with a *tal*-1 deletion, we propose two additional mechanisms that could play a role in the restriction of *tal*-1 deletions to T-ALL of the αβ lineage:

- 'Ectopic' (over)expression of the TAL-1 protein (due to *tal*-1 gene aberrations) forces the leukemic T-cells into the αβ lineage;
- Because the TAL-1 protein is only functional as a heterodimer, it might be that the TAL-1 partner (necessary for oncogenesis) is exclusively present in T-cells of the αβ lineage.

There is some support for these last hypothesized mechanisms. Although it has been shown that TAL-1 proteins in T-cell lines with or without a *tal*-1 deletion dimerize with the ubiquitously-expressed E2A proteins (36), inhibition experiments with anti-E2A monoclonal antibodies, which prevent dimerization of the E2A proteins, revealed that a minor part of the TAL-1 proteins is able to dimerize to other, yet unidentified proteins (36). This might imply that in addition to the ubiquitously expressed E2A protein, an αβ-lineage specific/inducing TAL-1 partner exists. If such a heterodimer is oncogenic, than this might explain the αβ-lineage restriction of T-ALL with *tal*-1 gene aberrations.

Deletion in *hprt* gene mimics *tal*-1 deletion

In normal T-cells, an intriguing deleting phenomenon has been discovered involving the housekeeping gene *hprt* on chromosome Xq26, that seems extremely homologous to the *tal*-1 deletions. In the *hprt* gene, three types of site-specific deletions can occur, which all damage coding exons of the gene (101,102). These three types of *hprt* deletions use a 5′ RSS in intron 1 consisting of a sole heptamer, but different 3′ RSS, which are located in intron 3 and consist of heptamer-nonamer sequences (Figure 1A) (101). The fusion regions of these *hprt* deletions also show N-regions, P-region nucleotide insertion and deletion of nucleotides and are therefore homologous to the Ig/TcR junctional regions and *tal*-1 breakpoint fusion regions (101,102). The *hprt* deletions occur at a low frequency of $\sim 10^{-7}$ in T-lymphocytes and are not oncogenic (103,104). These data suggest that oncogenic *tal*-1 deletions and non-oncogenic *hprt* deletions may be caused by a similar deletionally rearrangement process. Thus, this aberration in the *hprt* gene might be a useful read-out system in the search of a recombinase complex that is involved in TcR-δ deletion/*tal*-1 deletion.

Recently we identified a cell line in which *hprt* deletions occurred *in vitro*, which may indicate that this read-out system is valid. Furthermore, recently a *hprt* gene was found that was disrupted by a V-J exon of the TcR-α gene (105). Because the insertion was in the same region as the 5′ RSS of the *hprt* deletions, this aberration represents a direct link between TcR rearrangement and *hprt* deletion (105).

Conclusion

Recently, a lot of interesting data were published concerning the function of the TAL-1 protein (55,56,106-114). In normal cells this bHLH protein may, depending on the bHLH protein balance, repress or activate transcription (55). To date, still no decisive information is available about the role of the TAL-1 protein in the oncogenesis of differentiating thymocytes to T-ALL (77).

But, based on the data described above, we can conclude that the aberrations of the *tal*-1 gene in T-ALL (i.e. *tal*-1 deletions and t(1;14)) exhibit several intriguing differences and similarities, which may be related to the malignant transformation processes. On the one hand, the breakpoints in *tal*-1 deletions and t(1;14) occur at different sites in the *tal*-1 gene via different RSS and the two types of genetic aberrations might be caused by different recombinase complexes at different stages of T-cell differentiation. On the other hand, both aberrations result in 'ectopic' (over)expression of intact TAL-1 proteins and both are present in T-ALL of the TcR-αβ differentiation lineage (13).

However, further studies have to elucidate which of the proposed mechanisms cause the αβ lineage restriction of the *tal*-1 gene aberrations. Those studies on the TAL-1 protein will also expose its role in the oncogenesis of T-ALL, and may reveal its function in early T-cell differentiation.

REFERENCES

1. Finger LR, Harvey RC, Moore RCA, Showe LC, Croce CM. A common mechanism of chromosomal translocation in T- and B-cell neoplasia. Science 1986;234:982-985.

2. Boehm T, Rabbitts TH. The human T cell receptor genes are targets for chromosomal abnormalities in T cell tumors. Faseb J 1989;3:2344-2359.
3. Tycko B, Sklar J. Chromosomal translocations in lymphoid neoplasia: a reappraisal of the recombinase model. Cancer Cells 1990;2:1-8.
4. Rabbitts TH. Translocations, master genes, and differences between the origins of acute and chronic leukemias. Cell 1991;67:641-644.
5. Nichols J, Nimer SD. Transcription factors, translocations, and leukemia. Blood 1992;80:2953-2963.
6. Yunis JJ, Tanzer J. Molecular mechanisms of hematologic malignancies. Crit Rev Oncogen 1993;4:161-190.
7. Schatz DG, Oettinger MA, Schlissel MS. V(D)J recombination: molecular biology and regulation. Annu Rev Immunol 1992;10:359-383.
8. Hunger SP, Cleary ML. Chimaeric oncoproteins resulting from chromosomal translocations in acute lymphoblastic leukaemia. Sem Cancer Biol 1993;4:387-399.
9. Murre C, Schonleber McCaw P, Baltimore D. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, *MyoD*, and *myc* proteins. Cell 1989;56:777-783.
10. Bernard O, Guglielmi P, Jonveaux P, Cherif D, Gisselbrecht S, Mauchauffe M, Berger R, Larsen C-J, Mathieu-Mahul D. Two distinct mechanisms for the SCL gene activation in the t(1;14) translocation of T-cell leukemias. Genes Chrom Cancer 1990;1:194-208.
11. Chen Q, Cheng J-T, Tsai L-H, Schneider N, Buchanan G, Carroll A, Crist W, Ozanne B, Siciliano MJ, Baer R. The *tal* gene undergoes chromosome translocation in T cell leukemia and potentially encodes a helix-loop-helix protein. EMBO J 1990;9:415-424.
12. Brown L, Cheng J-T, Chen Q, Siciliano MJ, Crist W, Buchanan G, Baer R. Site-specific recombination of the *tal*-1 gene is a common occurrence in human T cell leukemia. EMBO J 1990;9:3343-3351.
13. Macintyre EA, Smit L, Ritz J, Kirsch IR, Strominger JL. Disruption of the SCL locus in T-lymphoid malignancies correlates with commitment to the T-cell receptor $\alpha\beta$ lineage. Blood 1992;80:1511-1520.
14. Breit TM, Mol EJ, Wolvers-Tettero ILM, Ludwig W-D, Van Wering ER, Van Dongen JJM. Site-specific deletions involving the *tal*-1 and *sil* genes are restricted to cells of the T-cell receptor $\alpha\beta$ lineage: T-cell receptor δ gene deletion mechanism affects multiple genes. J Exp Med 1993;177:965-977.
15. Stern M-H, Lipkowitz S, Aurias A, Griscelli C, Thomas G, Kirsch IR. Inversion of chromosome 7 in ataxia telangiectasia is generated by a rearrangement between T-cell receptor β and T-cell receptor γ genes. Blood 1989;74:2076-2080.
16. Rabbitts TH, Boehm T. Structural and functional chimerism results from chromosomal translocation in lymphoid tumors. Adv Immunol 1991;50:119-146.
17. Blackwell TK, Alt FW. Molecular characterization of the lymphoid V(D)J recombination activity. J Biol Chem 1989;264:10327-10330.
18. Kasai M, Maziarz RT, Aoki K, Macintyre E, Strominger JL. Molecular involvement of the *pvt-1* locus in a γ/δ T-cell leukemia bearing a variant t(8;14)(q24;q11) translocation. Mol Cell Biol 1992;12:4751-4757.
19. Kasai M, Aoki K, Matsuo Y, Minowada J, Maziarz RT, Strominger JL. Recombination hotspot associated factors specifically recognize novel target sequences at the site of interchromosomal rearrangements in T-ALL patients with t(8;14)(q24;q11) and t(1;14)(p32;q11). Int Immunol 1994;6:1017-1025.
20. Murre C, Schonleber McCaw P, Vaessin H, Caudy M, Jan LY, Jan YN, Cabrera CV, Buskin JN, Hauschka SD, Lassar AB, Weintraub H, Baltimore D. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 1989;58:537-544.
21. Kadesch T. Helix-loop-helix proteins in the regulation of immunoglobulin gene transcription. Immunol Today 1992;13:31-36.
22. Visvader J, Begley CG. Helix-loop-helix genes translocated in lymphoid leukemia. Trends Biol Sciences 1991;16:330-333.
23. Ferré-D'Amaré AR, Prendergast GC, Ziff EB, Burley SK. Recognition by Max of its cognate DNA through a dimeric b/HLH/Z domain. Nature 1993;363:38-45.
24. Kadesch T. Consequences of heteromeric interactions among helix-loop-helix proteins. Cell Growth Diff 1993;4:49-55.
25. Lasser AB, Buskin JN, Lockshon D, Davis RL, Apone S, Hauschka SD, Weintraub H. MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. Cell 1989;58:823-831.
26. Davis RL, Cheng PF, Lasser AB, Weintraub H. The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. Cell 1990;60:733-746.
27. Voronova A, Baltimore D. Mutations that disrupt DNA binding and dimer formation in the E47 helix-loop-helix protein map to distinct domains. Proc Natl Acad Sci USA 1990;87:4722-4726.
28. Lassar AB, David RL, Wright WE, Kadesch T, Murre C, Voronova A, Baltimore D, Weintraub H. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. Cell 1991;66:305-315.
29. Neuhold LA, Wold B. HLH forced dimers: tethering MyoD to E47 generates a dominant positive myogenic factor insulated from negative regulation by Id. Cell 1993;74:1033-1042.
30. Lenardo M, Pierce JW, Baltimore D. Protein-binding sites in Ig gene enhancers determine transcriptional activity and inducibility. Science 1987;236:1573-1577.
31. Kiledjian M, Su LK, Kadesch T. Identification and characterization of two functional domains within the murine heavy-chain enhancer. Mol Cell Biol 1988;8:145-149.
32. Blackwell TK, Kretzner L, Blackwood EM, Eisenman RN, Weintraub H. Sequence-specific DNA binding by the c-Myc protein. Science 1990;250:1149-1151.
33. Halazonetis TD, Kandil AN. Determination of the c-MYC DNA-binding site. Proc Natl Acad Sci USA 1991;88:6162-6166.
34. Murre C, Voronova A, Baltimore D. B-cell- and monocyte-specific E2-box-binding factors contain E12/E47-like subunits. Mol Cell Biol 1991;11:1156-1160.

35. Dang CV, Dolde C, Gillison ML, Jato GJ. Discrimination between related DNA sites by a single amino acid residue of Myc-related basic-helix-loop-helix proteins. Proc Natl Acad Sci USA 1992;89:599-602.
36. Hsu H-L, Huang L, Tsan JT, Funk W, Wright WE, Hu J-S, Kingston RE, Baer R. Preferred sequences for DNA recognition by the TAL1 helix-loop-helix proteins. Mol Cell Biol 1994;14:1256-1265.
37. Buskin JN, Hauschka SD. Identification of a myocyte nuclear factor which binds to the muscle-specific enhancer of the mouse muscle creatine kinase gene. Mol Cell Biol 1989;9:2627-2640.
38. Brennan TJ, Olson EN. Myogenin resides in the nucleus and acquires high affinity for a conserved enhancer element on heterodimerization. Genes Dev 1990;4:582-595.
39. Whelan J, Cordle SR, Henderson E, Weil PA, Stein R. Identification of a pancreatic β-cell insulin gene transcription factor that binds to and appears to activate cell-type-specific gene expression: its possible relationship to other cellular factors that bind to a common insulin gene sequence. Mol Cell Biol 1990;10:1564-1572.
40. Quong MW, Massari ME, Zwart R, Murre C. A new transcriptional-activation motif restricted to a class of helix-loop-helix proteins is functionally conserved in both yeast and mammalian cells. Mol Cell Biol 1993;13:792-800.
41. Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell 1990;61:49-59.
42. Wilson RB, Kiledjian M, Shen C-P, Benezra R, Zwollo P, Dymecki SM, Desiderio SV, Kadesch T. Repression of immunoglobulin enhancers by the helix-loop-helix protein Id: implications for B-lymphoid-cell development. Mol Cell Biol 1991;11:6185-6191.
43. Begley CG, Aplan PD, Denning SM, Haynes BF, Waldmann TA, Kirsch IR. The gene SCL is expressed during early hematopoiesis and encodes a differentiation-related DNA-binding motif. Proc Natl Acad Sci USA 1989;86:10128-10132.
44. Aplan PD, Begley CG, Bertness V, Nussmeier M, Ezquerra A, Coligan J, Kirsch IR. The SCL gene is formed from a transcriptionally complex locus. Mol Cell Biol 1990;10:6426-6435.
45. Bernard O, Lecointe N, Jonveaux P, Souyri M, Mauchauffé M, Berger R, Larsen CJ, Mathieu-Mahul D. Two site-specific deletions and t(1;14) translocation restricted to human T-cell acute leukemias disrupt the 5′ part of the *tal*-1 gene. Oncogene 1991;6:1477-1488.
46. Bernard O, Azogui O, Lecointe N, Mugneret F, Berger R, Larsen CJ, Mathieu-Mahul D. A third *tal*-1 promoter is specifically used in human T cell leukemias. J Exp Med 1992;176:919-925.
47. Visvader J, Begley CG, Adams JM. Differential expression of the *LYL*, *SCL* and *E2A* helix-loop-helix genes within the hemopoietic system. Oncogene 1991;6:187-194.
48. Green AR, Salvaris E, Begley CG. Erythroid expression of the 'helix-loop-helix' gene, SCL. Oncogene 1991;6:475-479.
49. Tanigawa T, Elwood NG, Metcalf D, Cary D, DeLuca E, Nicola NA, Begley CG. The SCL gene product is regulated by and differentially regulates cytokine responses during myeloid leukemic cell differentiation. Proc Natl Acad Sci USA 1993;90:7864-7868.
50. Kamps MP, Murre C, Sun X, Baltimore D. A new homeobox gene contributes the DNA binding domain of the t(1;19) translocation protein in pre-B ALL. Cell 1990;60:547-555.
51. Baer R. TAL1, TAL2 and LYL1: a family of basic helix-loop-helix proteins implicated in T cell acute leukemia. Sem Cancer Biol 1993;4:341-347.
52. Hsu H-L, Cheng J-T, Chen Q, Baer R. Enhancer-binding activity of the *tal*-1 oncoprotein in association with the E47/E12 helix-loop-helix proteins. Mol Cell Biol 1991;11:3037-3042.
53. Hsu H-L, Wadman I, Baer R. Formation of *in vivo* complexes between the TAL1 and E2A polypeptides of leukemic T cells. Proc Natl Acad Sci USA 1994;91:3181-3185.
54. Voronova AF, Lee F. The E2A and tal-1 helix-loop-helix proteins associate *in vivo* and are modulated by Id proteins during interleukin 6-induced myeloid differentiation. Proc Natl Acad Sci USA 1994;91:5952-5956.
55. Hsu H-L, Wadman I, Tsan JT, Baer R. Positive and negative transcriptional control by the TAL1 helix-loop-helix protein. Proc Natl Acad Sci USA 1994;91:5947-5951.
56. Doyle K, Zhang Y, Baer R, Bina M. Distinguishable patterns of protein-DNA interactions involving complexes of basic helix-loop-helix proteins. J Biol Chem 1994;16:12099-12105.
57. Begley CG, Aplan PD, Davey MP, Nakahara K, Tchorz K, Kurtzberg J, Hershfield MS, Haynes BF, Cohen DI, Waldmann TA, Kirsch IR. Chromosomal translocation in a human leukemic stem-cell line disrupts the T-cell antigen receptor δ-chain diversity region and results in a previously unreported fusion transcript. Proc Natl Acad Sci USA 1989;86:2031-2035.
58. Finger LR, Kagan J, Christopher G, Kurtzberg J, Hershfield MS, Nowell PC, Croce CM. Involvement of the *TCL5* gene on human chromosome 1 in T-cell leukemia and melanoma. Proc Natl Acad Sci USA 1989;86:5039-5043.
59. Carroll AJ, Crist WM, Link MP, Amylon MD, Pullen DJ, Ragab AH, Buchanan GR, Wimmer RS, Vietti TJ. The t(1;14)(p34;q11) is nonrandom and restricted to T-cell acute lymphoblastic leukemia: a pediatric oncology group study. Blood 1990;76:1220-1224.
60. Chen Q, Yang Y-C, Tsan JT, Xia Y, Ragab AH, Peiper SC, Carroll A, Baer R. Coding sequences of the *tal*-1 gene are disrupted by chromosome translocation in human T cell leukemia. J Exp Med 1990;172:1403-1408.
61. Xia Y, Brown L, Tsan JT, Yang CY-C, Siciliano MJ, Crist WM, Caroll AJ, Baer RJ. The translocation (1;14)(p34;q11) in human T-cell leukemia: chromosomal breakage 25 kilobase pairs downstream of the *TAL1* protooncogene. Genes Chrom Cancer 1992;4:211-216.
62. Bernard O, Barin C, Charrin C, Mathieu-Mahul D, Berger R. Characterization of translocation t(1;14)(p32;q11) in a T and in a B acute leukemia. Leukemia 1993;7:1509-1513.
63. Bernard O, Groettrup M, Mugneret F, Berger R, Azogui O. Molecular analysis of T-cell receptor transcripts in a human T-cell leukemia bearing a t(1;14) and an inv(7); cell surface expression of a TCR-β chain in the absence of α chain. Leukemia 1993;7:1645-1653.
64. Fitzgerald TJ, Neale GAM, Raimondi SC, Goorha RM. *c-tal*, a helix-loop-helix protein, is juxtaposed to the T-cell receptor-β chain gene by a reciprocal chromosomal translocation: t(1;7) (p32;q35). Blood 1991;78:2686-2695.
65. Aplan PD, Raimondi SC, Krisch IR. Disruption of the SCL gene by a t(1;3) translocation in a patient with T cell acute lymphoblastic leukemia. J Exp Med 1992;176:1303-1310.

66. Aplan PD, Lombardi DP, Ginsberg AM, Cossman J, Bertness VL, Kirsch IR. Disruption of the human SCL locus by 'illegitimate' V-(D)-J recombinase activity. Science 1990;250:1426-1429.
67. Jonsson OG, Kitchens RL, Baer RJ, Buchanan GR, Smith RG. Rearrangements of the *tal*-1 locus as clonal markers for T cell acute lymphoblastic leukemia. J Clin Invest 1991;87:2029-2035.
68. Aplan PD, Lombardi DP, Reaman GH, Sather HN, Hammond GD, Kirsch IR. Involvement of the putative hematopoietic transcription factor *SCL* in T-cell acute lymphoblastic leukemia. Blood 1992;79:1327-1333.
69. Bhatia K, Sprangler G, Advani S, Kamel A, Hamdy N, Iyer RS, Aplan P, Magrath IT. Molecular characterization of SCL rearrangements in T-cell ALL from India and Egypt. Int J Oncol 1993:2;725-730.
70. Bash RO, Crist WM, Shuster JJ, Link MP, Amylon M, Pullen J, Carroll AJ, Buchanan GR, Smith RG, Baer R. Clinical features and outcome of T-cell acute lymphoblastic leukemia in childhood with respect to alterations at the *TAL1* locus: a pediatric oncology group study. Blood 1993;81:2110-2117.
71. Kikuchi A, Hayashi Y, Kobayashi S, Hanada R, Moriwaki K, Yamamoto K, Fujimoto J, Kaneko Y, Yamamori S. Clinical significance of TAL1 gene alteration in childhood T-cell acute lymphoblastic leukemia and lymphoma. Leukemia 1993;7:933-938.
72. Janssen JWG, Ludwig W-D, Sterry W, Bartram CR. SIL-TAL1 deletion in T-cell acute lymphoblastic leukemia. Leukemia 1993;7:1204-1210.
73. Breit TM, Beishuizen A, Ludwig W-D, Mol EJ, Adriaansen HJ, Van Wering ER, Van Dongen JJM. *tal*-1 deletions in T-cell acute lymphoblastic leukemia as PCR target for detection of minimal residual disease. Leukemia 1993;7:2004-2011.
74. Aplan PD, Lombardi DP, Kirsch IR. Structural characterization of *SIL*, a gene frequently disrupted in T-cell acute lymphoblastic leukemia. Mol Cell Biol 1991;11:5462-5469.
75. Akira A, Okazaki K, Sakano H. Two pairs of recombination signals are sufficient to cause immunoglobulin V-(D)-J joining. Science 1987;238:1134-1138.
76. Hesse JE, Lieber MR, Mizuuchi K, Gellert M. V(D)J recombination: a functional definition of the joining signals. Genes Dev 1989;3:1053-1061.
77. Goldfarb AN, Greenberg JM. T-cell acute lymphoblastic leukemia and the associated basic helix-loop-helix gene *SCL/tal*. Leuk Lymph 1994;12:157-166.
78. Rockman S, Begley CG, Kannourakis G, Mann GJ, Dobrovic AN, Kefford RF, McGrath K. SCL gene in human tumors. Leukemia 1992;6:623-625.
79. Isobe M, Russo G, Haluska FG, Croce CM. Cloning of the gene encoding the δ subunit of the human T-cell receptor reveals its physical organization within the α-subunit locus and its involvement in chromosome translocations in T-cell malignancy. Proc Natl Acad Sci USA 1988;85:3933-3937.
80. Satyanarayana K, Hata S, Devlin P, Roncarolo MG, De Vries JE, Spits H, Strominger JL, Krangel MS. Genomic organization of the human T-cell antigen-receptor *α/δ* locus. Proc Natl Acad Sci USA 1988;85:8166-8170.
81. Van Dongen JJM, Comans-Bitter WM, Wolvers-Tettero ILM, Borst J. Development of human T lymphocytes and their thymus-dependency. Thymus 1990;16:207-234.
82. De Villartay J-P, Hockett RD, Coran D, Korsmeyer SJ, Cohen DI. Deletion of the human T-cell receptor δ-gene by a site-specific recombination. Nature 1988;335:170-174.
83. Hockett RD, De Villartay J-P, Pollock K, Poplack DG, Cohen DI, Korsmeyer SJ. Human T-cell antigen receptor (TCR) δ-chain locus and elements responsible for its deletion are within the TCR α-chain locus. Proc Natl Acad Sci USA 1988;85:9694-9698.
84. Breit TM, Wolvers-Tettero ILM, Bogers AJJC, De Krijger RR, Wladimiroff JW, Van Dongen JJM. Rearrangements of the human *TCRD*-deleting elements. Immunogenetics 1994;40:70-75.
85. Breit TM, Wolvers-Tettero ILM, Beishuizen A, Verhoeven M-AJ, Van Wering ER, Van Dongen JJM. Southern blot patterns, frequencies and junctional diversity of T-cell receptor δ gene rearrangements in acute lymphoblastic leukemia. Blood 1993;82:3036-3074.
86. Martin D, Huang R, LeBien T, Van Ness B. Induced rearrangement of κ genes in the BLIN-1 human pre-B cell line correlates with germline J-Cκ and Vκ transcription. J Exp Med 1991;173:639-645.
87. Schlissel MS, Corcoran LM, Baltimore D. Virus-transformed pre-B cells show ordered activation but not inactivation of immunoglobulin gene rearrangement and transcription. J Exp Med 1991;173:711-720.
88. Engler P, Haasch D, Pinkert CA, Doglio L, Glymour M, Brinster R, Storb U. A strain-specific modifier on mouse chromosome 4 controls the methylation of independent transgene loci. Cell 1991;65:939-947.
89. Engler P, Roth P, Kim JY, Storb U. Factors affecting the rearrangement efficiency of an Ig test gene. J Immunol 1991;146:2826-2835.
90. Tauchi T, Ohyashiki JH, Ohyashiki K, Saito M, Nakazawa S, Kimura N, Toyama K. Methylation status of T-cell receptor β-chain gene in B precursor acute lymphoblastic leukemia: correlation with hypomethylation and gene rearrangement. Cancer Res 1991;51:2917-2921.
91. Hsieh C-L, Lieber MR. CpG methylated minichromosomes become inaccessible for V(D)J recombination after undergoing replication. EMBO J 1992;11:315-325.
92. Hsieh C-L, McCloskey R.P, Lieber MR. V(D)J recombination on minichromosomes is not affected by transcription. J Biol Chem 1992;267:15613-15619.
93. Litz CE, McClure JS, Coad JE, Goldfarb AN, Brunning RD. Methylation status of the major breakpoint cluster region in Philadelphia chromosome negative leukemias. Leukemia 1992;6:35-41.
94. Tsukamoto N, Morita K, Karasawa M, Omine M. Methylation status of c-myc oncogene in leukemic cells: hypomethylaton in acute leukemia derived from myelodysplastic syndromes. Exp Hematol 1992;20:1061-1064.
95. Bird A. The essentials of DNA methylation. Cell 1992;70:5-8.
96. Ohyashiki JH, Ohyashiki K, Kawakubo K, Tauchi T, Nakazawa S, Kimura N, Toyama K. T-cell receptor β chain gene rearrangement in acute myeloid leukemia always occurs at the allele that contains the undermethylated Jβ1 region. Cancer Res 1992;52:6598-6602.
97. Ohyashiki JH, Ohyashiki K, Kawakubo K, Tauchi T, Shimamoto T, Toyama K. The methylation status of the major breakpoint cluster region in human leukemia cells, including Philadelphia chromosome-positive cells, is linked to the lineage of hematopoietic cells. Leukemia 1993;7:801-807.

98. Engler P, Weng A, Storb U. Influence of CpG methylation and target spacing on V(D)J recombination in a transgenic substrate. Mol Cell Biol 1993;13:571-577.
99. Breit TM, Wolvers-Tettero ILM, Van Dongen JJM. Lineage specific demethylation of *tal*-1 gene breakpoint region determines the frequency of *tal*-1 deletions in $\alpha\beta$ lineage T-cells. Oncogene 1994;9:1847-1853.
100. Burger C, Radbruch A. Protective methylation of immunoglobulin and T cell receptor (TcR) gene loci prior to induction of class switch and TcR recombination. Eur J Immunol 1990;20:2285-2291.
101. Fuscoe JC, Zimmerman LJ, Lippert MJ, Nicklas JA, O'Neill JP, Albertini RJ. V(D)J recombinase-like activity mediates *hprt* gene deletion in human fetal T-lymphocytes. Cancer Res 1991;51:6001-6005.
102. Fuscoe JC, Zimmerman LJ, Harrington-Brock K, Burnette L, Moore MM, Nicklas JA, O'Neill JP, Albertini RJ. V(D)J recombinase-mediated deletion of the *hprt* gene in T-lymphocytes from adult humans. Mutat Res 1992;283:13-20.
103. McGinniss MJ, Nicklas JA, Albertini RJ. Molecular analysis of in vivo *hprt* mutations in human T-lymphocytes: IV. studies in newborns. Environ Mol Mutagen 1989;14:229-237.
104. McGinniss MJ, Falta MT, Sullivan LM, Albertini RJ. In vivo *hprt* mutant frequencies in T-cells of normal human newborns. Mutat Res 1990;240:117-126.
105. Hou S-M. Novel types of mutation identified at the hprt locus of human T-lymphocytes. Mutat Res 1994;308:23-31.
106. Green AR, Lints T, Visvader J, Harvey R, Begley CG. *SCL* is coexpressed with *GATA-1* in hemopoietic cells but is also expressed in developing brain. Oncogene 1992;7:653-660.
107. Aplan PD, Nakahara K, Orkin SH, Kirsch IR. The *SCL* gene product: a positive regulator of erythroid differentiation. EMBO J 1992;11:4073-4081.
108. Cheng J-T, Cobb MH, Baer R. Phosphorylation of the TAL1 oncoprotein by the extracellular-signal-regulated protein kinase ERK1. Mol Cell Biol 1993;13:801-808.
109. Cheng J-T, Hsu H-L, Hwang L-Y, Baer R. Products of the *TAL1* oncogene: basic helix-loop-helix proteins phosphorylated at serine residues. Oncogene 1993;8:677-683.
110. Hwang L-Y, Siegelman M, Davis L, Oppenheimer-Marks N, Baer R. Expression of the *TAL1* proto-oncogene in cultured endothelial cells and blood vessels of the spleen. Oncogene 1993;8:3043-3046.
111. Elwood NJ, Cook WD, Metcalf D, Begley CG. SCL, the gene implicated in human T-cell leukaemia, is oncogenic in a murine T-lymphocyte cell line. Oncogene 1993;8:3093-3101.
112. Chiba T, Nagata Y, Kishi A, Sakamaki K, Miyajima A, Yamamoto M, Engel JD, Todokoro K. Induction of erythroid-specific gene expression in lymphoid cells. Proc Natl Acad Sci USA 1993;90:11593-11597.
113. Elwood NJ, Green AR, Melder A, Begley CG, Nicola N. The SCL protein displays cell-specific heterogeneity in size. Leukemia 1994;8:106-114.
114. Mouthon M-A, Bernard O, Mitjavila M-T, Romeo P-H, Vainchenker W, Mathieu-Mahul D. Expression of *tal*-1 and GATA-binding proteins during human hematopoiesis. Blood 1993;3:647-655.
115. Mellentin JD, Smith SD, Cleary ML. *Lyl*-1, a novel gene altered by chromosomal translocation in T cell leukemia, codes for a protein with a helix-loop-helix DNA binding motif. Cell 1989;58:77-83.
116. Green AR, Begley CG. SCL and related hemopoietic helix-loop-helix transcription factors. Int J Cell Cloning 1992;10:269-276.
117. Xia Y, Brown L, Yang CY-C, Tsan JT, Siciliano MJ, Espinosa III R, Le Beau MM, Baer RJ. *TAL2*, a helix-loop-helix gene activated by the (7;9)(q34;q32) translocation in human T-cell leukemia. Proc Natl Acad Sci USA 1991;88:11416-11420.
118. Spences CA, Groudine M. Control of *c-myc* regulation in normal and neoplastic cells. Adv Cancer Res 1991;56:1-48.
119. Kasai M, Maziarz RT, Aoki K, Macintyre E, Strominger JL. Molecular involvement of *pvt-1* locus in a γ/δ T-cell leukemia bearing a variant t(8;14)(q24;q11) translocation. Mol Cell Biol 1992;12:4751-4757.
120. Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD, Sklar J. *TAN-1*, the human homolog of the Drosophila *Notch* gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell 1991;66:649-661.
121. Murre C, Bain G, Van Dijk MA, Engel I, Furnari BA, Massari ME, Matthews JR, Quong MW, Rivera RR, Stuiver MH. Structure and function of helix-loop-helix proteins. Biochim Biophys Acta 1994;in press.
122. Bird AP. CpG-rich islands and the function of DNA methylation. Nature 1986;321:209-212.
123. Antequera F, Boyes J, Bird A. High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. Cell 1990;62:503-514.
124. Séité P, Hillion J, Leroux D, Berger R, Larsen C-J. Common sequence in chromosome translocations affecting B- and T-cell malignancies: a novel recombination site? Genes Chrom Cancer 1993;6:253-254.
125. Meerabux JM, Cotter FE, Kearney L, Nizetic D, Dhut S, Gibbons B, Lister TA, Young BD. Molecular cloning of a novel 11q23 breakpoint associated with non-Hodgkin's lymphoma. Oncogene 1994;9;893-898.

CHAPTER 4.2

SITE-SPECIFIC DELETIONS INVOLVING THE *tal*-1 AND *sil* GENES ARE RESTRICTED TO CELLS OF THE T-CELL RECEPTOR $\alpha\beta$ LINEAGE: T-cell receptor δ gene deletion mechanism affects multiple genes*

Timo M. Breit[1], Ellen J. Mol[1], Ingrid L.M. Wolvers-Tettero[1], Wolf-Dieter Ludwig[2], Elisabeth R. van Wering[3], and Jacques J.M. van Dongen[1]

1. Department of Immunology, University Hospital Dijkzigt/Erasmus University, Rotterdam, The Netherlands;
2. Department of Hematology/Oncology, Universitätsklinikum Steglitz, Berlin, Germany;
3. Dutch Childhood Leukemia Study Group, The Hague, The Netherlands.

SUMMARY

Site-specific deletions in the *tal*-1 gene are reported to occur in 12%-26% of T-ALL. So far two main types of *tal*-1 deletions have been described. Upon analysis of 134 T-ALL we have found two new types of *tal*-1 deletions. These four types of deletions juxtapose the 5′ part of the *tal*-1 gene to the *sil* gene promoter, thereby deleting all coding *sil* exons but leaving the coding *tal*-1 exons undamaged. The RSS and fusion regions of the *tal*-1 deletion breakpoints strongly resemble the RSS and junctional regions of Ig/TcR gene rearrangements, which implies that they are probably caused by the same V(D)J recombinase complex. Analysis of the 134 T-ALL suggested that the occurrence of *tal*-1 deletions is associated with the CD3 phenotype, because no *tal*-1 deletions were found in 25 TcR-$\gamma\delta^+$ T-ALL, whereas eight of the 69 $CD3^-$ T-ALL and 11 of the 40 TcR-$\alpha\beta^+$ T-ALL contained such a deletion. Careful examination of all TcR genes revealed that *tal*-1 deletions exclusively occurred in $CD3^-$ or $CD3^+$ T-ALL of the $\alpha\beta$ lineage with a frequency of 18% in T-ALL with one deleted TcR-δ allele, and a frequency of 34% in T-ALL with TcR-δ gene deletions on both alleles. Therefore, we conclude that $\alpha\beta$ lineage commitment of the T-ALL and especially the extent of TcR-δ gene deletions determines the chance of a *tal*-1 deletion. This suggests that *tal*-1 deletions are mediated via the same deletion mechanism as TcR-δ gene deletions.

INTRODUCTION

Recurrent chromosomal aberrations, such as translocations and inversions involving the Ig and TcR loci, are non-randomly associated with lymphoid malignancies. It is generally assumed that these chromosome aberrations are caused through "illegitimate" V(D)J recombinase activity by the enzyme system, which normally provides for the rearrangement processes in Ig and TcR gene complexes (1-5).

The reciprocal t(1;14)(p32;q11) is an example of a chromosome aberration that is

* Published in: J Exp Med 1993;177:965-977.

probably caused by "illegitimate" V(D)J recombination. This translocation is exclusively found in T-ALL and involves both the so-called *tal*-1 gene (also known as SCL or TCL-5) and the TcR-δ gene complex (6-10). Approximately 3% of pediatric T-ALL have a t(1;14) (11), and six of the seven translocation breakpoints analyzed to date cluster in the Dδ-Jδ region of the TcR-δ locus on chromosome 14 and in a one kb region of the 5′ part of the *tal*-1 locus on chromosome 1 (8-10).

The *tal*-1 gene is a transcriptionally complex locus in which the 5′ non-coding region has two distinct transcription initiation sites and a variable pattern of alternative exon utilization (12-14). At least six different forms of mRNA are expressed, predominantly in early hematopoietic cells (13-15). The TAL-1 protein contains a so-called helix-loop-helix DNA binding motif (HLH motif) (12,13,16,17), which is also found in several other proteins involved in control of cell proliferation or differentiation (18-21). Therefore, disregulation of the *tal*-1 gene expression by chromosomal aberrations may contribute to the leukemic transformation in T-ALL.

The 5′ part of the *tal*-1 locus can also be affected by a site-specific, submicroscopic deletion (*tal*-1 deletion) of ~90 kb, which occurs at high frequency in T-ALL only (14,22,23). As a result of this ~90 kb deletion the coding exons of the *tal*-1 gene are

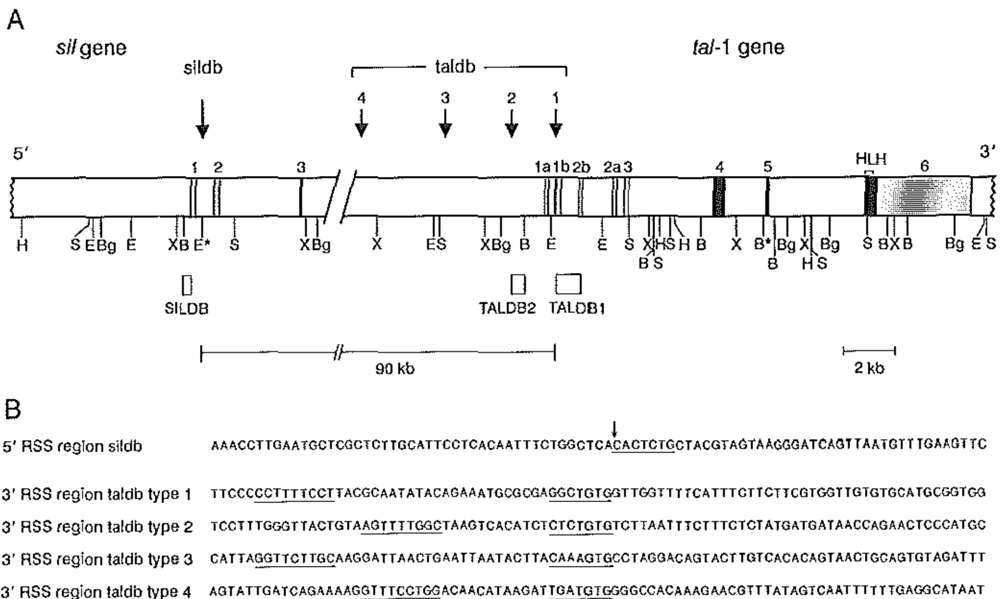


Figure 1. Restriction map and germline sequences of *tal*-1 deletion breakpoint regions. **(A)** Restriction map of the *tal*-1 locus and the 5′ part of the *sil* gene involved in the ~90 kb *tal*-1 deletion. The various types of *tal*-1 deletion breakpoints are indicated with arrows: sildb, breakpoint in *sil* gene; taldb, breakpoints in *tal*-1 gene. Non-coding exons are indicated as dotted boxes; solid boxes represent protein-coding exons. The HLH motif is indicated in *tal*-1 exon 6 (12,13). The relevant restriction sites are indicated: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sac*I; X, *Xba*I; *, polymorphic restriction site. Open boxes below the restriction map represent the probes used for Southern blot hybridization. **(B)** Germline sequences surrounding the breakpoints of the various types of *tal*-1 deletions. The heptamer sequence of the 5′RSS in the *sil* gene and heptamer-nonamer sequences of the 3′ RSS in the *tal*-1 gene are underlined. The arrow indicates the location of the breakpoints.

juxtaposed to the first non-coding exon of the recently described *sil* gene (24), which is therefore almost completely deleted (Figure 1A). The expressed *sil-tal-*1 fusion mRNA produces a normal TAL-1 protein, but is transcriptionally controlled by the *sil* gene promoter (14,25).

Also *tal-*1 deletions are assumed to be caused by the V(D)J recombinase system, because the breakpoints seem to cluster at heptamer-nonamer RSS (14,25), which are homologous to those used in the Ig and TcR rearrangement processes (4,5,26-28). Moreover, the fusion regions of the *tal-*1 deletion breakpoints show non-templated nucleotide addition (N-region), P-region nucleotides, and deletion of nucleotides by exonucleic nibbling from the flanking sequences, all of which are hallmarks of V(D)J recombination processes in Ig and TcR genes (5,26,29).

So far three types of *tal-*1 deletions are described. The two main types of *tal-*1 deletion use the same 5′ heptamer RSS, located between the first and second *sil* exon, but different 3′ heptamer-nonamer RSS in the 5′ part of the *tal-*1 locus, 1.7 kb apart of each other (14,25), whereas the third type (type C) has only been found in one patient and does not use any RSS (25). In our attempt to determine the occurrence of the *tal-*1 deletions in a series of 134 T-ALL, we identified two new types of *tal-*1 deletions that use the same 5′ heptamer RSS as type 1 and 2, but different 3′ heptamer-nonamer RSS.

In contrast to suggestions in other publications (14,25), the *tal-*1 deletions exclusively occurred in T-ALL of the αβ T-cell lineage and were especially correlated with TcR-δ gene deletions, which are characteristic for the αβ lineage.

MATERIALS AND METHODS

Cell samples

Cell samples were obtained from a non-random group of 134 T-ALL patients at initial diagnosis. To obtain sufficient numbers of $CD3^+$ T-ALL (especially TcR-$\gamma\delta^+$ T-ALL), we have selected T-ALL cell samples based on their CD3/TcR immunophenotype, resulting in 69 $CD3^-$ T-ALL (51% of the total series), 40 TcR-$\alpha\beta^+$ T-ALL (30%), and 25 TcR-$\gamma\delta^+$ T-ALL (19%). In random series of T-ALL, this immunophenotype distribution probably is ~70%, ~20%, and ~10%, respectively (30). MNC were isolated from peripheral blood, bone marrow, or pleural exudate by Ficoll-Paque (density, 1.077 g/ml: Pharmacia, Uppsala, Sweden) density centrifugation. The cell samples were frozen and stored in liquid nitrogen. Twelve T-cell lines were included as positive or negative controls (CEM, HPB-ALL, MOLT16, JURKAT, H9, HUT78, DND41, PEER, RPMI-8402, MOLT4, HSB-2, GH1) (31).

Immunologic marker analysis

The MNC of the T-ALL patients were analyzed for nuclear expression of TdT, for cytoplasmic expression of TcR-β (βF1), for cell membrane expression of T-cell markers CD1 (66IIC7), CD2 (Leu-5b), CD3 (Leu-4), CD4 (Leu-3a), CD5 (Leu-1), CD6 (OKT17), CD7 (3A1), and CD8 (Leu-2a), for HLA-DR antigen and for reactivity with the McAbs, BMA031 (anti-TcR-αβ), 11F2 (anti-TcR-γδ), and TCRδ1 (anti-TcR-δ). The rabbit anti-TdT antiserum was purchased from Supertechs (Bethesda, MD); the McAbs of the Leu series, anti-HLA-DR and 11F2, were obtained from Becton Dickinson (San Jose, CA); the CD1 antibody was obtained from Monosan/Sanbio (Nistelrode, The Netherlands); OKT17 from Ortho Diagnostic Systems (Raritan, NJ); the 3A1 hybridoma was from the American Type Culture Collection (Rockville, MD); TCRδ1 and βF1 were obtained from T Cell Diagnostics (Cambridge, MA). The McAb BMA031 was kindly provided by Dr. R. Kurrle (Behring, Marburg, Germany). Immunofluorescence stainings were evaluated with fluorescence microscopes (Carl Zeiss, Oberkochen, Germany) and/or a FACScan flowcytometer (Becton Dickinson).

Isolation of the *tal*-1 locus from a genomic library

Screening with the B2EE-2.0 probe (9) and TALDB2 probe of the CML-0 genomic library, constructed of *MboI* partial digested DNA from a CML patient cloned in the EMBL3 lambda replacement vector (32), yielded several phage inserts, which covered the whole *tal*-1 locus. The phage inserts were digested for restriction enzyme analysis and some genomic restriction fragments were subcloned in the pUC19 cloning vector.

Isolation of *sil* and *tal*-1 gene DNA probes

DNA probes were obtained by cloning the purified PCR amplification products of granulocyte DNA from a healthy donor using specific oligonucleotide primer sets. pUC19 was used as cloning vector (32). The oligonucleotide primer sets for the SILDB and TALDB2 probes, including the artificial tails containing restriction sites for cloning, are given in Table 1. All oligonucleotides were synthesized according to published *tal*-1 gene sequences (13,14,22) on a 392 DNA synthesizer (Applied Biosystems, Forster City, CA) with the solid-phase phosphotriester method and used without further purification. Probe TALDB1 was isolated by cloning a ~800 bp *MspI-MspI* fragment, containing the 3′ side of the *tal*-1 deletion type 1 (Figure 1A), from the CML-0 genomic library (33).

TABLE 1. Primers used in PCR and sequencing analysis of *tal*-1 deletions or isolation of DNA probes.

	Code	Position/Size[a]	Sequence[b]	Reference[c]
***tal*-1 deletion**			5′ 3′	
5′ all types	sildb	−155 bp	GGGGAGCTCGTGGGAGAAATTAAG	22
	sildb-seq	−111 bp	GGTATCATCTGAGCTAAGGTATGTG	22
3′ type 1	tal1db1	+155 bp	GCCTCGAAGGGTCCACATCTAC	14
	tal1db1-seq	+111 bp	CACACTCGGACACAGAGCCTG	14
	tal1db1-5′	+28 bp	TCACAATCCCACCGCATGCACA	14
3′ type 2	tal1db2	+152 bp	TTGTAAAATGGGGAGATAATGTCGAC	This paper
	tal1db2-seq	+110 bp	AACTTATATGACCTTTAAAAGG	This paper
3′ type 3	tal1db3	+58 bp	TGCATGCACTCTGATGAGCAGCC	This paper
	tal1db3-seq	+15 bp	ATCTACACTGCAGTTACTGTGTGAC	This paper
3′ type 4	tal1db4	+450 bp	GGATTATAGGTGCCTGTCACCAC	This paper
	tal1db4-seq	+34 bp	TACATCTTATAGTATGTAAATTATGCC	This paper
DNA probes				
SILDB	sildp5′	330 bp	CACAGGATCCTTGATCCTGGAGCGC	22
	sildp3′		CCGAAGCTTCCGCGGAGCTGAGGTCTG	22
TALDB2	tal1d2p5′	~575 bp	TGTAAGCTTTGGCTAAGTCACATCTCTC	14
	tal1d2p3′		CCTGTCAATAGGGACATAAATGCC	13

a. The position of the oligonucleotide primer is indicated upstream (−) or downstream (+) relative to the heptamer RSS. The sizes of the DNA probes are given. The position of the DNA probes are indicated in Figure 1A.
b. The underlined sequences represent the aspecific nucleotides, which generate restriction sites.
c. Sequence information used to design the oligonucleotide primers was derived from the indicated literature references or from our own sequence data.

Southern blot analysis

DNA was isolated from frozen MNC as described previously (32,34). A 15 μg sample was digested with the appropriate restriction enzymes (Pharmacia), size-fractionated in 0.7% agarose gels, and transferred to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) as described (32,34). *tal*-1 deletions were studied using ^{32}P random oligonucleotide-labeled B2EE-2.0 (9), TALDB1, TALDB2, and SILDB probes in *Eco*RI, *Hin*dIII, and *Bgl*II digests. TcR-β gene rearrangements were detected with the Jβ1, Jβ2, and Cβ probes (34,35) in *Eco*RI, and *Hin*dIII digests. The configuration of the TcR-γ genes was analyzed by use of the Jγ1.2, Jγ1.3, Jγ2.1, and Cγ probes (34,36) in *Eco*RI and *Kpn*I digests. The configuration of the TcR-δ genes was analyzed by use of the Vδ1, Vδ3, Jδ1, Jδ2, Cδ, δREC, and ψJα probes (34,37-40) in *Eco*RI, *Hin*dIII, and *Bgl*II digests.

PCR amplification analysis

PCR was essentially performed as described previously (32,41). A 0.1 μg sample of DNA, 12 pmol of the 5′ and the 3′ oligonucleotide primer, and 1 unit of *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) were used in each PCR reaction of 100 μl. The oligonucleotide primers are listed in Table 1. These oligonucleotides were designed according to our own sequence data and published *tal*-1 gene sequences (14) and *sil* gene sequences (22). The PCR reaction mixture was incubated at 94°C for 3 min, at 60°C for 2 min, and at 72°C for 3 min in a thermal cycler (Perkin-Elmer Cetus). After this initial cycle, denaturing, annealing, and extension steps were performed for another 39 cycles at 94°C for 1 min, at 60°C for 1 min, and at 72°C for 3 min, respectively. After the last cycle an additional extension step of 72°C for 7 min was executed. The PCR products of *tal*-1 deletion type 1 and type 2 were size-fractionated by 10% polyacrylamide gel-electrophoresis and visualized by ethidium bromide staining to demonstrate differences in size.

Sequence analysis

One μl of the original PCR product, 12 pmol of the limiting primer, 600 pmol of the opposite primer, and 5 units of *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus) were used in each asymmetric PCR reaction of 500 μl. The reaction mixture was incubated for a total of 25 to 30 cycles with the above-described regular temperature cycles. After the asymmetric amplification, the PCR product was precipitated twice in 50% ethanol plus 0.1 volume of 2 M NaAc, pH 5.6 (41). The dried pellet was resolved in 22 μl H_2O, half of which was used in the sequence reaction. Fifty pmol sequence primer was used in each reaction (sequence primers are indicated in Table 1). All sequence reactions were performed with the T7-sequencing kit (Pharmacia) following the manufacturer's instructions using ^{35}S radiolabeling, and run on a normal, denaturating 8% polyacrylamide sequence gel. All germline sequences and fusion regions of *tal*-1 deletions were sequenced in both directions.

RESULTS

Two new types of *tal*-1 deletions

Screening of 134 T-ALL by Southern blot analysis revealed two new types of *tal*-1 deletions in addition to the already described type 1 (type A), type 2 (type B), and type C

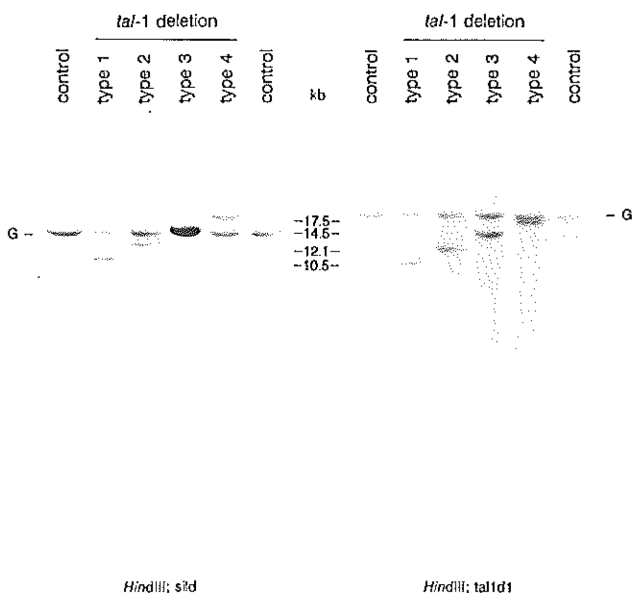


Figure 2. Southern blot analysis of the various types of *tal*-1 deletions. *Hin*dIII digests of DNA from patients with different types of *tal*-1 deletion: type 1, patient SL; type 2, patient PV; type 3, patient MB; type 4, patient BD. The Southern blot filter was successively hybridized with the SILDB and TALDB1 probes (Figure 1A). The sizes of the rearranged bands are indicated. G, germline band.

(14,25). We designated these new *tal*-1 deletions type 3 and type 4 (Figures 1A and 2). Based on the Southern blot data, it was concluded that the 3′ breakpoints of these two new *tal*-1 deletions were located upstream of the *tal*-1 locus, whereas the 5′ breakpoints were apparently identical to the 5′ breakpoints of *tal*-1 deletions type 1 and type 2 (Figures 1A and 2). To determine the exact location and the germline sequences of the new *tal*-1 deletion breakpoints, DNA of patient MB with *tal*-1 deletion type 3 was amplified by PCR using the sildb and tal1db2 oligonucleotide primers (Table 1), which resulted in an ~3 kb PCR product. After cloning and sequencing of this PCR product, a *tal*-1 deletion type 3 sequence primer was made (Table 1). A 4.3 kb *Xba*I-*Xba*I fragment isolated from a genomic library was sequenced by use of the tal1db3-seq primer, which provided the germline sequence of the *tal*-1 deletion type 3 breakpoint region (Figure 1B). Based on the sequencing data of the 3′ side of a 1.9 kb *Sal*I-*Xba*I subclone, the tal1db4 primer was made (Table 1). This primer in combination with the sildb primer resulted in an ~0.6 kb PCR product when DNA from patient BD with *tal*-1 deletion type 4 was amplified. Based on the direct sequencing data of this PCR product, a *tal*-1 deletion type 4 sequence primer was made (Table 1). Sequencing with this primer of the 1.9 kb *Sal*I-*Xba*I subclone provided the germline sequence of the *tal*-1 deletion type 4 breakpoint region (Figure 1B).

The sequence analysis showed that the *tal*-1 deletion type 3 and type 4 both used the same 5′ heptamer RSS as type 1 and type 2, but different 3′ heptamer-nonamer RSS (Figure 1B). The heptamer-nonamer RSS for *tal*-1 deletion type 2 is located ~1.7 kb upstream of the type 1 RSS (14,25), whereas the RSS of type 3 and type 4 are located ~4.5 kb and ~7.7 kb upstream of the type 1 RSS, respectively (Figure 1A). The RSS of all *tal*-1 deletions display a strong homology with the heptamer-nonamer RSS sequences of Ig and TcR genes.

Fusion regions of *tal*-1 deletion breakpoints

In total, 19 *tal*-1 deletions were found in the 134 T-ALL analyzed: 14 type 1, three type 2, one type 3, and one type 4 (Table 2). In addition, four T-cell lines (RPMI 8402, HSB-2, CEM, MOLT 16) contained a type 1 *tal*-1 deletion. PCR products containing the fusion regions of the *tal*-1 deletion breakpoints were obtained by amplification of the

TABLE 2. Frequency of *tal*-1 deletions in T-ALL.

		tal-1 deletions				
	number of patients	type 1 (n)	type 2 (n)	type 3 (n)	type 4 (n)	Total (n)
$CD3^-$ T-ALL	69	8.7% (6)	2.9% (2)	0% (0)	0% (0)	11.6% (8)
TcR-$\gamma\delta^+$ T-ALL	25	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)
TcR-$\alpha\beta^+$ T-ALL	40	20.0% (8)	2.5% (1)	2.5% (1)	2.5% (1)	27.5% (11)
Total	134	10.4% (14)	2.2% (3)	0.7% (1)	0.7% (1)	14.2% (19)

different *tal*-1 deletions from the 19 T-ALL and four T-cell lines (Figure 3A). Subsequent sequencing of the *tal*-1 deletion PCR products revealed the sequences of the breakpoint fusion regions (Figure 3B). All fusion regions contained randomly inserted nucleotides (N-region) except for patient MD, who had a fusion region consisting of two P-region nucleotides only. The average N-region insertion was 6.4 nucleotides, and a total of 14 P-region nucleotides were observed in the 23 fusion regions analyzed (19 T-ALL and four T-cell lines). Trimming occurred in the far majority of the *tal*-1 deletions. Thus, the fusion regions are highly homologous to the junctional regions of rearranged Ig and TcR genes.

Occurrence of *tal*-1 deletions is related to CD3 phenotype and TcR gene configuration

The overall percentage of *tal*-1 deletions in our series of T-ALL was 14.2% (19/134), but these *tal*-1 deletions appeared to be restricted to $CD3^-$ T-ALL (8/69) and TcR-$\alpha\beta^+$ T-ALL (11/40), whereas no *tal*-1 deletions were found in TcR-$\gamma\delta^+$ T-ALL (0/25) (Table 2). The presence or absence of *tal*-1 deletions was not associated with other immunophenotypic characteristics (Table 3).

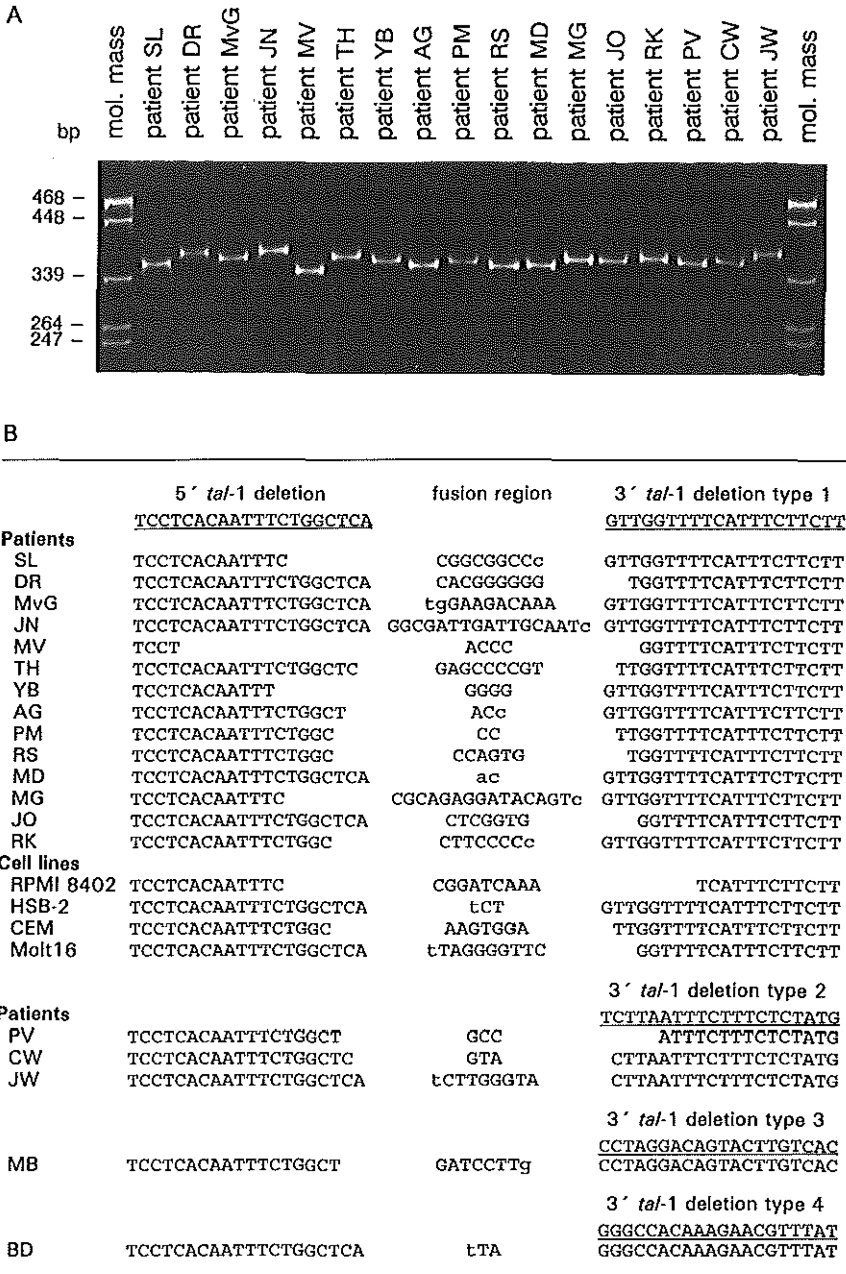
Since $CD3^-$ T-ALL theoretically represent precursor stages of both TcR-$\gamma\delta^+$ and TcR-$\alpha\beta^+$ T-ALL, we tried to use the configuration of the TcR-δ genes as an additional marker to determine whether the $CD3^-$ T-ALL group could be divided into γδ lineage or αβ lineage committed subgroups, and whether such a subdivision corresponded with the occurrence of *tal*-1 deletions. The configuration of the TcR-δ gene on each allele can potentially pass three consecutive stages: germline, rearranged, and deleted. Analysis of the TcR-δ gene configuration of the 19 T-ALL and four T-cell lines with *tal*-1 deletions revealed that all but one contained at least one deleted TcR-δ allele (Table 3): only one T-ALL (1/19) with a *tal*-1 deletion had no deletion of the TcR-δ gene, 31.6% (6/19) had one deleted TcR-δ allele with a rearrangement on the other allele, and 63.2% (12/19) had deletions of both TcR-δ alleles. Also in the four T-cell lines with a *tal*-1 deletion, a high frequency of TcR-δ gene deletions was found: seven of eight TcR-δ alleles were deleted (Table 3).

Further analysis of the eight TcR-δ gene rearrangements in the T-ALL with a *tal*-1 deletion showed that seven were complete Vδ-Jδ rearrangements, and one TcR-$\alpha\beta^+$ T-ALL (patient MG) contained a Vα-Jδ1 rearrangement (Table 4). Sequence analysis of the seven Vδ-Jδ junctional regions revealed that all these TcR-δ rearrangements in both $CD3^-$ T-ALL and TcR-$\alpha\beta^+$ T-ALL were out of frame and therefore non-functional (Figure 4). The rearranged TcR-δ gene in cell line RPMI 8402 is caused by a t(11;14)(p15;q11) and therefore represents a non-functional TcR-δ gene as well (39). This implies that all T-ALL and T-cell lines with a *tal*-1 deletion have deleted their TcR-δ genes (37/46 alleles) and/or contain non-functional TcR-δ gene rearrangements (9/46 alleles).

TcR-γ gene analysis of the T-ALL and T-cell lines with a *tal*-1 deletion revealed that 21.7% (10/46) and 78.3% (36/46) of the rearranged TcR-γ alleles involved the TcR-γ1 and TcR-γ2 locus, respectively (Table 3), which represents a normal rearrangement pattern, as found in the total group of 134 analyzed T-ALL. Analysis of the TcR-β configuration of the 19 T-ALL and four T-cell lines revealed rearrangements in all cases, but without any preferential pattern.

***tal*-1 deletions coincide with TcR-δ gene deletions**

Based on the above described results, we decided to determine the incidence of TcR-δ



	5′ *tal*-1 deletion	fusion region	3′ *tal*-1 deletion type 1
	TCCTCACAATTTCTGGCTCA		GTTGGTTTTCATTTCTTCTT
Patients			
SL	TCCTCACAATTTC	CGGCGGCCc	GTTGGTTTTCATTTCTTCTT
DR	TCCTCACAATTTCTGGCTCA	CACGGGGGG	TGGTTTTCATTTCTTCTT
MvG	TCCTCACAATTTCTGGCTCA	tgGAAGACAAA	GTTGGTTTTCATTTCTTCTT
JN	TCCTCACAATTTCTGGCTCA	GGCGATTGATTGCAATc	GTTGGTTTTCATTTCTTCTT
MV	TCCT	ACCC	GGTTTTCATTTCTTCTT
TH	TCCTCACAATTTCTGGCTC	GAGCCCCGT	TTGGTTTTCATTTCTTCTT
YB	TCCTCACAATTT	GGGG	GTTGGTTTTCATTTCTTCTT
AG	TCCTCACAATTTCTGGCT	ACc	GTTGGTTTTCATTTCTTCTT
PM	TCCTCACAATTTCTGGC	CC	TTGGTTTTCATTTCTTCTT
RS	TCCTCACAATTTCTGGC	CCAGTG	TGGTTTTCATTTCTTCTT
MD	TCCTCACAATTTCTGGCTCA	ac	GTTGGTTTTCATTTCTTCTT
MG	TCCTCACAATTTC	CGCAGAGGATACAGTc	GTTGGTTTTCATTTCTTCTT
JO	TCCTCACAATTTCTGGCTCA	CTCGGTG	GGTTTTCATTTCTTCTT
RK	TCCTCACAATTTCTGGC	CTTCCCCc	GTTGGTTTTCATTTCTTCTT
Cell lines			
RPMI 8402	TCCTCACAATTTC	CGGATCAAA	TCATTTCTTCTT
HSB-2	TCCTCACAATTTCTGGCTCA	tCT	GTTGGTTTTCATTTCTTCTT
CEM	TCCTCACAATTTCTGGC	AAGTGGA	TTGGTTTTCATTTCTTCTT
Molt16	TCCTCACAATTTCTGGCTCA	tTAGGGGTTC	GGTTTTCATTTCTTCTT

			3′ *tal*-1 deletion type 2
Patients			TCTTAATTTCTTTCTCTATG
PV	TCCTCACAATTTCTGGCT	GCC	ATTTCTTTCTCTATG
CW	TCCTCACAATTTCTGGCTC	GTA	CTTAATTTCTTTCTCTATG
JW	TCCTCACAATTTCTGGCTCA	tCTTGGGTA	CTTAATTTCTTTCTCTATG

			3′ *tal*-1 deletion type 3
			CCTAGGACAGTACTTGTCAC
MB	TCCTCACAATTTCTGGCT	GATCCTTg	CCTAGGACAGTACTTGTCAC

			3′ *tal*-1 deletion type 4
			GGGCCACAAAGAACGTTTAT
BD	TCCTCACAATTTCTGGCTCA	tTA	GGGCCACAAAGAACGTTTAT

Figure 3. PCR and sequence analysis of *tal*-1 deletion fusion regions. (A) PCR products obtained via amplification of the DNA from patients with a *tal*-1 deletion type 1 or type 2 were size fractionated in a ethidium bromide-stained 10% polyacryl amide gel. The PCR reaction was performed using the sildb primer and either the tal1db1 primer (type 1) or tal1db2 primer (type 2). (B) Sequences of the fusion regions of all *tal*-1 deletions are aligned with the known (underlined) *sil* and *tal* germline sequences. Lower case characters at the end of a fusion region represent P-region nucleotides (29). All other nucleotides of the fusion region represent N-region nucleotides.

TABLE 3. Characteristics of 19 T-ALL and four T cell lines with a *tal*-1 deletion.

		tal-1 deletion type 1																		type 2			type 3	type 4
		SL	DR	MvG	JN	MV	TH	YB	AG	PM	RS	MD	MG	JO[a]	RK	Tcl1[b]	Tcl2[b]	Tcl3[b]	Tcl4[b]	PV	CW	JW	MB	BD
Immunologic markers[c]																								
TdT		+	+	+	+	70%	32%	71%	+	67%	+	59%	+	59%	+	+	–	–	+	45%	+	+	+	+
HLA-DR	(L243)	–	–	–	–	–	–	NT	–	NT	–	–	–	NT	–	–	–	–	–	–	NT	NT	–	–
CD1	(66IIC7)	65%	–	–	–	–	55%	–	–	–	21%	–	–	±	–	–	–	–	–	21%	–	–	–	50%
CD2	(Leu-5b)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	20%	+	+	+	+	+
CD3	(Leu-4)	–	–	–	–	–	–	35%	45%	46%	64%	72%	+	+	+	–	–	–	+	–	–	59%	+	+
CD4	(Leu-3a)	+	–	–	–	73%	68%	–	–	+	34%	18%	–	±	+	–	–	+	–	39%	+	34%	48%	37%
CD5	(Leu-1)	+	+	+	+	+	71%	+	+	+	+	+	+	+	+	+	+	64%	+	+	+	+	+	+
CD6	(OKT17)	73%	NT	+	71%	–	63%	+	43%	+	NT	28%	35%	±	NT	25%	72%	20%	+	+	+	36%	NT	35%
CD7	(3A1)	+	+	+	+	+	74%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CD8	(Leu-2a)	73%	–	–	–	73%	61%	–	34%	+	53%	17%	–	–	–	–	–	–	–	71%	42%	36%	32%	+
TcR-αβ	(BMA031)	–	–	–	–	–	–	23%	49%	23%	NT	59%	+	+	61%	–	–	–	+	–	–	42%	NT	35%
TcR-γδ	(11F2)	NT	–	–	–	–	–	–	NT	–	NT	–	–	–	–	–	–	–	–	–	–	–	NT	–
Cyβ	(βF1)	NT	+	+	NT	+	60%	+	NT	+	NT	+	+	+	NT	+	20%	73%	+	32%	+	+	NT	+
Southern blot analysis[d]																								
TcR-δ	genes	D/R	D/R	D/D	D/D	D/R	D/D	D/D	D/D	D/D	D/D	D/R	D/R	D/D	D/D	D/T	D/D	D/D	D/D	R/R	D/R	D/D	D/D	D/D
TcR-γ1	genes	D/D	D/D	D/D	D/D	D/R	D/D	D/R	D/D	D/D	D/D	D/D	D/R	D/D	D/D	D/D	D/D	D/D	D/D	D/R	R/R	R/R	D/D	R/R
TcR-γ2	genes	R/R	R/R	R/R	R/R	R/G	R/R	R/G	R/R	R/R	R/R	R/R	R/G	R/R	R/R	R/R	R/R	R/R	R/R	R/G	G/G	G/G	R/R	G/G
TcR-β1	genes	G/G	R/R	D/R	R/R	R/R	D/D	D/R	D/R	D/R	R/R	D/R	D/G	D/D	R/G	D/R	R/R	D/R	D/R	R/G	D/R	R/R	D/R	D/D
TcR-β2	genes	R/R	R/G	R/G	R/G	G/G	R/R	R/R	R/G	R/R	R/G	R/G	R/G	R/R	R/G	R/G	G/G	R/R	R/G	R/R	R/G	R/G	R/G	R/R

a. Immunofluorescence data of patient JO were difficult to interpret due to high background. ±, percentages positivity between 15 and 75%.
b. T-cell lines; Tcl1 = RPMI 8402; Tcl2 = HSB-2; Tcl3 = CEM; Tcl4 = MOLT 16 (31).
c. Immunologic marker analysis: +, ≥75% of the cells are positive; –, ≤15% of the cells are positive; positivity between 15 and 75% is indicated. NT, not tested.
d. Southern blot analysis: interpretation of the results using the described TcR probes. G, allele in germline configuration; R, rearrangement of the involved allele; T, translocation of involved allele; D, deletion of the involved allele.

TABLE 4. TcR-δ rearrangements and configuration of deleting elements in 19 T-ALL and four T-cell lines with a *tal*-1 deletion[a].

T-ALL patients and cell lines[b]	CD3 phenotype	TcR-δ rearrangements	deleting elements δREC	deleting elements ψJα
PV	CD3$^-$	Vδ1-Jδ1/Vδ1-Jδ1	D/D	G/G
MV	CD3$^-$	δREC-ψJα/Vδ3-Jδ2	R/G	R/G
DR	CD3$^-$	D/Vδ3-Jδ1	D/G	D/G
SL	CD3$^-$	D/Vδ1-Jδ1	D/D	D/G
CW	CD3$^-$	D/Vδ1-Jδ1	D/D	D/G
HSB-2	CD3$^-$	D/δREC-ψJα	R/R	D/R
RPMI 8402	CD3$^-$	D/t(11;14)	D/G	D/D
MvG	CD3$^-$	D/D	D/D	D/D
JN	CD3$^-$	D/D	D/D	D/D
TH	CD3$^-$	D/D	D/D	D/D
CEM	CD3$^-$	D/D	D/D	D/D
MD	$\alpha\beta^+$	D/Vδ1-Jδ1	D/D	D/G
MG	$\alpha\beta^+$	D/Vα-Jδ1	D/D	D/D
RK	$\alpha\beta^+$	D/δREC-ψJα	D/R	D/R
BD	$\alpha\beta^+$	D/D/δREC-ψJα[c]	D/D/R[c]	D/D/R[c]
YB	$\alpha\beta^+$	D/D	D/D	D/D
AG	$\alpha\beta^+$	D/D	D/D	D/D
PM	$\alpha\beta^+$	D/D	D/G	D/D
RS	$\alpha\beta^+$	D/D	D/G	D/D
JO	$\alpha\beta^+$	D/D	D/D	D/D
JW	$\alpha\beta^+$	D/D	D/D	D/D
MB	$\alpha\beta^+$	D/D	D/D	D/D
Molt 16	$\alpha\beta^+$	D/D	D/D	D/D

a. Gene configuration; G, allele in germline configuration; R, rearranged allele; D, deleted allele.
b. The T-ALL and T-cell lines are ordered according to their immunophenotype (first CD3$^-$, followed by TcR-$\alpha\beta^+$) and the configuration of their TcR-δ genes (i.e. from Vδ-Jδ rearrangement and δREC-ψJα rearrangement to deletion).
c. δREC-ψJα rearrangement present in a small subpopulation.

gene rearrangements and deletions in the total group of 134 T-ALL. The results allowed us to divide the 134 T-ALL in subgroups on the basis of their TcR-δ gene configuration in addition to their CD3 phenotype, as presented in Table 5. It became clear that in the CD3$^-$ T-ALL group almost all *tal*-1 deletions (7/8) cluster in a small subgroup (39% of all CD3$^-$ T-ALL), which is defined by containing at least one deleted TcR-δ allele. Since TcR-$\alpha\beta^+$ T-ALL also contain at least one deleted TcR-δ allele, virtually all *tal*-1 deletions coincide with TcR-δ gene deletions. In addition, it is remarkable that the far majority of *tal*-1 deletions in TcR-$\alpha\beta^+$ T-ALL (9/11) cluster in the major subgroup, which is defined by deletion of both TcR-δ alleles (63% of all TcR-$\alpha\beta^+$ T-ALL). Only two TcR-$\gamma\delta^+$ T-ALL (2/25) had one deleted TcR-δ gene, and no *tal*-1 deletions were found in this CD3$^+$ subgroup. The overall results show that the frequency of *tal*-1 deletions in T-ALL with both TcR-δ alleles deleted (34.3%) is about twice that of *tal*-1 deletions in T-ALL with one TcR-δ allele deleted (17.6%) (Table 5). This suggests that not the CD3 immunophenotype, but the extend of TcR-δ gene deletion determines the chance of having a *tal*-1 deletion. This is further supported by the finding that only one *tal*-1 deletion was found in the 65 T-ALL without a TcR-δ gene deletion, in contrast to 18 of the 69 T-ALL with one or two deleted TcR-δ genes.

Patients	Vδ1	Junctional region	Jδ1
	CTCTTGGGGAACT		ACACCGATAAACT
SL	CTCTTGGGGA	CCCCCGGAAATAGTaGGACGGA	ACCGATAAACT
MD	CTCTTGG	CTTTCTCTCCGGgtACTGGGGGATTTgt	ACACCGATAAACT
PV	CTCTTGGG	CAATTTGGTTCAGTCATTTATGACCCCAAGGGGATAATT	CACCGATAAACT
PV	CTC	GACGCCGC	ACACCGATAAACT
CW	CTCTTGGGGA	GCTGAGATCCCCGTCCTCCCAGGGAGCGCGGGGACCTAt	ACACCGATAAACT
	Vδ3		
	ACTGTGCCTTTAG		
DR	ACTGTGCCTTTA	AGGACTGGGCCGGGGCGGGATGGGGG	T
			Jδ2
			CTTTGACAGCACA
MV	ACTGTGCCTTT	CTTACTGCCGGGACTTAGGAGGGGAG	GACAGCACA

Figure 4. Junctional region sequences of non-functional TcR-δ rearrangements in T-ALL with a *tal*-1 deletion. Sequences of the junctional regions of the TcR-δ rearrangements are aligned with the known (underlined) Vδ and Jδ germline sequences (37). All rearrangements are out of frame. Underlined sequences in the junctional regions represent Dδ nucleotides. Lower case characters represent P-region nucleotides, and all other junctional region nucleotides represent N-region nucleotides. Overlined sequences are stopcodons when read in the correct reading frame.

Configuration of δREC and ψJα gene segments in T-ALL with a *tal*-1 deletion

The TcR-δ deletion mechanism, which is responsible for the deletion of non-functional TcR-δ rearrangements, is assumed to use two so-called deleting elements δREC and ψJα (40,42). The specific δREC-ψJα rearrangement was found in three T-ALL and one T-cell line with a *tal*-1 deletion (Table 4). In one T-ALL (patient BD) this rearrangement was present only in a small subpopulation of the leukemic cells (Table 4). 80.4% (37/46) of the δREC alleles and 78.3% (36/46) of the ψJα alleles were deleted in the T-ALL and T-cell lines with a *tal*-1 deletion. Only two TcR-α/δ loci were left with both deleting elements in germline configuration on the same allele (Table 4). It is noteworthy that both these two TcR-α/δ loci contained a TcR-δ gene rearrangement of the Vδ3 gene segment, which can only be obtained via inversion of the TcR-δ locus (Table 4). This inversion might inhibit δREC–ψJα rearrangements. This would imply that further TcR-δ gene deletions by specific δREC-ψJα rearrangements are impossible in the T-ALL with a *tal*-1 deletion.

DISCUSSION

Five types of *tal*-1 deletions

So far two main types of *tal*-1 deletions have been reported, type 1 and type 2 (14,25). Here we describe two new types of *tal*-1 deletions, designated type 3 and type 4. Whereas type 1 and 2 were found in relatively high frequencies (10.4% and 2.2%, respectively) in our series of T-ALL, type 3 and 4 were each observed only once (0.7%). The rare type C *tal*-1 deletion described by Aplan et al. (25) is an unusual deletion because it does not use any RSS (Aplan's type A and type B are type 1 and type 2, respectively).

TABLE 5. Frequency of *tal*-1 deletions in T-ALL subgroups defined by CD3 phenotype and TcR-δ gene configuration.

	TcR-δ gene configuration[a]				
	G/G	R/G	R/R	D/R	D/D
***tal*-1 deletions in**					
$CD3^-$ T-ALL (8/69)	0% (0/7)	0% (0/5)	3.3% (1/30)	23.5% (4/17)	30.0% (3/10)
TcR-$\gamma\delta^+$ T-ALL (0/25)	– (0/0)	– (0/0)	0% (0/23)	0% (0/2)	– (0/0)
TcR-$\alpha\beta^+$ T-ALL (11/40)	– (0/0)	– (0/0)	– (0/0)	13.3% (2/15)	36.0% (9/25)
Total T-ALL (19/134)	0% (0/7)	0% (0/5)	1.9% (1/53)	17.6% (6/34)	34.3% (12/35)

a. TcR-δ configuration: G, allele in germline configuration; R, rearranged allele; D, deleted allele.

All types of *tal*-1 deletions result in a complete deletion of the coding exons of the *sil* locus, but leave the *tal*-1 coding exons undamaged. Thus, the oncogenic effect of the *tal*-1 deletions is not the result of an alteration of the TAL-1 protein, but an aberrant expression of the normal TAL-1 protein, which may contribute to the leukemic transformation of immature T-cells into T-ALL.

RSS used in *tal*-1 deletions

The *tal*-1 deletions type 1, type 2, type 3, and type 4 use the same 5' RSS, which consists of a heptamer sequence only and is located between the first and second non-coding *sil* exons. The 3' RSS used in these *tal*-1 deletions consists of different heptamer-nonamer sequences, with spacers of 24 nucleotides (type 1 and type 3) or 12 nucleotides (type 2 and type 4). All four 3' RSS are located in the non-coding 5' part of the *tal*-1 locus. These 3' RSS are highly homologous to the consensus RSS used in regular Ig and TcR gene rearrangement processes (Figure 5) (27,28).

Based on several remarkable observations, there has been a lot of speculation on the exact mechanism causing the *tal*-1 deletions: for instance, the fact that the 3' RSS consists of a heptamer-nonamer sequence, whereas the 5' RSS consists only of a heptamer with homology to the consensus heptamer of ~70% (5/7 nucleotides). It can be anticipated that such a small heptamer sequence with no demand for absolute homology will be present at various locations in the 5' region of the *sil* locus. Nevertheless only one "specific" heptamer is used in all types of *tal*-1 deletions. In addition, the 3' RSS of the *tal*-1 deletion type 2 displays the highest homology with the consensus RSS of Ig and TcR genes, but this type of *tal*-1 deletion represents only a minority of the total number of *tal*-1 deletions. Hence, there are other (sequence) factors that contribute to the development of a *tal*-1 recombination event.

	heptamer	nonamer	(spacer)	heptamer	
Ig/TcR consensus	CACAGTG	GGTTTTTGT	(12/23)	CACTGTG	
tal-1 deletion	---TC--	CC----CC-	(24)	GG-----	type 1
		A-----G-C	(12)	-T-----	type 2
		----C---C	(24)	--AA---	type 3
		-T--CC--G	(12)	TGA----	type 4
hprt gene deletion	---T--A	T--A-----	(12)	GTG----	class I
		CT----AT-	(23)	-T-----	class II
		T-----G--	(23)	TTTA---	class III

Figure 5. RSS used in rearrangement processes of Ig, TcR, *tal*-1, and *hprt* genes. The RSS used in the various types of *tal*-1 deletions or *hprt* deletions are aligned with the consensus heptamer-nonamer sequence of Ig and TcR genes (25,27,43). -, nucleotide homologous to the RSS consensus sequence.

Sequences homologous to the RSS of Ig and TcR genes do not only occur in the *sil* and *tal*-1 genes, but also in other genes, and may lead to recombination and thereby deletion. An example of such site-specific deletions is observed in blood T-lymphocytes and involves the housekeeping gene *hprt* on chromosome Xq26 (43). Three types of deletions have been observed in the *hprt* gene, designated classes I–III, and all three damage the gene. The three types of *hprt* deletions use the same 5′ RSS, which is located in intron 1 and consists of a sole heptamer, but different 3′ RSS, which are located in intron 3 and consist of heptamer-nonamer sequences with spacers of different sizes (Figure 5). The fusion regions of these *hprt* deletions show N-regions, P-region nucleotide insertion, and deletion of nucleotides by trimming of the flanking sequences, and are therefore homologous to the Ig and TcR junctional regions and *tal*-1 breakpoint fusion regions (43). The *hprt* deletions occur at a low frequency of $\sim 10^{-7}$ and are not oncogenic (44,45).

Fusion regions of *tal*-1 deletion breakpoints

Because fusion regions of *tal*-1 deletions and *hprt* deletions strongly resemble junctional regions of normal Ig and TcR gene rearrangements, the fusion region nucleotide insertion and deletion of all *tal*-1 and *hprt* deletions described to date were compared to the junctional regions of TcR-δ and TcR-γ gene rearrangements (Table 6). Remarkably, the average insertion observed in the *tal*-1 breakpoint fusion regions (7.2 nucleotides) and the *hprt* breakpoint fusion regions (5.7 nucleotides) were comparable to the average insertion of the TcR-γ gene rearrangement (7.3 nucleotides), but lower than that of the TcR-δ gene rearrangements (28.3 nucleotides), due to the use of Dδ gene segments in the latter rearrangement (Table 6) (46). However, average nucleotide deletion of the *tal*-1 deletions (5.4 nucleotides) and the *hprt* deletions (5.8 nucleotides) were less extensive than in TcR-γ gene rearrangements (9.1 nucleotides), but were comparable to TcR-δ gene rearrangements (5.0 nucleotides) (Table 6) (46). Although the average nucleotide deletion of *tal*-1 deletion type 1 and type 2 were identical (5.6 nucleotides), the nucleotide deletion at the 5′ flanking side and 3′ flanking side differed markedly between the two types of *tal*-1 deletions. In *tal*-1 deletion type 1, the average nucleotide deletion of the 5′ flanking side (3.8 nucleotides) was more than twice that of the 3′ flanking side (1.8 nucleotides), whereas in *tal*-1 deletion type 2 the reversed situation was observed with the average nucleotide deletion at the 5′ flanking side (2.0 nucleotides), being approximately half of that at the 3′ flanking side (3.6 nucleotides). This difference is most probably related to the different sizes of the spacers in the 3′ RSS of these two types of *tal*-1 deletions. The

TABLE 6. Junctional diversity of TcR-δ and TcR-γ gene rearrangements and fusion region diversity of *tal*-1 and *hprt* deletions.

Rearrangement/deletion (no. of alleles analyzed)	No. of inserted nucleotides		No. of deleted nucleotides	
	mean	range	mean	range
TcR-δ gene rearrangements[a]				
Vδ-Jδ (n=45)	28.3	5–47	5.0	0–20
TcR-γ gene rearrangements[a]				
Vγ-Jγ (n=30)	7.3	0–25	9.1	1–27
tal-1 deletion fusion regions[b]				
type 1 (n=46)	7.2	0–17	5.6	0–24
type 2 (n=10)	7.5	3–15	5.6	1–14
type 3 (n=1)	8		2	
type 4 (n=1)	3		0	
hprt deletion fusion regions[c]				
Class I (n=15)	5.3	0–10	5.2	0–27
Class II (n=2)	10.0	8–12	9.5	3–16
Class III (n=1)	3		8	

a. Data from Reference 46.
b. Combined results of this paper and References 14,22, and 25.
c. Data from Reference 43.

type 1 heptamer-nonamer contains a 24 bp spacer and therefore resembles the RSS of a TcR V gene segment, whereas the type 2 heptamer-nonamer with a 12 bp spacer resembles the RSS of a TcR J gene segment (4,27). So the "J gene-like" side is trimmed twice as much as the "V gene-like" side in both types of *tal*-1 deletions. This resembles the TcR-γ rearrangements where deletion by trimming of the Jγ gene segments is about twice that of the Vγ gene segments (46). Also, in *hprt* deletions this heptamer-spacer-nonamer-related nucleotide deletion is observed, where the "J gene-like" side of the *hprt* deletion class I is trimmed over twice as much as the other side. These combined data suggest that the size of the spacer induces direction to the activity of the recombination enzyme complex.

tal-1 deletions are restricted to the TcR-αβ lineage

tal-1 deletions are restricted to malignancies of the T-cell lineage since they have not been discovered in any other hematopoietic malignancy tested (8,22,25). However, the frequencies of the *tal*-1 deletions in T-ALL differ markedly between the reported studies, from 12% (14) to 26% (22). This difference may be caused by the compilation of the analyzed series of T-ALL. The high frequency of 26% *tal*-1 deletions may be an overestimation caused by an over-representation of TcR-$\alpha\beta^+$ T-ALL (22), because in our series such a high frequency of *tal*-1 deletions (27.5%) was only found in the group of TcR-$\alpha\beta^+$ T-ALL. However, in the study by Aplan et al. (25), almost all *tal*-1 deletions (10/11) were found in $CD3^-$ T-ALL, whereas only half of our *tal*-1 deletions (8/19) were found in $CD3^-$ T-ALL.

In our study, *tal*-1 deletions were detected only in TcR-$\alpha\beta^+$ (27.5%) and $CD3^-$ T-ALL

(11.6%), but not in TcR-$\gamma\delta^+$ T-ALL. This suggested that the occurrence of *tal*-1 deletions is restricted to T-ALL of the $\alpha\beta$ differentiation lineage. Therefore, we wished to investigate whether the $CD3^-$ T-ALL with a *tal*-1 deletion represented precursors of TcR-$\alpha\beta^+$ T-ALL or precursors of TcR-$\gamma\delta^+$ T-ALL. One of the present theories concerning the separation of the $\alpha\beta$ and $\gamma\delta$ differentiation pathways assumes that all T-cells that do not productively rearrange their TcR-γ and/or TcR-δ genes are capable of differentiation into TcR-$\alpha\beta$ committed T-cells by deletion of the TcR-δ locus, which is embedded in the TcR-α locus (30,40,42). If so, the configuration of the TcR-δ genes in $CD3^-$ T-ALL can be used cautiously as an $\alpha\beta/\gamma\delta$ lineage marker. Therefore, we divided the $CD3^-$ T-ALL into two subgroups on basis of their TcR-δ gene configuration. The first subgroup consisted of $CD3^-$ T-ALL (n = 42) without deletion of the TcR-δ locus but with TcR-δ gene rearrangement in most of them, and therefore resembled T-ALL of the $\gamma\delta$ lineage (Table 5). The other $CD3^-$ T-ALL subgroup (n = 27) had one or both TcR-δ alleles deleted and therefore may represent an early stage of the $\alpha\beta$ lineage. Almost all *tal*-1 deletions in $CD3^-$ T-ALL (7/8), were found in this putative $\alpha\beta$ lineage $CD3^-$ subgroup, and only one was found in the putative $\gamma\delta$ lineage $CD3^-$ subgroup. However, sequencing of the junctional regions revealed that all TcR-δ rearrangements in $CD3^-$ T-ALL with a *tal*-1 deletion were non-functional. Therefore these T-ALL could never express a TcR-δ chain and consequently belonged to the $\alpha\beta$ lineage. Thus, all *tal*-1 deletions appeared to be restricted to T-ALL of the $\alpha\beta$ lineage. Interestingly, the reported *hprt* deletions, which are comparable to the *tal*-1 deletions, were found in T-cell clones derived from mature blood T-lymphocytes (45). Although the precise TcR-$\alpha\beta$/TcR-$\gamma\delta$ phenotype of these T-cell clones was not reported, their CD4/CD8 phenotype strongly suggests that they belonged to the $\alpha\beta$ lineage (45). This would be in line with the restriction of *tal*-1 deletions to the $\alpha\beta$ lineage.

The finding that all *tal*-1 deletions were detected in the TcR-$\alpha\beta^+$ T-ALL or $CD3^-$ T-ALL of the $\alpha\beta$ lineage may be caused by a combination of two mechanisms. The first mechanism is based on the theory that in Ig or TcR gene recombination both recombining elements must be transcriptionally active (2,4). If *tal*-1 deletions are indeed caused by "illegitimate" V(D)J recombination, the simultaneous expression of the *sil* gene and *tal*-1 gene may be a prerequisite for recombination and thus deletion. This is supported by the findings that *tal*-1 expression in the few cases tested was restricted to TcR-$\alpha\beta^+$ T-ALL and $CD3^-$ T-ALL of $\alpha\beta$ lineage, whereas *sil* expression was not restricted to a particular subgroup of T-ALL (24; Breit et al., unpublished results). The second mechanism is based on the theory that once both TcR-δ alleles are rearranged, but no TcR-$\gamma\delta$ expression occurs due to non-functional rearrangements of TcR-γ and/or TcR-δ genes, the rearranged TcR-δ genes will be deleted via a special deletion mechanism involving the δREC and ψJα recombination elements (40,42). TcR-δ gene deletion prepares the allele for subsequent rearrangement of TcR-α gene segments and thereby forces the T-cells to differentiate into the $\alpha\beta$ lineage (30). One might speculate that a special TcR-δ gene deleting recombinase complex is present only in T-cells of the $\alpha\beta$ lineage and is also responsible for the *tal*-1 deletions and *hprt* deletions. This is supported by the finding that the frequency of *tal*-1 deletions increases with the number of deleted TcR-δ alleles. Where in T-ALL without TcR-δ gene deletions the frequency of *tal*-1 deletions was just 1.5% (1/65), in T-ALL with one deleted TcR-δ allele this frequency was 17.6% (6/34) and in T-ALL with TcR-δ gene deletions on both alleles the frequency of *tal*-1 deletions was substantially higher, 34.3% (12/35). Interestingly,

further deletion of the remaining (non-functional) TcR-δ genes in the T-ALL with a *tal*-1 deletion was not possible via δREC-ψJα rearrangements in most of them because of deletion of the δREC and/or ψJα gene segments (Table 4). Additional support for our speculation is found in the observation that the *hprt* deletions probably exclusively occur in TcR-$\alpha\beta^+$ T-lymphocytes, which generally have TcR-δ gene deletions on both alleles.

Rearrangement studies in which lymphoid cell lines of different lineages are transvected with extrachromosomal vectors containing the TcR-δ gene deleting elements and/or *sil*-*tal*-1 gene constructs might prove whether indeed a special TcR-δ gene deleting recombinase complex exists and whether this enzyme complex is involved in *tal*-1 deletions.

Based on our data, we hypothesize that the multiple enzymes of the Ig/TcR gene recombinase complex are differentially expressed, related to the differentiation lineage and differentiation stage of the lymphoid cells. The mechanisms regulating such putative differential expression could also determine the occurrence of oncogenic and non-oncogenic rearrangements and deletions in other genes with RSS homologous to the Ig and TcR genes. This would explain the restriction of particular chromosome aberrations to specific types of lymphoid leukemias, such as *tal*-1 deletions in T-ALL of the $\alpha\beta$ lineage.

After submission of this manuscript, Macintyre et al. (47) published a study on 39 T-ALL patients and concluded also that the occurrence of *tal*-1 deletions correlated with commitment to the $\alpha\beta$ lineage.

ACKNOWLEDGMENTS. The authors gratefully acknowledge Prof. Dr. R. Benner, Dr. H. Hooijkaas, Dr. A. Beishuizen, and Dr. H.J. Adriaansen for their continuous support; Dr. R. Kurrle (Behring, Marburg, Germany) for kindly providing the BMA031 antibody; Dr. T.H. Rabbitts, Dr. T. Quertermous, Dr. P. van de Elsen, Prof. Dr. R.J. Baer, and Prof. Dr. S.J. Korsmeyer for kindly providing the B2EE-2.0, TcR-γ, and TcR-δ probes; Dr. G.C. Grosveld and Dr. C. Troelstra for kindly providing the CML-0 genomic library; Mr. T.M. van Os for excellent assistance in the preparation of the figures; and Ms. A.D. Korpershoek for her secretarial support; Dr. D. Campana, Dr. K. Hählen, Dr. J.C. Kluin-Nelemans, Dr. R.J. van de Griend, and Dr. C.E. van der Schoot for kindly providing T-ALL cell samples; The Dutch Childhood Leukemia Study Group (DCLSG) kindly provided 32 of the 134 leukemia cell samples. Board members of the DCLSG are Drs. J.P.M. Bökkerink, M.V.A. Bruin, P.J. van Dijken, K. Hählen, W.A. Kamps, E.F. van Leeuwen, F.A.E. Nabben, A. Postma, J.A. Rammeloo, I.M. Risseeuw-Appel, G.A.M. de Vaan, E.Th. van 't Veer-Korthof, A.J.P. Veerman, F.C. de Waal, M. van Weel-Sipman, and R.S. Weening.

REFERENCES

1. Finger LR, Harvey RC, Moore RCA, Showe LC, Croce CM. A common mechanism of chromosomal translocation in T- and B-cell neoplasia. Science 1986;234:982-985.
2. Boehm T, Rabbitts TH. A chromosomal basis of lymphoid malignancy in man. Eur J Biochem 1989;185:1-17.
3. Tycko B, Sklar J. Chromosomal translocations in lymphoid neoplasia: a reappraisal of the recombinase model. Cancer Cells 1990;2:1-8.
4. Blackwell TK, Alt FW. Molecular characterization of the lymphoid V(D)J recombination activity. J Biol Chem 1989;264:10327-10330.
5. Schatz DG, Oettinger MA, Schlissel MS. V(D)J recombination: molecular biology and regulation. Annu Rev Immunol 1992;10:359-383.
6. Begley CG, Aplan PD, Davey MP, Nakahara K, Tchorz K, Kurtzberg J, Hershfield MS, Haynes BF, Cohen DI, Waldmann TA, Kirsch IR. Chromosomal translocation in a human leukemic stem-cell line disrupts the T-cell antigen receptor δ-chain diversity region and results in a previously unreported fusion transcript. Proc Natl Acad Sci USA 1989;86:2031-2035.

7. Finger LR, Kagan J, Christopher G, Kurtzberg J, Hershfield MS, Nowell PC, Croce CM. Involvement of the *TCL5* gene on human chromosome 1 in T-cell leukemia and melanoma. Proc Natl Acad Sci USA 1989;86:5039-5043.
8. Bernard O, Guglielmi P, Jonveaux P, Cherif D, Gisselbrecht S, Mauchauffe M, Berger R, Larsen C-J, Mathieu-Mahul D. Two distinct mechanisms for the *SCL* gene activation in the t(1;14) translocation of T-cell leukemias. Genes Chrom Cancer 1990;1:194-208.
9. Chen Q, Cheng J-T, Tsai L-H, Schneider N, Buchanan G, Carroll A, Crist W, Ozanne B, Siciliano MJ, Baer R. The *tal* gene undergoes chromosome translocation in T cell leukemia and potentially encodes a helix-loop-helix protein. EMBO J 1990;9:415-424.
10. Chen Q, Ying-Chuan Yang C, Tsou Tsan J, Xia Y, Ragab AH, Peiper SC, Carroll A, Baer R. Coding sequences of the *tal*-1 gene are disrupted by chromosome translocation in human T cell leukemia. J Exp Med 1990;172:1403-1408.
11. Carroll AJ, Crist WM, Link MP, Amylon MD, Pullen DJ, Ragab AH, Buchanan GR, Wimmer RS, Vietti TJ. The t(1;14)(p34;q11) is nonrandom and restricted to T-cell acute lymphoblastic leukemia: a pediatric oncology group study. Blood 1990;76:1220-1224.
12. Begley CG, Aplan PD, Denning SM, Haynes BF, Waldmann TA, Kirsch IR. The gene SCL is expressed during early hematopoiesis and encodes a differentiation-related DNA-binding motif. Proc Natl Acad Sci USA 1989;86:10128-10132.
13. Aplan PD, Begley CG, Bertness V, Nussmeier M, Ezquerra A, Coligan J, Kirsch IR. The SCL gene is formed from a transcriptionally complex locus. Mol Cell Biol 1990;10:6426-6435.
14. Bernard O, Lecointe N, Jonveaux P, Souyri M, Mauchauffé M, Berger R, Larsen CJ, Mathieu-Mahul D. Two site-specific deletions and t(1;14) translocation restricted to human T-cell acute leukemias disrupt the 5' part of the *tal*-1 gene. Oncogene 1991;6:1477-1488.
15. Green AR, Salvaris E, Begley CG. Erythroid expression of the "helix-loop-helix" gene, SCL. Oncogene 1991;6:475-479.
16. Murre C, Schonleber McCaw P, Baltimore D. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, *MyoD*, and *myc* proteins. Cell 1989;56:777-783.
17. Murre C, Schonleber McCaw P, Vaessin H, Caudy M, Jan LY, Jan YN, Cabrera CV, Buskin JN, Hauschka SD, Lassar AB, Weintraub H, Baltimore D. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 1989;58:537-544.
18. Hsu H-L, Cheng J-T, Chen Q, Baer R. Enhancer-binding activity of the *tal*-1 oncoprotein in association with the E47/E12 helix-loop-helix proteins. Mol Cell Biol 1991;11:3037-3042.
19. Visvader J, Begley CG, Adams JM. Differential expression of the *LYL*, *SCL* and *E2A* helix-loop-helix genes within the hemopoietic system. Oncogene 1991;6:187-194.
20. Visvader J, Begley CG. Helix-loop-helix genes translocated in lymphoid leukemia. TIBS 1991;16:330-333.
21. Green AR, Lints T, Visvader J, Harvey R, Begley CG. *SCL* is coexpressed with *GATA-1* in hemopoietic cells but is also expressed in developing brain. Oncogene 1992;7:653-600.
22. Brown L, Cheng J-T, Chen Q, Siciliano MJ, Crist W, Buchanan G, Baer R. Site-specific recombination of the *tal*-1 gene is a common occurrence in human T cell leukemia. EMBO J 1990;9:3343-3351.
23. Aplan PD, Lombardi DP, Ginsberg AM, Cossman J, Bertness VL, Kirsch IR. Disruption of the human SCL locus by "illegitimate" V-(D)-J recombinase activity. Science 1990;250:1426-1429.
24. Aplan PD, Lombardi DP, Kirsch IR. Structural characterization of *SIL*, a gene frequently disrupted in T-cell acute lymphoblastic leukemia. Mol Cell Biol 1991;11:5462-5469.
25. Aplan PD, Lombardi DP, Reaman GH, Sather HN, Hammond GD, Kirsch IR. Involvement of the putative hematopoietic transcription factor *SCL* in T-cell acute lymphoblastic leukemia. Blood 1992;79:1327-1333.
26. Alt FW, Baltimore D. Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-JH fusions. Proc Natl Acad Sci USA 1982;79:4118-4122.
27. Akira A, Okazaki K, Sakano H. Two pairs of recombination signals are sufficient to cause immunoglobulin V-(D)-J joining. Science 1987;238:1134-1138.
28. Hesse JE, Lieber MR, Mizuuchi K, Gellert M. V(D)J recombination: a functional definition of the joining signals. Genes Dev 1989;3:1053-1061.
29. Lafaille JJ, DeCloux A, Bonneville M, Takagaki Y, Tonegawa S. Junctional Sequences of T cell receptor γδ genes: implications for γδ T cell lineages and for a novel intermediate of V-(D)-J joining. Cell 1989;59:859-870.
30. Van Dongen JJM, Comans-Bitter WM, Wolvers-Tettero ILM, Borst J. Development of human T lymphocytes and their thymus-dependency. Thymus 1990;16:207-234.
31. Minowada J. Leukemia cell lines. Cancer Rev 1988;10:1-18.
32. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning, a laboratory manual, 2nd edn. Cold Spring Harbor: Cold Spring Harbor Laboratory, 1989.

33. De Klein A, Hagemeijer A, Bartram CR, Houwen R, Hoefsloot L, Carbonell F, Chan L, Barnett M, Greaves M, Kleihauer E, Heisterkamp N, Groffen J, Grosveld G. *bcr* rearrangement and translocation of the c-*abl* oncogene in Philadelphia positive acute lymphoblastic leukemia. Blood 1986;68:1369-1375.
34. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of immunoglobulin and T cell receptor genes. Part I: Basic and technical aspects. Clin Chim Acta 1991;198:1-92.
35. Duby AD, Klein KA, Murre C, Seidman JG. A novel mechanism of somatic rearrangement predicted by a human T-cell antigen receptor β-chain complementary DNA. Science 1985;228:1204-1206.
36. Quertermous T, Strauss WM, Van Dongen JJM, Seidman JG. Human T cell γ chain joining regions and T cell development. J Immunol 1987;138:2687-2690.
37. Loh EY, Cwirla S, Serafini AT, Phillips JH, Lanier LL. Human T-cell-receptor δ chain: genomic organization, diversity, and expression in populations of cells. Proc Natl Acad Sci USA 1988;85:9714-9718.
38. Baer R, Boehm T, Yssel H, Spits H, Rabbitts TH. Complex rearrangements within the human Jδ-Cδ/Jα-Cα locus and aberrant recombination between Jα segments. EMBO J 1988;7:1661-1668.
39. Boehm T, Baer R, Lavenir I, Forster A, Waters JJ, Nacheva E, Rabbitts TH. The mechanism of chromosomal translocation t(11;14) involving the T-cell receptor Cδ locus on human chromosome 14q11 and a transcribed region of chromosome 11p15. EMBO J 1988;7:385-394.
40. De Villartay J-P, Hockett RD, Coran D, Korsmeyer SJ, Cohen DI. Deletion of the human T-cell receptor δ-gene by a site-specific recombination. Nature 1988;335:170-174.
41. Breit TM, Wolvers-Tettero ILM, Hählen K, Van Wering ER, Van Dongen JJM. Extensive junctional diversity of $\gamma\delta$ T-cell receptors expressed by T-cell acute lymphoblastic leukemias: implications for the detection of minimal residual disease. Leukemia 1991;5:1076-1086.
42. Hockett RD, De Villartay J-P, Pollock K, Poplack DG, Cohen DI, Korsmeyer SJ. Human T-cell antigen receptor (TCR) δ-chain locus and elements responsible for its deletion are within the TCR α-chain locus. Proc Natl Acad Sci USA 1988;85:9694-9698.
43. Fuscoe JC, Zimmerman LJ, Lippert MJ, Nicklas JA, O'Neill JP, Albertini RJ. V(D)J recombinase-like activity mediates *hprt* gene deletion in human fetal T-lymphocytes. Cancer Res 1991;51:6001-6005.
44. McGinniss MJ, Nicklas JA, Albertini RJ. Molecular analysis of in vivo *hprt* mutations in human T-lymphocytes: IV. studies in newborns. Environ Mol Mutagen 1989;14:229-237.
45. McGinniss MJ, Falta MT, Sullivan LM, Albertini RJ. In vivo *hprt* mutant frequencies in T-cells of normal human newborns. Mutat Res 1990;240:117-126.
46. Breit TM, Wolvers-Tettero ILM, Van Dongen JJM. Receptor diversity of human T-cell receptor $\gamma\delta$ expressing cells. Prog Histochem Cytochem 1992;26:182-193.
47. Macintyre EA, Smit L, Ritz J, Kirsch IR, Strominger JL. Disruption of the SCL locus in T-lymphoid malignancies correlates with commitment to the T-cell receptor $\alpha\beta$ lineage. Blood 1992;80:1511-1520.

CHAPTER 4.3

LINEAGE SPECIFIC DEMETHYLATION OF *tal*-1 GENE BREAKPOINT REGION DETERMINES THE FREQUENCY OF *tal*-1 DELETIONS IN $\alpha\beta$ LINEAGE T-CELLS*

Timo M. Breit, Ingrid L.M. Wolvers-Tettero, and Jacques J.M. van Dongen

Department of Immunology, University Hospital Dijkzigt/Erasmus University, Rotterdam, The Netherlands.

ABSTRACT

tal-1 deletions are caused by a site specific recombination, which exclusively occurs in 12-26% of T-ALL. In a previous study on a large series of T-ALL we demonstrated an apparent preferential occurrence of *tal*-1 deletions in CD3$^-$ and CD3$^+$ $\alpha\beta$ lineage T-ALL with TcR-δ gene deletions on one or both alleles. In the present study we investigated whether accessibility of the *tal*-1 deletion breakpoint regions influences the preferential occurrence in specific T-ALL subgroups. Because DNA methylation is assumed to determine accessibility of DNA for recombination, the methylation status of the *tal*-1 deletion type 1 breakpoint regions (sildb and taldb1) was studied. Although the sildb were completely demethylated in all T-ALL, preferential (de)methylation configurations of the taldb1 were observed in the analyzed 119 T-ALL. Most TcR-$\alpha\beta^+$ T-ALL contained completely demethylated taldb1 (77%), whereas in most TcR-$\gamma\delta^+$ T-ALL partial or complete methylation occurred (42% and 47% respectively). In T-ALL subgroups defined by different TcR-δ gene configurations also preferential taldb1 (de)methylation patterns were seen, which was most prominent in T-ALL with both TcR-δ genes deleted (84% complete demethylation). The previously observed preferential occurrence of *tal*-1 deletion type 1 in TcR-$\alpha\beta^+$ versus CD3$^-$ T-ALL and in T-ALL with both versus one TcR-δ genes deleted, disappeared when we restricted to T-ALL with completely demethylated taldb1. Moreover, all T-ALL with a *tal*-1 deletion type 1 (n = 15) contained completely demethylated taldb1. We therefore conclude that complete demethylation of taldb1 is a prerequisite for *tal*-1 deletions type 1 and that the differences in *tal*-1 deletion frequencies observed in the various T-ALL subgroups are caused by differences in the (de)methylation status of taldb1 in these subgroups.

INTRODUCTION

The *tal*-1 gene on human chromosome 1p32 is frequently affected in T-ALL. Translocations t(1;14)(p32;q11), t(1;7)(p32;p35), and t(1;3)(p34;p21) recombine the *tal*-1 gene to the TcR-δ gene complex (1-5), the TcR-β gene complex (6), and a yet unidentified

* Published in: Oncogene 1994;9:1847-1853.

transcriptional unit on chromosome 3p21 (7), respectively. In addition, 12-26% of T-ALL contain a site-specific, so-called *tal*-1 deletion (8-13), in which the coding exons of the *tal*-1 gene (14,15) are juxtaposed to the first non-coding exons of the *sil* gene (16). As a result of this ~90 kb deletion, the *sil* coding exons are deleted and the expressed *sil-tal*-1 fusion mRNA in principle produces a normal TAL-1 protein, which is transcriptionally controlled by the *sil* gene promoter.

The site-specificity of *tal*-1 deletions is caused by the fact that they are mediated via RSS, which are homologous to the consensus heptamer-nonamer sequences in Ig and TcR genes (17,18). To date, six types of *tal*-1 deletions are described, five of which use the same 5′ RSS consisting of a sole heptamer located in the first *sil* intron, and different 3′ RSS consisting of heptamer-nonamer sequences located in the 5′ region of the *tal*-1 locus (8-12) (Figure 1). The two main types, type 1 and type 2 (also called type A and B, respectively) occur most frequently, whereas the four other types thus far have each been observed only once (8,10-13).

In an earlier study we showed that *tal*-1 deletions were correlated with CD3 phenotype, because no *tal*-1 deletions were found in TcR-$\gamma\delta^+$ T-ALL, whereas 12% of the CD3$^-$ T-ALL and 28% of the TcR-$\alpha\beta^+$ T-ALL contained such a deletion (12,19). The normal counterparts of T-ALL, i.e. the cortical thymocytes, differentiate from CD3$^-$ stages to CD3$^+$ stages with either TcR-$\gamma\delta$ or TcR-$\alpha\beta$ expression. The TcR-δ gene plays a pivotal role in the $\gamma\delta$ versus $\alpha\beta$ lineage commitment of thymocytes, because it is located within the TcR-α locus (20,21). Functionally rearranged TcR-δ genes coincides with germline TcR-α genes, but functional rearrangement of TcR-α genes needs deletion of the TcR-δ genes. Therefore deletion of the TcR-δ genes either by the specific rearrangement of the TcR-δ deleting elements δREC and ψJα (22,23), or by a Vα-Jα rearrangement, commits the thymocyte to the $\alpha\beta$ lineage. Based on this fact, we used the TcR-δ configuration to subdivide the T-ALL (12,24). This revealed that *tal*-1 deletions exclusively occurred in CD3$^-$ or CD3$^+$ T-ALL of the $\alpha\beta$ lineage with a frequency of 18% in T-ALL with one deleted TcR-δ allele, and 34% in T-ALL with TcR-δ gene deletions on both alleles (12,19).

tal-1 deletions are assumed to be caused by "illegitimate" V(D)J recombination, because they strongly resemble Ig and TcR rearrangements (25,26). The deletions not only use heptamer-nonamer RSS, their breakpoint fusion regions also show non-templated nucleotide addition (N-region), P-region nucleotides, and deletion of nucleotides by

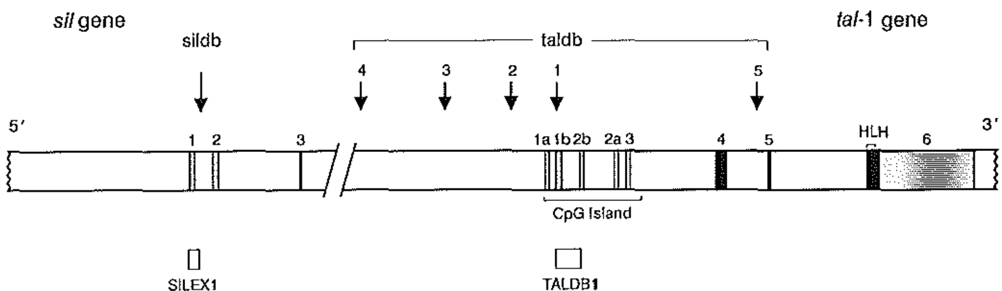


Figure 1. Schematic representation of the *tal*-1 deletion breakpoint regions in the *sil* gene and *tal*-1 gene, sildb and taldb, respectively (8,9). The various types of *tal*-1 deletion breakpoints are indicated with arrows (10-13). *tal*-1 deletion type 5 is described by Bash et al. (13) as *tal1*^{d3}. The CpG island is located according to Aplan et al. (15). Open boxes below the bar represent the probes used for Southern blot hybridization (12).

exonucleic nibbling from the flanking sequences (25-28). V(D)J recombination in general has at least three basic requirements: the availability of appropriate RSS (17,18), the presence of a functional recombinase enzyme complex (29), and the accessibility of the rearranging DNA sequences (30). RSS are present in the deletion breakpoint regions of the *sil* and *tal*-1 genes (sildb and taldb) (Figure 1) and mRNA transcripts of the recombinase activating genes RAG1 and RAG2 are found in T-ALL (31). We therefore investigated whether the accessibility of either the sildb and/or taldb influences the frequency of *tal*-1 deletions in the T-ALL subgroups as defined by CD3 phenotype and TcR-δ gene configuration.

Although early studies assumed that transcription of the rearranging gene segments is essential for V(D)J recombination (32,33), increasing evidence shows that only demethylation of these gene segments is required (34-38). Moreover, it has been proven with artificial extrachromosomal substrates as well as in leukemias that hypomethylated DNA sequences rearrange more frequently than methylated sequences (34-40). Also, breakpoint regions involved in translocations exhibit specific demethylation patterns (41-43). Therefore, we wished to determine whether the DNA (de)methylation status (i.e. DNA accessibility) of the *tal*-1 deletion breakpoint regions can explain the differences in the frequencies of *tal*-1 deletion between the various T-ALL subgroups and the absence of *tal*-1 deletions in precursor B-ALL and acute myeloid leukemia (AML) (12,44). For this purpose we analyzed 119 T-ALL (including 20 T-ALL with a *tal*-1 deletion), 16 precursor B-ALL, and 51 AML by Southern blot analysis with the isoschizomeric restriction enzymes *Msp*I and *Hpa*II, which are insensitive and sensitive to DNA methylation, respectively (45). By hybridizing DNA probes to the *tal*-1 deletion breakpoint regions, we could study lineage specific (de)methylation of these breakpoint regions and its effect on the frequency of *tal*-1 deletions in T-ALL subgroups of the αβ lineage.

MATERIALS AND METHODS

Cell samples

Leukemia cell samples were obtained from 119 T-ALL patients, 16 precursor B-ALL, and 51 AML at initial diagnosis. Of almost all acute leukemias the CD3 phenotype, TcR-δ gene configuration and the *tal*-1 deletions (15 type 1, 3 type 2, 1 type 3, and 1 type 4) were described previously, as well as the relationship between these characteristics (12,24,44,46). Thymocyte cell samples were obtained as described before from thymi of seven children (<16 years) undergoing cardiac surgery (44,47). Four T-cell lines containing a *tal*-1 deletion type 1 were also analyzed: CEM, HSB-2, Molt 16, and RPMI-8402 (48).

Analysis of demethylation status

The demethylation status of the *tal*-1 deletion breakpoint regions, sildb and taldb was determined by parallel digestions with the isoschizomeric restriction enzymes *Msp*I and *Hpa*II (Pharmacia, Uppsala, Sweden) (45). 15 μg of high molecular weight DNA was digested with *Msp*I, *Msp*I/*Eco*RI, *Hpa*II or *Hpa*II/*Eco*RI, using 25 Units of each restriction enzyme in a one hour incubation at 37°C. Digested DNA was size-fractionated in 0.7% agarose gels and transferred to Nytran-13N nylon membranes (Schleicher & Schuell Inc., Dassel, Germany) as described (47). The DNA methylation status was analyzed by hybridization with the ^{32}P random-labeled 0.8 kb TALDB1 probe (12), and the 0.5 kb SILEX1 probe (Figure 1). The SILEX1 probe is a *Msp*I-*Msp*I subclone of the *Bam*HI cloned PCR product (~0.6 kb) obtained by amplification of control DNA using the sildp5′(12) and silex1p3′ (cacagGATCCCTTATACTACGTAGCAGAGTG) oligonucleotide primers as described (12).

We defined three possible configurations for the demethylation status: complete (+), partial (±), and

absent (–) demethylation. Examples of the various demethylation configurations are presented in Figure 2. Complete demethylation results in a single *Hpa*II band of the same size as seen in the *Msp*I digest of the same patient. Absence of demethylation (i.e. complete methylation) results in *Hpa*II bands which all are larger than the *Msp*I band. Partial demethylation represents all banding patterns in between complete and absent demethylation and results in a *Hpa*II band of the same size as well as larger bands as compared to the *Msp*I digest.

RESULTS AND DISCUSSION

Demethylation of the *sil* and *tal*-1 gene

The 119 T-ALL, 16 precursor B-ALL, 51 AML and seven thymocyte samples were analyzed for the demethylation status of the *tal*-1 deletion breakpoint regions, sildb and taldb, which are located in the 5′ regions of the *sil* gene and the *tal*-1 gene, respectively (8-12) (Figure 1). The sildb displayed complete demethylation in all T-ALL, precursor B-ALL, AML, and thymocyte samples. This implies that the 5′ RSS of the *tal*-1 deletion located in the *sil* gene is accessible for recombination during all stages of lymphoid differentiation, which is in line with the broad expression of the SIL protein during hematopoiesis. Therefore, any preference for the occurrence of *tal*-1 deletions has to be found in the accessibility of the taldb.

Only the breakpoint region of *tal*-1 deletion type 1 (taldb1) was analyzed, because it is located in a so-called CpG island and contains therefore several *Hpa*II restriction sites, whereas no such restriction sites are present in the *tal*-1 deletion type 2 breakpoint region (taldb2) (15,49). *tal*-1 deletion type 3 and type 4 breakpoint regions (taldb3 and taldb4) were not analyzed, because they each were only observed in a single patient. Analysis of the taldb1 revealed that in the total group of T-ALL, all three demethylation configurations were present in approximately equal percentages (Table 1) (Figure 2). Hence, there are variations in the demethylation status and therefore probably differences in accessibility of the 3′ RSS of the *tal*-1 deletion type 1.

The majority of the analyzed precursor B-ALL (75%) and AML (90%) displayed complete demethylation of taldb1, while the rest showed predominantly partial demethylation (Table 1). In all polyclonal thymocyte samples the taldb1 was completely demethylated (Figure 2).

It should be stressed that there is most probably no direct correlation between demethylation of the taldb1 and expression of the *tal*-1 gene, because the 5′ region of the gene -including the taldb1- contains a CpG island (49,50). In general, CpG islands are non-methylated, even when the gene they belong to is inactive (49,51), which probably also applies to the *tal*-1 gene. This might explain the demethylation of taldb1 in the majority of precursor B-ALL and AML.

Demethylation in CD3 phenotypic T-ALL subgroups

Dividing the T-ALL in subgroups according to their CD3 phenotype revealed variations in preferential demethylation configurations of the taldb1 between these subgroups. The subgroup frequencies are summarized in Table 1. In $CD3^-$ T-ALL, all configurations of demethylation were present in roughly equal frequencies, whereas TcR-$\gamma\delta^+$ T-ALL demonstrated a strong preference for partial (42%) or absent (47%) demethylation. Most

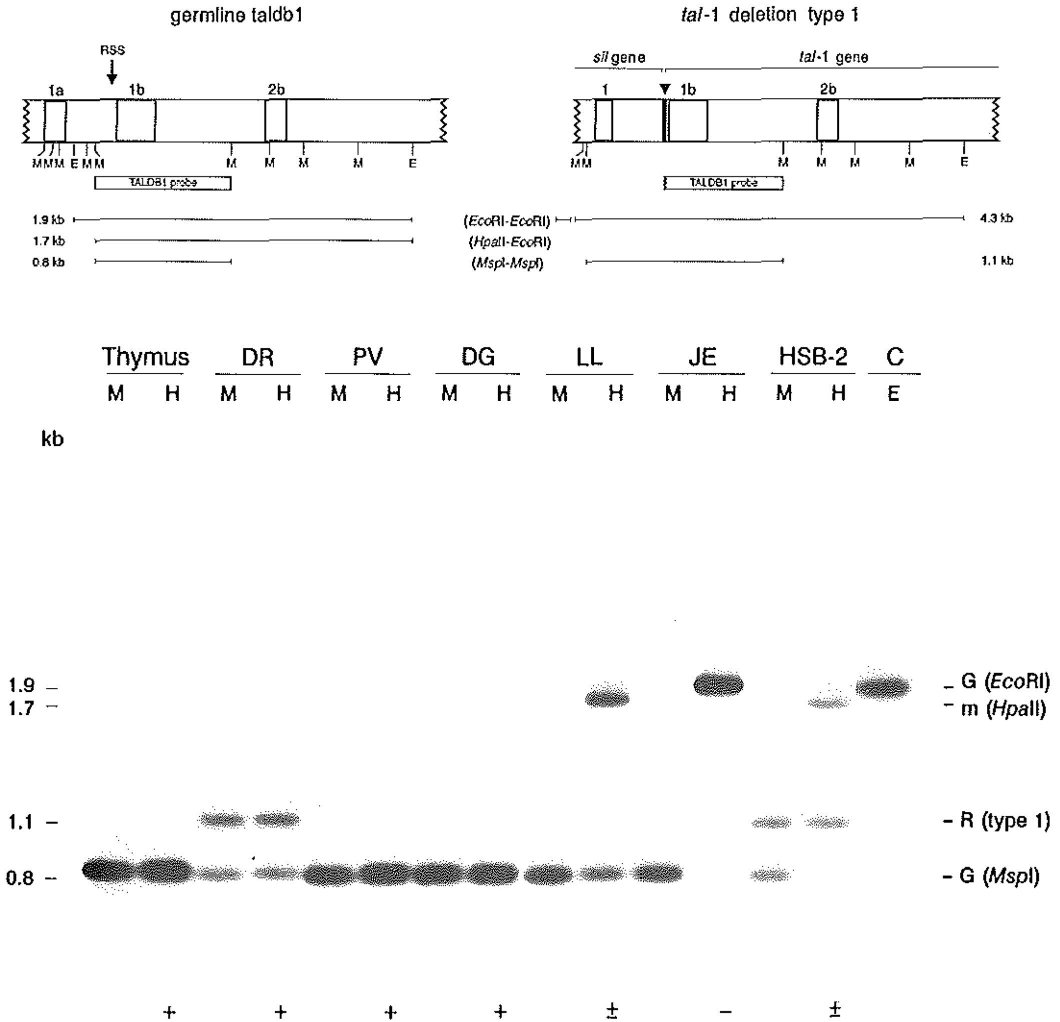


Figure 2. Southern blot demethylation patterns of the *tal*-1 deletion breakpoint region type 1 in T-ALL. **Top:** Restriction maps of the germline taldb1 (left) and rearranged taldb1 (right) for *MspI/HpaII* (M) and *EcoRI* (E). Indicated are the position of the RSS (↓) in the germline taldb1 region and the fusion region (▾) of the *tal*-1 deletion type 1. The restriction fragments that are generated by *MspI* and *EcoRI* digests, or by the methylation sensitive *HpaII/EcoRI* digest are shown. **Bottom:** Hybridization with the TALDB1 probe of parallel DNA digestions using isoschizomeric restriction enzymes *MspI* (M) and *HpaII* (H) in combination with *EcoRI* (E). Lanes 1 and 2, thymic cell sample; lanes 3 and 4, T-ALL patient DR with a *tal*-1 deletion type 1; lanes 5 and 6, T-ALL patient PV with a *tal*-1 deletion type 2; lanes 7 and 8, T-ALL patient DG; lanes 9 and 10, T-ALL patient LL; lanes 11 and 12, T-ALL patient JE; lines 13 and 14, T-cell line HSB-2 with a *tal*-1 deletion type 1; lane 15, control DNA digested with *EcoRI* only. Indicated are; the germline bands (G) of *MspI* and *EcoRI*, the band caused by methylation (m) of 3′ *HpaII* sites, and the rearranged band (R) of *tal*-1 deletion type 1. Given at the bottom of the figure are the various configurations of the *tal*-1 gene demethylation status; complete (+), partial (±), and absent (–) demethylation.

TABLE 1. Demethylation status of taldb1 and frequency of *tal*-1 deletions type 1 in AML, precursor B-ALL, and T-ALL subgroups defined by CD3 phenotype or TcR-δ gene configuration.

				CD3 phenotype of T-ALL			TcR-δ gene configuration of T-ALL[a]			
	AML (n=51)	precursor B-ALL (n=16)	Total T-ALL (n=119)	$CD3^-$ (n=65)	TcR-$\gamma\delta^+$ (n=19)	TcR-$\alpha\beta^+$ (n=35)	G/G (n=6)	R/G, R/R (n=55)	D/R (n=27)	D/D (n=31)
demethylation status taldb1										
+ (complete)	90.2% (46)	75.0% (12)	39.5% (47)	27.7% (18)	10.5% (2)	77.1% (27)	33.3% (2)	12.7% (7)	44.4% (12)	83.9% (26)
± (partial)	7.8% (4)	25.0% (4)	32.8% (39)	40.0% (26)	42.1% (8)	14.3% (5)	33.3% (2)	49.1% (27)	22.2% (6)	12.9% (4)
− (absent)	2.0% (1)	0% (0)	27.7% (33)	32.3% (21)	47.4% (9)	8.6% (3)	33.3% (2)	38.2% (21)	33.3% (9)	3.2% (1)
frequency of *tal*-1 deletion type 1										
+ (complete demethylation)[b]	0% (0/46)	0% (0/12)	31.9% (15/47)	33.3% (6/18)	0% (0/2)	33.3% (9/27)	0% (0/2)	0% (0/7)	50.0% (6/12)	34.6% (9/26)
Total group[b]	0% (0/51)	0% (0/16)	12.6% (15/119)	9.2% (6/65)	0% (0/19)	25.7% (9/35)	0% (0/6)	0% (0/55)	22.2% (6/27)	29.0% (9/31)

a. TcR-δ configuration: G, allele in germline configuration; R, rearranged allele; D, deleted allele.

b. Frequencies of *tal*-1 deletions type 1 in the AML, precursor B-ALL, and T-ALL subgroups defined by CD3 phenotype or TcR-δ gene configuration and in the subgroups of these acute leukemias defined by complete demethylation of taldb1. Subgroups with partial or absent demethylated taldb1 did not contain any *tal*-1 deletions type 1.

strikingly was the finding that in the majority (77%) of the TcR-$\alpha\beta^+$ T-ALL the taldb1 was completely demethylated. Because T-ALL are presumed to originate from cortical thymocytes, this observation is in line with the completely demethylated taldb1 in the thymocyte samples, which predominantly consist of TcR-$\alpha\beta^+$ thymocytes (52). Thus, in CD3$^+$ T-ALL there is a clear difference in the taldb1 demethylation status associated with $\alpha\beta/\gamma\delta$ TcR phenotype.

Demethylation in TcR-δ gene configuration T-ALL subgroups

When the TcR-δ gene configuration is introduced as differentiation marker, it is possible to identify T-ALL with different allelic combinations of germline (G), rearranged (R), and deleted (D) TcR-δ genes: i.e. G/G, R/G, R/R, D/R, and D/D. These T-ALL subgroups also revealed different frequencies of taldb1 demethylation configurations (Table 1). When both TcR-δ alleles are in germline configuration, there was no preference for a specific demethylation configuration. In the T-ALL subgroup with at least one rearranged TcR-δ allele, but no TcR-δ gene deletion (R/G and R/R), there was a preference for partial (49%) or absent (38%) demethylation configurations. The subgroup with deletion of one TcR-δ allele showed a slight preference (44%) for complete demethylation, whereas the subgroup with both TcR-δ alleles deleted demonstrated a marked preference (84%) for complete demethylation of the taldb1. All eighteen T-ALL with an incomplete TcR-δ gene rearrangement (Dδ-Dδ, Dδ-Jδ, or Vδ-Dδ) on at least one allele did not contain completely demethylated taldb1, irrespectively of the TcR-δ gene configuration of the other allele (results not shown).

Combining the three markers: taldb1 demethylation, CD3 phenotype, and TcR-δ gene configuration enhances the already discovered correlations. This is most obvious in the TcR-$\alpha\beta^+$ T-ALL with both TcR-δ alleles deleted, of which almost all (91%; 20/22) contained completely demethylated taldb1.

As for now, it appears that in relative immature T-ALL with still unrearranged TcR genes all stages of taldb1 methylation occur, whereas TcR-δ gene rearrangement is correlated with methylation in the *tal*-1 gene, which might be caused by the mechanism of so-called protective methylation (53). This hypermethylation is found in germline Ig and TcR genes prior to rearrangement processes to protect them from uninduced recombination (53), and might therefore also be involved in protection of other genes. TcR-δ gene deletion and/or TcR-α gene rearrangement however is correlated with demethylation in the *tal*-1 gene.

Demethylation in precursor B-ALL and AML

The 16 analyzed precursor B-ALL existed of two null-ALL, 12 common ALL and two pre B-ALL. In the null ALL the taldb1 were completely demethylated, whereas in common ALL 83% was completely and 17% was partially demethylated. In pre B-ALL only partially demethylated taldb1 were found. There was no correlation between TcR-δ gene rearrangement/deletion and taldb1 demethylation in precursor B-ALL, which is in line with the lack of correlation between TcR-δ gene rearrangement and immunophenotype in precursor B-ALL. However, in this small series of precursor B-ALL the taldb1 demethylation seems to decrease parallel to phenotypic maturation, suggesting that protective methylation of the *tal*-1 gene occurs in late stages of precursor B-cell differentiation.

In the 51 analyzed AML only five (10%) contained (partially) methylated taldb1, whereas all others contained completely demethylated taldb1. TcR-δ gene rearrangements in AML are rare (5%) (46), but all three analyzed AML with TcR-δ gene rearrangements contained partially demethylated taldb1. Of AML without TcR-δ gene rearrangements, the majority (96%; 46/48) contained completely demethylated taldb1. Therefore, TcR-δ gene rearrangement in AML seems to be correlated with taldb1 methylation, which may be caused by the mechanism of protective methylation.

tal-1 deletions and taldb1 demethylation status

Because the frequencies of demethylated taldb1 in the various T-ALL subgroups seem to parallel the observed frequencies of *tal*-1 deletion type 1, the correlation between these two variables was investigated. Strikingly, all 15 T-ALL with a *tal*-1 deletion type 1 had completely demethylated taldb1 on both alleles (Table 1) (Figure 2). This was also true for the T-ALL containing a *tal*-1 deletion type 2 and type 3 (Figure 1). The T-ALL with a *tal*-1 deletion type 4 had a partial demethylation configuration of taldb1 caused by methylation of the 3′ *Hpa*II restriction site on the unaffected allele. For now, it is uncertain what the meaning is of taldb1 demethylation for recombination of upstream located breakpoints. An analogous observation of allele specific partial demethylation was made in the four T-ALL derived T-cell lines containing a *tal*-1 deletion type 1, where methylation was seen exclusively on the non-affected *tal*-1 allele and only involved the 3′ *Hpa*II restriction sites. This is probably caused by *de novo* methylation, which is a general mechanism for cell lines to shut off non-essential genes (54).

In *tal*-1 deletions it is impossible to determine whether the demethylation of the taldb1 on the affected allele was already present at the moment of recombination, or resulted from the joining to the constitutive demethylated sildb. However, altogether our data strongly support the hypothesis that demethylation of the taldb1 is a prerequisite for recombination. If this is true, it can be interpreted as evidence for the existence of a T-cell specific recombinase complex, because the majority of precursor B-ALL and AML also have completely demethylated taldb1, but never contain a *tal*-1 deletion (44).

tal-1 deletions and taldb1 demethylation status in CD3 phenotypic T-ALL subgroups

Determination of the *tal*-1 deletion type 1 frequencies in T-ALL subgroups defined by their CD3 phenotype and taldb1 demethylation status revealed equal percentages in $CD3^-$ T-ALL (33%;6/18) and TcR-$\alpha\beta^+$ T-ALL (33%;9/27) subgroups with completely demethylated taldb1 (Table 1). This is in sharp contrast to the frequency of tal-1 deletion type 1 in the total group of $CD3^-$ T-ALL (9%) and TcR-$\alpha\beta^+$ (26%) (Table 1). It can be concluded that the differences in taldb1 demethylation (Table 1) caused the variation in *tal*-1 deletion type 1 frequencies between these CD3 phenotypic subgroups. The absence of *tal*-1 deletions in the TcR-$\gamma\delta^+$ subgroup may be explained by the fact that only two of the 19 tested TcR-$\gamma\delta^+$ T-ALL had completely demethylated taldb1 (Table 1).

tal-1 deletions and taldb1 demethylation status in TcR-δ gene configuration T-ALL subgroups

Finally, *tal*-1 deletion type 1 frequencies were determined in T-ALL subgroups defined by TcR-δ gene configuration and taldb1 demethylation (Table 1). As shown before, no *tal*-1

deletions were present in the T-ALL with both TcR-δ genes in germline configuration, which might be explained by the lack of recombinase activity in those immature T-ALL. However, in the T-ALL subgroups with one or two TcR-δ alleles deleted, the results were comparable to those in the CD3 phenotypic subgroups. The *tal*-1 deletion type 1 frequencies in the D/R and D/D subgroups (22%; 6/27 and 29%; 9/31, respectively) were reversed when we restricted to T-ALL with completely demethylated taldb1 (50%; 6/12 and 35%; 9/26, respectively) (Table 1). So, the differential demethylation of the taldb1 caused the initial observed differences in *tal*-1 deletion frequencies. This observation is supported by the finding that T-ALL with an incomplete TcR-δ rearrangement never contained a *tal*-1 deletion, probably because these T-ALL did not contain completely demethylated taldb1 (results not shown).

Comparing the average *tal*-1 deletion type 1 frequency in T-ALL with completely demethylated taldb1 and at least one deleted TcR-δ allele (39%; 15/38) to the total absence of these *tal*-1 deletions in T-ALL with only rearranged TcR-δ genes (0/7), demonstrated a significant difference between these subgroups (Table 1). Formally, we cannot rule out the possibility that the overexpression of the TAL-1 protein as a result of a *tal*-1 deletion forces the affected thymocyte and thus the eventual T-ALL into the αβ lineage by TcR-δ gene deletion and/or TcR-α gene rearrangement.

CONCLUSION

To explain the absence of *tal*-1 deletions type 1 in T-ALL without TcR-δ gene deletion, we postulate the existence of a special recombinase complex for TcR-δ gene deletion and/or TcR-α gene rearrangement, which is exclusively present in immature thymocytes committed to the αβ lineage. These thymocytes probably are characterized by two non-functionally Vδ-(Dδ)-Jδ rearrangements or at least one TcR-δ gene deletion. Whether this specific recombinase complex seizes at the TcR-δ gene deleting elements (δREC and ψJα) (22,23), or represents just a specific TcR-α rearrangement recombinase complex has to be elucidated. A hypothetical scheme linking together all components and processes involved in *tal*-1 deletions, is presented in Figure 3. In this scheme the putative TcR-δ gene deletion (TcR-α gene rearrangement) recombinase complex is responsible for TcR-δ gene deletion, which commits the thymocyte to the αβ lineage. As side effect this specific recombinase

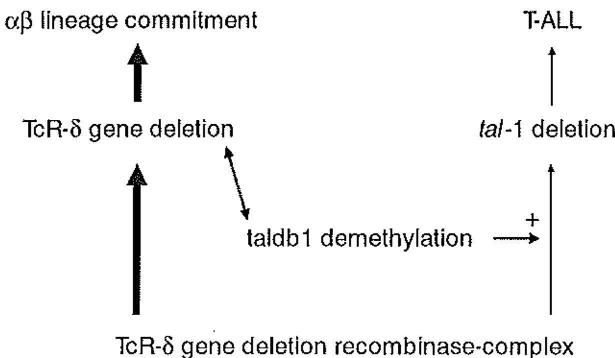


Figure 3. Hypothetical scheme linking together all components and processes involved in the *tal*-1 deletion. For explanation see Conclusion.

complex is able to generate a deletion involving the *tal*-1 gene on chromosome 1, which probably induces the thymocyte to become a malignant cell. The latter conclusion is based on the presumed oncogenic character of the aberration and the fact that *tal*-1 deletions are not detectable in normal thymocytes and blood T-lymphocytes (44,55). The occurrence of *tal*-1 deletion type 1 is dependent on the accessibility (= demethylation) of the taldb1 for the recombinase complex, which on its turn is correlated to TcR-δ gene deletion. Whether the demethylation status of the *tal*-1 gene or expression of the TAL-1 protein regulates in any way the deletion of the TcR-δ gene and thereby indirectly determines the T-cell differentiation lineage or vice versa, has to be resolved by further studies.

ACKNOWLEDGMENTS. The authors gratefully acknowledge Prof. Dr. R. Benner and Dr. H. Hooijkaas for their continuously support; Mr. T.M. van Os for excellent assistance in the preparation of the figures; and Ms. A.D. Korpershoek for her secretarial support; Prof. Dr. E. Bos and Dr. A.J.J.C. Bogers for kindly providing the thymic samples; Dr. E.J. Harthoorn-Lasthuizen, Dr. C. van der Heul, Dr. A.C.J.M. Holdrinet, Dr. M.C. Kappers-Klunne, Prof. Dr. B. Löwenberg, Dr. W-D. Ludwig, Dr. W.E. Terpstra, Dr. M.B. van 't Veer, and the Dutch Childhood Leukemia Study Group (DCLSG) for kindly providing leukemia cell samples.

REFERENCES

1. Begley CG, Aplan PD, Davey MP, Nakahara K, Tchorz K, Kurtzberg J, Hershfield MS, Haynes BF, Cohen DI, Waldmann TA, Kirsch IR. Chromosomal translocation in a human leukemic stem-cell line disrupts the T-cell antigen receptor δ-chain diversity region and results in a previously unreported fusion transcript. Proc Natl Acad Sci USA 1989;86:2031-2035.
2. Bernard O, Guglielmi P, Jonveaux P, Cherif D, Gisselbrecht S, Mauchauffe M, Berger R, Larsen C-J, Mathieu-Mahul D. Two distinct mechanisms for the *SCL* gene activation in the t(1;14) translocation of T-cell leukemias. Genes Chromosomes & Cancer 1990;1:194-208.
3. Chen Q, Cheng J-T, Tsai L-H, Schneider N, Buchanan G, Carroll A, Crist W, Ozanne B, Siciliano MJ, Baer R. The *tal* gene undergoes chromosome translocation in T cell leukemia and potentially encodes a helix-loop-helix protein. EMBO J 1990;9:415-424.
4. Chen Q, Ying-Chuan Yang C, Tsou Tsan J, Xia Y, Ragab AH, Peiper SC, Carroll A, Baer R. Coding sequences of the *tal*-1 gene are disrupted by chromosome translocation in human T cell leukemia. J Exp Med 1990;172:1403-1408.
5. Carroll AJ, Crist WM, Link MP, Amylon MD, Pullen DJ, Ragab AH, Buchanan GR, Wimmer RS, Vietti TJ. The t(1;14)(p34;q11) is nonrandom and restricted to T-cell acute lymphoblastic leukemia: a pediatric oncology group study. Blood 1990;76:1220-1224.
6. Fitzgerald TJ, Neale GAM, Raimondi SC, Goorha RM. *c-tal*, a helix-loop-helix protein, is juxtaposed to the T-cell receptor-β chain gene by a reciprocal chromosomal translocation: t(1;7) (p32;q35). Blood 1991;78:2686-2695.
7. Aplan PD, Raimondi SC, Krisch IR. Disruption of the SCL Gene by a t(1;3) translocation in a patient with T cell acute lymphoblastic leukemia. J Exp Med 1992;176:1303-1310.
8. Brown L, Cheng J-T, Chen Q, Siciliano MJ, Crist W, Buchanan G, Baer R. Site-specific recombination of the *tal*-1 gene is a common occurrence in human T cell leukemia. EMBO J 1990;9:3343-3351.
9. Aplan PD, Lombardi DP, Ginsberg AM, Cossman J, Bertness VL, Kirsch IR. Disruption of the human SCL locus by "illegitimate" V-(D)-J recombinase activity. Science 1990;250:1426-1429.
10. Bernard O, Lecointe N, Jonveaux P, Souyri M, Mauchauffé M, Berger R, Larsen CJ, Mathieu-Mahul D. Two site-specific deletions and t(1;14) translocation restricted to human T-cell acute leukemias disrupt the 5' part of the *tal*-1 gene. Oncogene 1991;6:1477-1488.
11. Aplan PD, Lombardi DP, Reaman GH, Sather HN, Hammond GD, Kirsch IR. Involvement of the putative hematopoietic transcription factor *SCL* in T-cell acute lymphoblastic leukemia. Blood 1992;79:1327-1333.
12. Breit TM, Mol EJ, Wolvers-Tettero ILM, Ludwig W-D, Van Wering ER, Van Dongen JJM. Site-specific deletions involving the *tal*-1 and *sil* genes are restricted to cells of the T cell receptor α/β lineage: T cell receptor δ gene deletion mechanism affects multiple genes. J Exp Med 1993;177:965-977.

13. Bash RO, Crist WM, Shuster JJ, Link MP, Amylon M, Pullen J, Carroll AJ, Buchanan GR, Smith RG, Baer R. Clinical features and outcome of T-cell acute lymphoblastic leukemia in childhood with respect to alterations at the *TAL1* locus: a pediatric oncology group study. Blood 1993;81:2110-2117.
14. Begley CG, Aplan PD, Denning SM, Haynes BF, Waldmann TA, Kirsch IR. The gene SCL is expressed during early hematopoiesis and encodes a differentiation-related DNA-binding motif. Proc Natl Acad Sci USA 1989;86:10128-10132.
15. Aplan PD, Begley CG, Bertness V, Nussmeier M, Ezquerra A, Coligan J, Kirsch IR. The SCL gene is formed from a transcriptionally complex locus. Mol Cell Biol 1990;10:6426-6435.
16. Aplan PD, Lombardi DP, Kirsch IR. Structural characterization of *SIL*, a gene frequently disrupted in T-cell acute lymphoblastic leukemia. Mol Cell Biol 1991;11:5462-5469.
17. Akira A, Okazaki K, Sakano H. Two pairs of recombination signals are sufficient to cause immunoglobulin V-(D)-J joining. Science 1987;238:1134-1138.
18. Hesse JE, Lieber MR, Mizuuchi K, Gellert M. V(D)J recombination: a functional definition of the joining signals. Genes Dev 1989;3:1053-1061.
19. Macintyre EA, Smit L, Ritz J, Kirsch IR, Strominger JL. Disruption of the SCL locus in T-lymphoid malignancies correlates with commitment to the T-cell receptor $\alpha\beta$ lineage. Blood 1992;80:1511-1520.
20. Isobe M, Russo G, Haluska FG, Croce CM. Cloning of the gene encoding the δ subunit of the human T-cell receptor reveals its physical organization within the α-subunit locus and its involvement in chromosome translocations in T-cell malignancy. Proc Natl Acad Sci USA 1988;85:3933-3937.
21. Satyanarayana K, Hata S, Devlin P, Roncarolo MG, De Vries JE, Spits H, Strominger JL, Krangel MS. Genomic organization of the human T-cell antigen-receptor α/δ locus. Proc Natl Acad Sci USA 1988;85:8166-8170.
22. De Villartay J-P, Hockett RD, Coran D, Korsmeyer SJ, Cohen DI. Deletion of the human T-cell receptor δ-gene by a site-specific recombination. Nature 1988;335:170-174.
23. Hockett RD, De Villartay J-P, Pollock K, Poplack DG, Cohen DI, Korsmeyer SJ. Human T-cell antigen receptor (TCR) δ-chain locus and elements responsible for its deletion are within the TCR α-chain locus. Proc Natl Acad Sci USA 1988;85:9694-9698.
24. Breit TM, Wolvers-Tettero ILM, Beishuizen A, Verhoeven M-AJ, Van Wering ER, Van Dongen JJM. Southern blot patterns, frequencies and junctional diversity of T-cell receptor δ gene rearrangements in acute lymphoblastic leukemia. Blood 1993;82:3063-3074.
25. Blackwell TK, Alt FW. Molecular characterization of the lymphoid V(D)J recombination activity. J Biol Chem 1989;264:10327-10330.
26. Schatz DG, Oettinger MA, Schlissel MS. V(D)J recombination: molecular biology and regulation. Annu Rev Immunol 1992;10:359-363.
27. Alt FW, Baltimore D. Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-JH fusions. Proc Natl Acad Sci USA 1982;79:4118-4122.
28. Lafaille JJ, DeCloux A, Bonneville M, Takagaki Y, Tonegawa S. Junctional Sequences of T cell receptor $\gamma\delta$ genes: implications for $\gamma\delta$ T cell lineages and for a novel intermediate of V-(D)-J joining. Cell 1989;59:859-870.
29. Oettinger MA. Activation of V(D)J recombination by *RAG1* and *RAG2*. TIG 1992;8:413-416.
30. Alt FW, Blackwell TK, Yancopoulos GD. Development of the primary antibody repertoire. Science 1987;238:1079-1087.
31. Bories JC, Cayuela JM, Loiseau P, Sigaux F. Expression of human recombination activating genes (RAG1 en RAG2) in neoplastic lymphoid cells: correlation with cell differentiation and antigen receptor expression. Blood 1991;78:2053-2061.
32. Martin D, Huang R, LeBien T, Van Ness B. Induced rearrangement of κ genes in the BLIN-1 human pre-B cell line correlates with germline J-C_κ and V_κ transcription. J Exp Med 1991;173:639-645.
33. Schlissel MS, Corcoran LM, Baltimore D. Virus-transformed pre-B cells show ordered activation but not inactivation of immunoglobulin gene rearrangement and transcription. J Exp Med 1991;173:711-720.
34. Engler P, Roth P, Kim JY, Storb U. Factors affecting the rearrangement efficiency of an Ig test gene. J Immunol 1991;146:2826-2835.
35. Engler P, Haasch D, Pinkert CA, Doglio L, Glymour M, Brinster R, Storb U. A strain-specific modifier on mouse chromosome 4 controls the methylation of independent transgene loci. Cell 1991;65:939-947.
36. Hsieh C-L, McCloskey RP, Lieber MR. V(D)J recombination on minichromosomes is not affected by transcription. J Biol Chem 1992;267:15613-15619.
37. Hsieh C-L, Lieber MR. CpG methylated minichromosomes become inaccessible for V(D)J recombination after undergoing replication. EMBO J 1992;11:315-325.
38. Engler P, Weng A, Storb U. Influence of CpG methylation and target spacing on V(D)J recombination in a transgenic substrate. Mol Cell Biol 1993;13:571-577.

39. Tauchi T, Ohyashiki JH, Ohyashiki K, Saito M, Nakazawa S, Kimura N, Toyama K. Methylation status of receptor β-chain gene in B precursor acute lymphoblastic leukemia: correlation with hypomethylation and rearrangement. Cancer Res 1991;51:2917-2921.
40. Ohyashiki JH, Ohyashiki K, Kawakubo K, Tauchi T, Nakazawa S, Kimura N, Toyama K. T-cell receptor β chain rearrangement in acute myeloid leukemia always occurs at the allele that contains the undermethylated Jβ1 re Cancer Res 1992;52:6598-6602.
41. Litz CE, McClure JS, Coad JE, Goldfarb AN, Brunning RD. Methylation status of the major breakpoint cluster r in Philadelphia chromosome negative leukemias. Leukemia 1992;6:35-41.
42. Tsukamoto N, Morita K, Karasawa M, Omine M. Methylation status of c-*myc* oncogene in leukemic hypomethylaton in acute leukemia derived from myelodysplastic syndromes. Exp Hematol 1992;20:1061-
43. Ohyashiki JH, Ohyashiki K, Kawakubo K, Tauchi T, Shimamoto T, Toyama K. The methylation status of the breakpoint cluster region in human leukemia cells, including Philadelphia chromosome-positive cells, is linked lineage of hematopoietic cells. Leukemia 1993;7:801-807.
44. Breit TM, Beishuizen A, Ludwig W-D, Mol EJ, Adriaansen HJ, Van Wering ER, Van Dongen JJM. *tal*-1 deleti T-cells acute lymphoblastic leukemia as PCR target for detection of minimal residual disease. Leu 1993;7:2004-2011.
45. Waalwijk C, Flavell RA. MspI, an isoschizomer of HpaII which cleaves both unmethylated and methylated HpaII Nucleic Acids Res 1978;5:3231-3236.
46. Adriaansen HJ, Soeting PWC, Wolvers-Tettero ILM, Van Dongen JJM. Immunoglobulin and T-cell receptor rearrangements in acute non-lymphocytic leukemias: analysis of 54 cases and a review of the literature. Leu 1991;5:744-751.
47. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of immunoglobulin and T cell receptor genes. Part I: bas technical aspects. Clin Chim Acta 1991;198:1-92.
48. Minowada J. Leukemia cell lines. Cancer Rev 1988;10:1-18.
49. Bird AP. CpG-rich islands and the function of DNA methylation. Nature 1986;321:209-212.
50. Tazi J, Bird A. Alternative chromatin structure at CpG islands. Cell 1990;60:909-920.
51. Bird A. The essentials of DNA methylation. Cell 1992;70:5-8.
52. Van Dongen JJM, Comans-Bitter WM, Wolvers-Tettero ILM, Borst J. Development of human T lymphocyte their thymus-dependency. Thymus 1990;16:207-234.
53. Burger C, Radbruch A. Protective methylation of immunoglobulin and T cell receptor (TcR) gene loci p induction of class switch and TcR recombination. Eur J Immunol 1990;20:2285-2291.
54. Antequera F, Boyes J, Bird A. High levels of De Novo methylation and altered chromatin structure at CpG i in cell lines. Cell 1990;62:503-514.
55. Johnsson OG, Kitchens RL, Baer RJ, Buchanan GR, Smith RG. Rearrangements of the *tal*-1 locus as clonal m for T cell acute lymphoblastic leukemia. J Clin Invest 1991;87:2029-2035.

CONCLUDING REMARKS

CHAPTER 5

CONCLUDING REMARKS*

The most important process during the differentiation from prothymocyte to mature T-lymphocyte is the regulation of the expression and selection of TcR molecules, which takes place in the thymus (1). To obtain functional TcR-$\gamma\delta$ or TcR-$\alpha\beta$ molecules, immature thymocytes must rearrange the V, (D) and J gene segments of the relevant TcR gene complexes (2). Several proteins, such as the products of the recombination activating genes RAG-1 and RAG-2 and the enzyme TdT, participate in the V(D)J rearrangement processes (3). The studies presented in this thesis encompass the identification of several phenotypic and genotypic characteristics of human early T-cell differentiation (4). In general, the experimental work was performed with the analysis of T-ALL (Chapters 2.2, 2.3, 3.2, 3.5, 4.2, and 4.3), and the proposed hypotheses were tested in normal human thymocytes or T-lymphocytes whenever possible (Chapters 2.5, 3.3, and 3.4).

T-ALL MODEL

A lymphoid leukemia is generally considered to consist of clonal cells which are "frozen" in a specific stage of lymphoid differentiation. The leukemic cells of T-ALL are assumed to originate from malignantly transformed cortical thymocytes, because T-ALL as well as cortical thymocytes express TdT, which is absent in normal extrathymic T-cells (5). Since T-ALL can originate from any maturing cortical thymocyte, T-ALL provide a human T-cell differentiation model in which each T-ALL represents the malignant (clonal) counterpart of a normal thymocyte in a specific differentiation stage (6).

In our studies on a large series of ~140 T-ALL, we observed many variations in phenotypic and genotypic characteristics, which may reflect important changes during T-cell differentiation. In the T-ALL model it is essential to identify the crucial characteristics, which can be used to arrange the various T-ALL in a progressive differentiation model for cortical thymocytes. We will summarize and discuss several important characteristics, including the expression of CD3, CD4, and CD8 molecules, the configuration of the TcR-δ and TcR-γ genes and the methylation status of a specific part in the promoter region of the *tal*-1 gene on chromosome 1.

TcR-δ gene configuration

TcR-$\gamma\delta$ expression requires functionally rearranged TcR-δ and TcR-γ genes on at least one allele, whereas for TcR-$\alpha\beta$ expression, the TcR-δ gene must be deleted on at least one allele, because it is located in between the Vα and the Jα gene segments (7,8). For this

* Parts of this chapter are published in: Res Immunol 1994;145:139-143 and 155-156.

reason it is assumed that the TcR-δ gene plays an important role in T-cell differentiation (9). We have therefore chosen to divide our T-ALL in subgroups on the basis of their TcR-δ gene configuration (10). The configuration of the TcR-δ gene on each allele can potentially pass through three consecutive stages: germline (G), rearranged (R), and deleted (D). Of the six possible allelic combinations of TcR-δ configuration, five were present in the 138 analyzed T-ALL (i.e. G/G, R/G, R/R, D/R and D/D). We assume the T-ALL in the subgroups with G/G and D/D configurations to be derived from relatively immature and mature thymocytes, respectively (Table 1).

TABLE 1. Characteristics of T-ALL subgroups as defined by their TcR-δ gene configuration[a].

	TcR-δ gene configuration				
	G/G	R/G	R/R	D/R	D/D
TcR-δ rearrangements[b]	(0)	(5)	(84)	(23)	(0)
Ri	–	40	30	17	–
Rc	–	60	70	83	–
TcR/CD3 phenotype[c]	(7)	(6)	(57)	(35)	(34)
$TcR^-/CD3^-$	100	100	58	51	26
TcR-$\gamma\delta^+/CD3^+$	0	0	42	3	0
TcR-$\alpha\beta^+/CD3^+$	0	0	0	46	74
CD4/CD8 phenotype[c,d]	(6)	(3)	(54)	(27)	(30)
$CD4^-/CD8^-$	83	33	30	11	17
$CD4^+/CD8^+$	0	67	37	48	57
$CD4^+/CD8^-$	0	0	26	26	7
$CD4^-/CD8^+$	17	0	7	15	20
taldb1 demethylation[c]	(6)	(5)	(50)	(27)	(31)
complete	33	20	12	44	84
partial	33	40	50	22	13
absent	33	40	38	33	3

a. Abbreviations used: G, germline; R, rearranged; Ri, incomplete rearrangement; Rc, complete rearrangement; D, deletion.
b. Relative allelic frequencies in percentages. The number of analyzed alleles is in parentheses.
c. Relative ALL frequencies in percentages. The number of analyzed ALL is in parentheses.
d. – <25%; and + ≥25% of the ALL cells positive.

TcR-δ gene rearrangements

The TcR-δ gene rearrangements occur in three conformations: Ri, incomplete rearrangement (i.e. Dδ-Dδ, Dδ-Jδ, and Vδ-Dδ); Rc, complete rearrangement (i.e. Vδ-Jδ); and Ru, unidentifiable rearrangement (i.e. Vα-Jδ and translocations) (10). Because Ri and Rc probably represent consecutive conformations, the relative frequencies of Ri and Rc in the various T-ALL subgroups were analyzed (Table 1). The highest frequency of Ri (40%) in the R/G subgroup and the high frequency of Rc (83%) in the D/R subgroup indicates that during T-cell differentiation, initially TcR-δ alleles contain incomplete rearrangements, followed by complete rearrangements. This also suggests that TcR-δ gene deletion only starts when

both alleles contain complete (non-functional?) rearrangements. However, circular excision products derived from normal thymocytes provide evidence that also incompletely rearranged alleles and even germline TcR-δ alleles can be deleted (5).

TcR/CD3 phenotype

Three distinct TcR/CD3 phenotypes occur in T-ALL: TcR$^-$/CD3$^-$, TcR-$\gamma\delta^+$/CD3$^+$, and TcR-$\alpha\beta^+$/CD3$^+$. It is obvious that the TcR-δ gene configuration is closely correlated with the TcR/CD3 phenotype. In the G/G and R/G subgroups, no CD3$^+$ T-ALL were present, and in the D/R and D/D subgroups, the TcR-$\gamma\delta^+$/CD3$^+$ T-ALL were virtually absent (Table 1). In the D/R subgroup there was an almost equal distribution of TcR$^-$/CD3$^-$ and TcR-$\alpha\beta^+$/CD3$^+$ T-ALL. However, in the D/D subgroup this distribution was skewed to TcR-$\alpha\beta^+$/CD3$^+$ T-ALL (Table 1). The remaining R/R subgroup only contained TcR$^-$/CD3$^-$ and TcR-$\gamma\delta^+$/CD3$^+$ T-ALL in comparable frequencies.

It is noteworthy that the G/G and R/G subgroups are small and that no TcR-$\gamma\delta^+$/CD3$^+$ T-ALL were present in the R/G subgroup. These observations suggest that TcR-δ gene rearrangement starts on both alleles. Thymocytes with R/R genotype may become TcR-$\gamma\delta^+$/CD3$^+$ or may proceed into the $\alpha\beta$ lineage by deleting their rearranged TcR-δ genes to obtain TcR-α rearrangements. These TcR-δ gene deletions and TcR-α rearrangements might start on one allele, because in the D/R subgroup almost half of the T-ALL are TcR-$\alpha\beta^+$/CD3$^+$, whereas in the D/D subgroup three-quarters of the T-ALL express TcR-$\alpha\beta$. The remaining D/D T-ALL which are still CD3$^-$, might have non-functional Vα-Jα rearrangements, and their normal thymic counterparts will probably be eliminated in the thymus.

CD4/CD8 phenotype

Both CD4 and CD8 are variably expressed on differentiating thymocytes. Several differentiation schemes concerning the CD4/CD8 phenotype are proposed for human and murine thymocytes, in which the most immature thymocytes are CD4$^-$/CD8$^-$, whereas the mature T-cells are CD4$^+$/CD8$^-$, CD4$^-$/CD8$^+$ or again CD4$^-$/CD8$^-$. The thymocytes with CD4$^+$/CD8$^+$ phenotype are assumed to represent intermediate differentiation stages (11-13). This simplified scheme of CD4/CD8 expression can also be found in our T-ALL (Table 1).

The majority of T-ALL without TcR-δ gene rearrangements showed the CD4$^-$/CD8$^-$ phenotype, which frequency declined in the more mature T-ALL subgroups. In the total group of T-ALL the CD4$^+$/CD8$^+$ phenotype was most frequent (43%). Its frequency was at its height in the more mature T-ALL subgroups, as was the case with the CD4$^-$/CD8$^+$ phenotype. The prominent presence of the CD4$^+$/CD8$^+$ phenotype may be caused by the oncogenic transformation of the T-ALL cells or may imply that the original cortical thymocytes still had to enter the CD4 or CD8 lineage (14). It is also of interest that the CD4/CD8 expression of TcR-$\gamma\delta^+$ T-ALL is quite diverse: CD4$^+$/CD8$^-$ (43%), CD4$^+$/CD8$^+$ (29%), CD4$^-$/CD8$^-$ (25%), and CD4$^-$/CD8$^+$ (4%). It can be concluded that the differential expression of the CD4 and CD8 molecules on thymocytes is a complex issue, which needs extensive studies to be resolved.

Demethylation of the *tal*-1 promotor region

In T-ALL, the *tal*-1 gene on chromosome 1 is frequently involved in chromosome aberrations, such as t(1;14)(p32;q11) (15) and *tal*-1 deletions (16). These *tal*-1 deletions are present in approximately 12-26% of the T-ALL and juxtapose the *tal*-1 gene to the 5′ non-coding part of the upstream located *sil* gene, thereby deleting the intermediate 90 kb DNA sequences (17). This process is mediated by "illegitimate" V(D)J recombinase activity, as indicated by the fact that *tal*-1 deletions demonstrate extreme homology to TcR gene rearrangements (16). We have proven that *tal*-1 deletions are restricted to T-ALL of the $\alpha\beta$ lineage, with a preferential occurrence in the TcR-δ D/D subgroup (34%) that was about twice the frequency of the D/R subgroup (18%) (18). Additionally, we were able to prove that this preference is the result of preferential demethylation of the *tal*-1 deletion breakpoint region in the *tal*-1 gene (taldb1), i.e. differential accessibility of the DNA for the recombinase complex (19). Our studies revealed that in T-ALL, the taldb1 is differentially demethylated in the various TcR-δ gene configuration subgroups (Table 1). Overall, it shows that in the R/R subgroup the majority of T-ALL contain partly or non-demethylated taldb1. In contrast, the D/R T-ALL and especially D/D T-ALL subgroups predominantly contain completely demethylated taldb1. This differential (de)methylation may be caused by a general mechanism of so-called "protective methylation" (20). This mechanism may transiently methylate the DNA before rearrangement, to protect it from unwanted recombination. Whether the demethylation of the taldb1 located in the *tal*-1 promotor region has anything to do with T-cell differentiation is hard to say. A possible role for the TAL-1 protein is dubious, since so far no expression is reported during thymocyte differentiation.

Conclusions of the T-ALL studies

Our T-ALL studies on some, quite diverse, phenotypic and genotypic characteristics prove that the most promising correlations are observed, if we use the TcR-δ gene configuration as standard differentiation marker in addition to the TcR/CD3 phenotype to divide the T-ALL in subgroups. We therefore conclude that the TcR-δ gene configuration is an excellent marker for early T-cell differentiation. It provides information whether the thymocyte is: a) relatively immature (G subgroups); b) in the $\gamma\delta$ lineage (R/R subgroup); or c) already $\alpha\beta$-lineage-committed (D subgroups).

Although the phenotypic and genotypic characteristics of T-ALL discussed here provide some insight in the possible mechanisms of thymocyte differentiation, we need to be cautious in extrapolating our T-ALL data to normal thymocyte differentiation, because it is unclear whether malignant transformation of the thymocyte might induce immunophenotypic and/or immunogenotypic changes. Moreover, the T-ALL cells may (partially) mature so that the assumption of a "frozen" differentiation stage may not be valid. Nevertheless, we feel that the T-ALL model is a useful model to study normal thymocyte differentiation. We have summarized most of our data in the schematic diagram in Figure 1. An intriguing new characteristic presented here is the methylation and demethylation of a specific part of the genome, i.e. the promotor region of the *tal*-1 gene. If this is the result of a general process involving many genes, than this might be an important additional marker in early T-cell differentiation.

The here and elsewhere described studies (5) propose a linear pathway of T-cell differentiation, based on the expression of several membrane proteins. However, we are

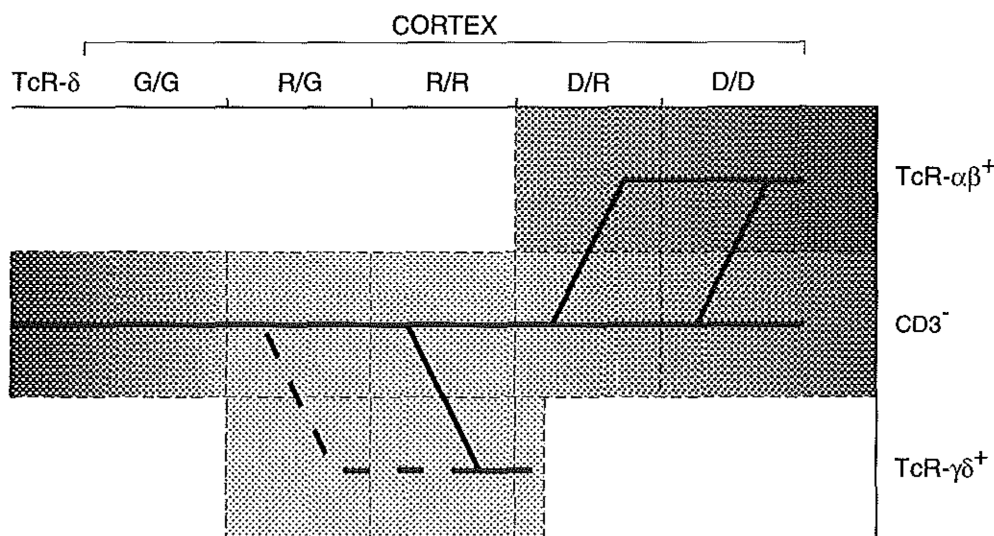


Figure 1. Schematic diagram of human early T-cell differentiation based on phenotypic and genotypic data of T-ALL subgroups, as defined by their TcR-δ gene configuration. The intensity of shading represents the demethylation status of the taldb1: dark = demethylated; and light = methylated.

still unable to fit *all* our T-ALL samples in these differentiation schemes. This may be a result of the uncertainties of the T-ALL model, but it could also be that the proposed differentiation schemes are oversimplified. For instance, almost all schemes are basically monolinear and unidirectional, ignoring the possibility that several lineages may differentiate parallel to each other, or that differentiating cells may revert to an earlier differentiation stage. When the functions of the proteins involved in T-cell differentiation processes (membrane, cytoplasmic, and nuclear proteins) are better known and also taken into account, the picture might become more clear.

NORMAL HUMAN T-CELLS

To discern the influences of the leukemic transformation from the "real" differentiation processes, the observations made in the T-ALL should be checked in normal human T-cells whenever possible. It is obvious that experiments with normal differentiating human T-cells have their own limitations e.g. with respect to polyclonality of the cell samples. Although the majority of the studies presented in this thesis deal with T-ALL, we were able to test some of the T-ALL derived hypotheses in normal T-cells .

Most of our studies on thymocytes focussed on the processes on DNA level that are involved in the bifurcation into the TcR-$\alpha\beta$ and TcR-$\gamma\delta$ lineage. From our studies and those of De Villartay et al.,.(9,21,22), it has become clear that the TcR-α/δ locus plays a decisive role in these lineage commitment processes. Because rearrangements of the TcR-δ deleting elements (δREC and $\psi J\alpha$) are prominently present in normal differentiating thymocytes and

represent an intermediate stage between TcR-δ and TcR-α gene rearrangements (5), we concentrated in our studies on this particular rearrangement. Not only were we able to determine that δREC-ψJα rearrangement is restricted to a certain thymocyte differentiation stage (Chapter 3.3), but we also found indications for the existence of a specific TcR-δ deleting recombinase complex (18,19).

The most important obstacle in the analysis of gene rearrangements in normal differentiating thymocyte cell samples is their polyclonality. Our most recent study involved the isolation of a particular immature thymocyte population (Chapter 3.3) and demonstrated that cell sorting experiments are essential to study normal early T-cell differentiation in detail. The consecutive phenotypic stages in thymocyte differentiation are being slowly unravelled. With the thereby developed insights and tools (i.e. McAb), it must be possible to purify thymocytes in various stages of differentiation. Analysis of these thymic subpopulations for stage specific rearrangement processes and/or proteins will provide the conclusive information necessary to understand human early T-cell differentiation.

PERSPECTIVES

Human early T-cell differentiation can be studied at different levels:

1. the DNA level, which concerns the various TcR gene rearrangements;
2. the DNA-protein level, which involves the regulation of the rearrangement processes as well as the transcription of T-cell-specific genes;
3. the level of signal transduction pathways, which deals with the signalling from membrane proteins to the nucleus;
4. the membrane protein level, including the antigen specific TcR, the CD3 signaling proteins, the accessory molecules, and the differentiation stage-specific membrane proteins;
5. the level of interaction between thymocytes and their micro-environment, for the induction of proliferation, differentiation, and selection, which includes the interactions of thymocytes with thymic stroma, cytokines, and antigen presenting cells.

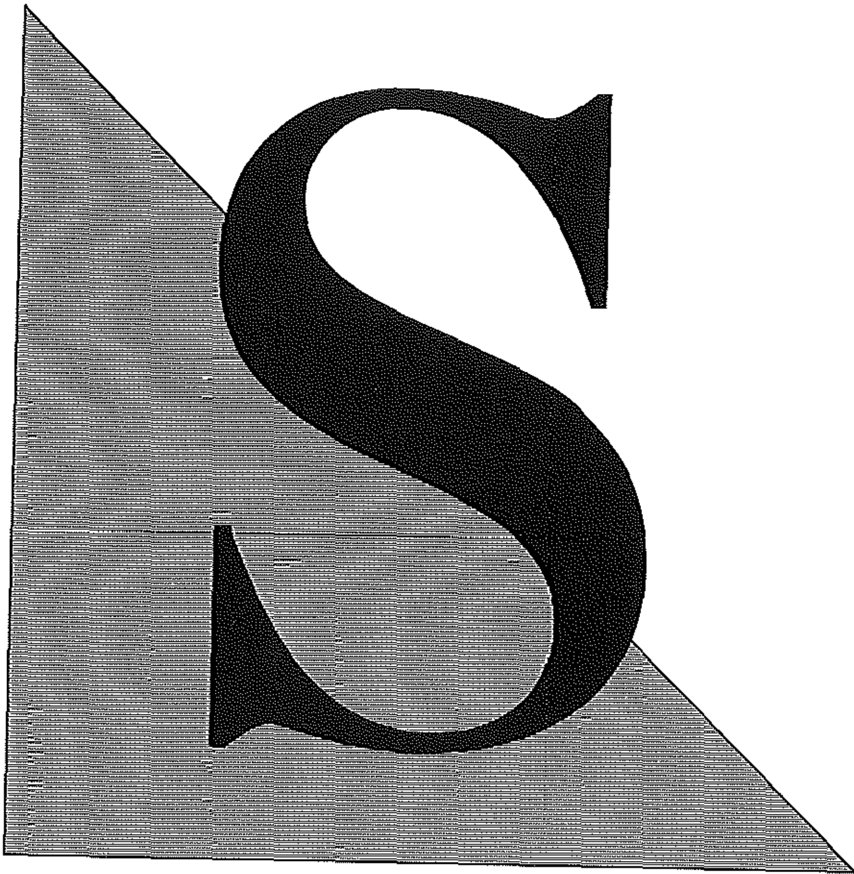
Although the components of the different levels have to work harmoniously together when T-cells differentiate towards maturity, T-cell research is still in its infancy and therefore most levels are studied independently. Of course, interaction between components of different levels can only be determined after these components have been identified and placed in the T-cell differentiation pathways. But research at the various levels of T-cell differentiation is progressing steadily and integration of knowledge concerning the different levels is carefully initiated at some points. Although the majority of immunological research, and therefore also a lot of the T-cell differentiation research, deals only with *human* and *murine* T-cell differentiation, much can be learned from the analogous processes in other species. Combining the knowledge of the different levels and different species, now comprises one of the biggest challenges in T-cell research and might lead to surprisingly new insights and innovative ways for future studies on early T-cell differentiation.

REFERENCES

1. Davis MM. T cell receptor gene diversity and selection. Ann Rev Biochem 1990;59:475-496.
2. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988;334:395-402.
3. Lewis SM. The mechanism of V(D)J joining: lessons from molecular, immunological, and comparative analyses. Adv Immunol 1994;56:27-150.
4. Breit TM, Wolvers-Tettero ILM, Van Dongen JJM. Phenotypic and genotypic characteristics of human early T-cell differentiation: the T-cell acute lymphoblastic leukaemia model. Forum in Immunology: "Human early T-cell differentiation". Res Immunol 1994;145:139-143 and 155-156.
5. Van Dongen JJM, Comans-Bitter WM, Wolvers-Tettero ILM, Borst J. Development of human T lymphocytes and their thymus-dependency. Thymus 1990;16:207-234.
6. Van Dongen JJM. Human T cell differentiation: basic aspects and their clinical applications. Thesis, Department of Immunology, Erasmus University Rotterdam, 1990.
7. Isobe M, Russo G, Haluska FG, Croce CM. Cloning of the gene encoding the δ subunit of the human T-cell receptor reveals its physical organization within the α-subunit locus and its involvement in chromosome translocations in T-cell malignancy. Proc Natl Acad Sci USA 1988;85:3933-3937.
8. Satyanarayana K, Hata S, Devlin P, Grazia Roncarolo M, De Vries JE, Spits H, Strominger JL, Krangel MS. Genomic organization of the human T-cell antigen-receptor *α/δ* locus. Proc Natl Acad Sci USA 1988;85:8166-8170.
9. De Villartay J-P, Hockett RD, Coran D, Korsmeyer SJ, Cohen DI. Deletion of the human T-cell receptor *δ*-gene by a site-specific recombination. Nature 1988;335:170-174.
10. Breit TM, Wolvers-Tettero ILM, Beishuizen A, Verhoeven M-AJ, Van Wering ER, Van Dongen JJM. Southern blot patterns, frequencies and junctional diversity of T-cell receptor *δ* gene rearrangements in acute lymphoblastic leukemia. Blood 1993;10:3063-3074.
11. Scollay R. T-cell subset relationship in thymocyte development. Curr Opin Immunol 1991;3:204-209.
12. Nikolić-Žugić J. Phenotypic and functional stages in the intrathymic development of *αβ* T cells. Immunol Today 1991;12:65-70.
13. Shortman K. Cellular aspects of early T-cell development. Curr Opin Immunol 1992;4:140-146.
14. Chan SH, Cosgrove D, Waltzinger C, Benoist C, Mathis D. Another view of the selective model of thymocyte selection. Cell 1993;73:225-236.
15. Bernard O, Guglielmi P, Jonveaux P, Cherif D, Gisselbrecht S, Mauschauffe M, Berger R, Larsen C-J, Mathieu-Mahul D. Two distinct mechanisms for the *SCL* gene activation in the t(1;14) translocation of T-cell leukemias. Genes Chrom Cancer 1990;1:194-208.
16. Brown L, Cheng J-T, Chen Q, Siciliano MJ, Crist W, Buchanan G, Baer R. Site-specific recombination of the *tal*-1 gene is a common occurrence in human T cell leukemia. EMBO J 1990;9:3343-3351.
17. Aplan PD, Lombardi DP, Ginsberg AM, Cossman J, Bertness VL, Kirsch IR. Disruption of the human SCL locus by "illegitimate" V-(D)-J recombinase activity. Science 1990;250:1426-1429.
18. Breit TM, Mol EJ, Wolvers-Tettero ILM, Ludwig W-D, Van Wering ER, Van Dongen JJM. Site-specific deletions involving the *tal*-1 and *sil* genes are restricted to cells of the T cell receptor *α/β* lineage: T cell receptor *δ* gene deletion mechanism affects multiple genes. J Exp Med 1993;177:965-977.
19. Breit TM, Wolvers-Tettero ILM, Van Dongen JJM. Lineage specific demethylation of *tal*-1 gene breakpoint region determines the frequency of *tal*-1 deletions in *αβ* lineage T-cells. Oncogene 1994;9:1847-1853.
20. Burger C, Radbruch A. Protective methylation of immunoglobulin and T cell receptor (TcR) gene loci prior to induction of class switch and TcR recombination. Eur J Immunol 1990;20:2285-2291.
21. De Villartay J-P, Lewis D, Hockett R, Waldmann TA, Korsmeyer SJ, Cohen DI. Deletional rearrangement in the human T-cell receptor α-chain locus. Proc Natl Acad Sci USA 1987;84:8608-8612.
22. De Chasseval R, De Villartay J-P. Functional characterization of the promoter for the human germ-line T cell receptor Jα (TEA) transcript. Eur J Immunol 1993;23:1294-1298.

SUMMARY

SAMENVATTING



SUMMARY

The immune system can recognize and destroy the large variety of potential pathogens that are present in the environment and may enter the body. The effector cells of the adaptive immune system are lymphocytes and phagocytes. Specific recognition of foreign antigens is mediated by B- and T-lymphocytes. The antigen-specific receptors of B-cells are membrane-bound immunoglobulin (Ig) molecules, whereas T-cells use membrane-bound T-cell receptor (TcR) molecules. The TcR is a heterodimer and occurs in two configurations: TcR-$\alpha\beta$ and TcR-$\gamma\delta$. The majority of human T-lymphocytes expresses TcR-$\alpha\beta$, whereas a minority expresses TcR-$\gamma\delta$. Most of the characterized cell-mediated immune responses are carried out by TcR-$\alpha\beta^+$ T-cells. The exact function of TcR-$\gamma\delta^+$ T-cells is still unknown.

Early T-cell differentiation takes place in the thymus and is characterized by assembly and expression of the TcR molecules. Because TcR molecules must be capable of discriminating foreign antigen from self antigen, TcR expressing thymocytes undergo selection processes. The TcR molecules are selected for the specificity of their variable domains. In contrast to the constant domain (C), the variable domain is not present as a complete exon in germline DNA. It is assembled by joining one variable (V) to one joining (J) gene segment. In addition small diversity (D) gen segments can be present between the rearranged V and J gene segments. The germline TcR gene complexes contain multiple V, (D), and J gene segments. Different combinations of these gene segments provide an enormous antigen receptor repertoire using only a limited amount of germline DNA. The receptor repertoire is increased because the gene segments are imperfectly joined during the rearrangement processes. At the site of the junction, random insertion and deletion of nucleotides make up the junctional region of the rearrangement. Although a lot is known about the rearranging genes, the V(D)J recombination processes and the regulation of these processes are still poorly understood.

In our studies on T-cell differentiation we frequently used T-cell acute lymphoblastic leukemias (T-ALL), because the leukemic cells of a T-ALL are assumed to originate from malignantly transformed immature thymocytes. A T-ALL therefore consists of clonal cells which are "frozen" in a specific stage of T-cell differentiation.

Repertoire studies on the human TcR-$\gamma\delta$ (**Chapter 2**) were performed by analyzing TcR-$\gamma\delta^+$ T-ALL, thymocytes and T-lymphocytes. TcR-$\gamma\delta^+$ thymocytes and TcR-$\gamma\delta^+$ T-ALL are characterized by a restricted combinatorial repertoire and an extended junctional diversity. Their combinatorial repertoire is limited, because of preferential usage of TcR-γ gene segments (VγI-Jγ2.3) and TcR-δ gene segments (Vδ1-Jδ1). Long junctional regions with random nucleotides cause their extended junctional diversity. In man, the far majority of the peripheral blood TcR-$\gamma\delta^+$ T-lymphocytes expresses a TcR with Vγ9-Jγ1.2/Vδ2-Dδ3-Jδ1 chains. In these T-cells we identified a selection marker at a fixed position in the Vδ2-Jδ1 junctional region that consisted of homologous amino acid residues. Therefore, we can conclude that the actual repertoire of the human TcR-$\gamma\delta^+$ T-cells is restricted by selection for preferential combinations of V and J segments, as well as for preferential occurrence of amino acid residues in the junctional regions. It may be that these repertoire restrictions will become a lead to the function of TcR-$\gamma\delta^+$ T-cells.

Studies on the role of TcR gene rearrangements in T-cell differentiation mainly focussed on rearrangements of the TcR-δ gene (**Chapter 3**). This gene is located within the TcR-α gene complex between the Vα and Jα gene segments. Therefore, rearrangement of a Vα gene segment to a Jα gene segment will delete the intermediately located TcR-δ gene. For this reason TcR-δ and TcR-α gene rearrangements cannot coincide on the same allele. TcR-α gene rearrangements are preceded by the so-called TcR-δ deleting rearrangement in which the δREC gene segment is linked to the ψJα gene segment to form a non-functional rearrangement. The TcR-δ gene is deleted by this rearrangement because the δREC and ψJα gene segments flank this gene. The order of rearrangements of the TcR-α/δ locus thus is: rearrangement of the TcR-δ gene segments; deletion of the TcR-δ gene via rearrangement of the deleting elements, δREC and ψJα; and rearrangement of the TcR-α gene segments. It is plausible that the TcR-α/δ locus plays a decisive role in the divergence of the TcR-αβ and TcR-γδ lineages.

Studies of TcR gene rearrangements require suitable tools to analyze the involved genes. We constructed twenty DNA probes for Southern blot analysis of the TcR-δ gene. In this way the restriction-enzyme pattern for every possible TcR-δ gene rearrangement could be determined. Hence, we were able to analyze the TcR-δ gene rearrangements in human T-cell differentiation by use of a large series of T-ALL and normal polyclonal thymocyte populations. Two pathways of sequential TcR-δ gene rearrangements in post-natal thymocytes were proposed: a fetal-like pathway that mainly results in Vδ2-Dδ3-Jδ1 rearrangements and a post-natal pathway that mainly results in Vδ1-Dδ2-Dδ3-Jδ1 rearrangements. The δREC-ψJα rearrangement was observed as a predominant rearrangement in thymocytes and revealed homology to TcR-α gene rearrangements because it rarely contained a Dδ gene segment in its junctional region. The discovery of several T-ALL and a T-cell line with an active TcR-δ deletion recombinase complex may lead to new studies on this particular rearrangement.

Errors of the V(D)J recombinase complex may cause that DNA sequences, other than the Ig/TcR gene segments, are joined to each other. This may result in a chromosomal translocation, an inversion, or a deletion. In T-cells, these aberrations may cause a step in the oncogenic transformation of a thymocyte into a leukemic cell. Most of the translocations in T-ALL involve a TcR gene. However, there is also a submicroscopical deletion on chromosome 1 that links the *tal*-1 gene to the promoter of the upstream located *sil* gene (**Chapter 4**). Although this aberration deletes the major part of the *sil* gene and leaves the coding exons of the *tal*-1 gene unaffected, it is called the *tal*-1 deletion. There are five types of *tal*-1 deletions. Based on homology with TcR gene rearrangements it is assumed that they are caused by "illegitimate" V(D)J recombinase activity. The *tal*-1 deletions are exclusively observed in T-ALL with a frequency of 15-25%. We discovered that *tal*-1 deletions do not occur in TcR-$\gamma\delta^+$ T-ALL and occur most frequently in TcR-$\alpha\beta^+$ T-ALL. In case of TcR$^-$ T-ALL, the *tal*-1 deletion were most frequently found in T-ALL with both TcR-δ genes deleted. Therefore, the *tal*-1 deletions seem to be restricted to T-ALL of the TcR-αβ lineage. The lineage restriction of this aberration could be explained by the DNA methylation status of the *tal*-1 deletion breakpoints, because demethylation was also most frequently seen in T-ALL of the TcR-αβ lineage. Demethylation of gene segments is probably a prerequisite for recombination, and the *tal*-1 deletions were observed exclusively in T-ALL with completely demethylated *tal*-1 breakpoint regions. However, demethylation could only

partly explain the lineage restricted occurrence of the *tal*-1 deletion, because in the T-ALL that were non-$\alpha\beta$ lineage and completely demethylated, no *tal*-1 deletions occurred. This and the association between TcR-δ gene deletion and *tal*-1 deletion, lead to the proposal of a specific TcR-δ gene deleting recombinase complex.

In conclusion, we can say that the studies presented in this thesis add a new dimension to the established T-cell differentiation schemes, which are based on phenotypic markers. This dimension is the changing genotype of the thymocyte during differentiation. In general the configuration of the TcR-α/δ locus appears to be a reliable and useful marker for T-cell differentiation (**Chapter 5**), especially for determination of TcR-$\alpha\beta$ or TcR-$\gamma\delta$ lineage commitment. Our studies also suggest that specific sites in the DNA occur at which the regulation of TcR gene rearrangements takes place. Identification of these regulatory elements and the involved DNA-binding proteins should be the next target in T-cell differentiation research.

SAMENVATTING

Het immuunsysteem is in staat om de enorme verscheidenheid aan potentiële pathogenen welke aanwezig zijn in het milieu te herkennen en te vernietigen. Twee typen immuuncellen spelen hierbij een belangrijke rol, de B-lymfocyten en T-lymfocyten, die buitengewoon specifiek lichaamsvreemde antigenen kunnen herkennen. Membraangebonden immunoglobuline (Ig) moleculen zijn de antigeen-specifieke receptoren van B-cellen, terwijl de T-cellen gebruik maken van membraangebonden T-cel receptor (TcR) moleculen. Er zijn twee typen TcR moleculen: TcR-$\alpha\beta$ en TcR-$\gamma\delta$. Het merendeel van de T-lymfocyten heeft een TcR-$\alpha\beta$ molecuul op de celmembraan. Deze TcR-$\alpha\beta^+$ T-lymfocyten voeren vrijwel alle bekende T-cel immuunreakties uit. De functie van TcR-$\gamma\delta$ T-lymfocyten is vooralsnog onbekend.

De thymus is het orgaan waarin de onrijpe T-cellen zich als thymocyten ontwikkelen tot rijpe T-lymfocyten. Deze differentiatie wordt gekenmerkt door de samenstelling en expressie van de TcR moleculen. Omdat het essentieel is dat de TcR moleculen "vreemd" van "zelf" antigenen kunnen onderscheiden, worden thymocyten op basis van de TcR op hun membraan geselecteerd. Dit betreft vooral selectie op grond van de antigeen-specificiteit van de variabele domeinen van hun TcR. In tegenstelling tot het constante domein (C) van een TcR, is het variabele domein niet aanwezig als een compleet exon in het kiemlijn DNA. Het wordt samengesteld door het aan elkaar koppelen van één variabel (V) en één verbindings (J) gensegment. Daarbij kan er een klein diversiteit (D) gensegment aanwezig zijn tussen de herschikte V en J gensegment. De kiemlijn TcR gencomplexen bestaan uit series V, (D), en J gensegmenten. De verschillende combinaties van deze gensegmenten veroorzaken een enorme diversiteit van de antigeenreceptor met maar een geringe hoeveelheid DNA sequenties. Het receptor repertoire wordt verder vergroot doordat de gensegmenten niet altijd op precies dezelfde manier aan elkaar gekoppeld worden tijdens de herschikkingsprocessen. Op de koppelingsplaatsen worden nucleotiden ingevoegd en gedeleteerd en vormen zo verbindingsregionen die "junctional regions" worden genoemd. Hoewel al veel kennis is van de herschikkende genen, is er van de V(D)J herschikkingsprocessen en de regulatie van deze processen nog maar weinig bekend.

In ons T-cel differentiatie onderzoek hebben we veel gebruik gemaakt van T-cel acute lymfoblastaire leukemieën (T-ALL) omdat aangenomen wordt dat de leukemiecellen van een T-ALL ontstaan zijn uit een kwaadaardig getransformeerde, onrijpe thymocyt. Een T-ALL bestaat uit een klonale celpopulatie, waarvan de cellen zijn blijven steken in één bepaald stadium van de T-cel differentiatie.

Repertoire studies van de humane TcR-$\gamma\delta$ (**Hoofdstuk 2**) zijn uitgevoerd met behulp van TcR-$\gamma\delta^+$ T-ALL, thymocyten en T-lymfocyten. TcR-$\gamma\delta^+$ thymocyten en TcR-$\gamma\delta^+$ T-ALLs worden gekarakteriseerd door een beperkt combinatierepertoire en een uitgebreide "junctional" diversiteit. Het combinatierepertoire is beperkt, omdat er een preferentieel gebruik is van TcR-γ gensegmenten (Vγ1-Jγ2.3) en TcR-δ gensegmenten (Vδ1-Jδ1). Lange "junctional regions" met willekeurige ingevoegde nucleotiden veroorzaken de uitgebreide "junctional" diversiteit. In de mens brengt de overgrote meerderheid van de perifere bloed

T-lymfocyten een TcR tot expressie die bestaat uit Vγ9-Jγ1.2/Vδ2-Dδ3-Jδ1 ketens. In deze T-cellen hebben we een selectie marker geïdentificeerd op een vaste plaats in de Vδ2-Jδ1 "junctional region", welke bestond uit op elkaar lijkende aminozuren. Daarom kunnen we concluderen dat het werkelijke repertoire van humane TcR-γδ$^+$ T-cellen beperkt wordt op door selectie op voorkeurscombinaties van V en J segmenten alsmede op eiwit niveau op voorkeur voor bepaalde aminozuren in de "junctional regions". Het zou kunnen, dat deze restricties in het TcR-γδ repertoire een aanwijzing kunnen geven voor verder onderzoek naar de functie van TcR-γδ$^+$ T-cellen.

Onderzoek is verricht naar de rol van TcR genherschikkingen in de T-cel differentiatie (**Hoofdstuk 3**). Het onderzoek was voornamelijk gericht op herschikkingen van het TcR-δ gen. Dit gen is gelokaliseerd in het TcR-α gencomplex, tussen de Vα en Jα gensegmenten. Een herschikking van een Vα gensegment naar een Jα gensegment zal dientengevolge het tussenliggende TcR-δ gen deleteren. Hierdoor is het uitgesloten dat TcR-δ en TcR-α genherschikkingen tegelijkertijd voorkomen op één allel. TcR-α genherschikkingen worden voorafgegaan door een herschikking waarbij het δREC gensegment wordt gekoppeld aan het ψJα gensegment. Doordat de δREC en ψJα gensegmenten aan weerskanten van het TcR-δ gen zijn gelegen, resulteert deze niet-functionele herschikking in de deletie van het TcR-δ gen. De volgorde van herschikking van het TcR-α/δ locus is dus: herschikking van de TcR-δ gensegmenten; deletie van het TcR-δ gen via herschikking van de deleterende elementen, δREC en ψJα; en herschikking van de TcR-α gensegmenten. Door deze volgorde van herschikkingen is het aannemelijk dat het TcR-α/δ locus een beslissende rol speelt in de splitsing van de TcR-αβ and TcR-γδ differentiatielijnen.

Onderzoek naar TcR genherschikkingen is uitsluitend mogelijk met de juiste materialen voor de analyse van de betrokken genen. Voor dit doel hebben we twintig DNA probes gemaakt die gebruikt kunnen worden voor Southern blot analyse van het TcR-δ gen. Met deze probes was het mogelijk het restrictie-enzympatroon van alle mogelijke TcR-δ genherschikking te bepalen. Aldus waren we in staat om de TcR-δ genherschikkingen te analyseren die voorkomen tijdens de humane T-cel differentiatie. Dit onderzoek is uitgevoerd met behulp van een groot aantal T-ALL en normale, polyklonale thymocyten populaties. Binnen TcR-δ genherschikkingen van post-natale thymocyten konden twee hoofdlijnen worden onderscheiden: een onrijpe ("foetale") hoofdlijn die voornamelijk resulteert in Vδ2-Dδ3-Jδ1 herschikkingen en een post-natale hoofdlijn die voornamelijk resulteert in Vδ1-Dδ2-Dδ3-Jδ1 herschikkingen. De δREC-ψJα herschikking komt voor als een prominente herschikking in thymocyten en vertoonde overeenkomst met TcR-α genherschikking omdat deze zelden een Dδ gensegment bevatte in de "junctional region". De ontdekking van verscheidene T-ALL en een T-cellijn met een actief TcR-δ deletie enzymcomplex zou kunnen leiden tot nieuw onderzoek naar deze speciale herschikking.

Fouten van het V(D)J herschikkings enzymcomplex kunnen DNA sequenties, anders dan Ig/TcR gensegmenten, aan elkaar koppelen. Hierdoor kunnen translocaties, inversies of deleties ontstaan. In T-cellen kunnen deze afwijkingen bijdragen aan de oncogene transformatie van een thymocyt tot een leukemiecel. Bij het overgrote deel van de translocaties in T-ALL is een TcR gen betrokken. Maar er bestaat ook een submicroscopische deletie in chromosoom 1 waardoor het *tal*-1 gen gekoppeld wordt aan de eerder op het DNA gelegen promotor van het *sil* gen (**Hoofdstuk 4**). Alhoewel de afwijking het grootste gedeelte van het *sil* gen deleteert zonder de coderende exonen van

het *tal*-1 gen te beschadigen, wordt deze *tal*-1 deletie genoemd. Er zijn vijf typen *tal*-1 deleties. Op basis van de overeenkomst met TcR genherschikkingen wordt aangenomen dat ze worden veroorzaakt door oneigenlijke V(D)J herschikkingsactiviteit. *tal*-1 deleties komen uitsluitend voor in T-ALL, en wel met een frequentie van 15-25%. Het bleek bovendien dat de *tal*-1 deleties niet voorkomen in TcR-$\gamma\delta^+$ T-ALL, maar voornamelijk in TcR-$\alpha\beta^+$ T-ALL. In het geval van TcR^- T-ALL worden de *tal*-1 deleties voornamelijk gevonden in de T-ALL waarin beide TcR-δ genen gedeleteerd zijn. Hieruit concluderen we dat het voorkomen van deze afwijking is beperkt tot T-ALL van de TcR-$\alpha\beta$ differentiatielijn. Deze restrictie kon worden verklaard door de mate van DNA demethylatie van het *tal*-1 deletie breukpunt. Demethylatie van gensegmenten is waarschijnlijk een voorwaarde voor recombinatie. De *tal*-1 deleties kwamen uitsluitend voor in T-ALL met compleet gedemethyleerde *tal*-1 breukpunt gebieden. Toch kan demethylatie de differentiatielijnrestrictie van deze *tal*-1 deleties maar gedeeltelijk verklaren, omdat in T-ALL die niet behoorde tot de $\alpha\beta$ lineage en bovendien compleet gedemethyleerde *tal*-1 genen bevatten, toch geen *tal*-1 deleties voorkwamen. Dit feit en de associatie tussen TcR-δ gendeletie en *tal*-1 deletie, leidde tot de postulatie dat er een specifiek TcR-δ deletie complex bestaat.

Concluderend kunnen we stellen dat het onderzoek zoals beschreven in dit proefschrift een nieuwe dimensie heeft toegevoegd aan de reeds bekende T-cel differentiatie schema's welke gebaseerd zijn op fenotypische kenmerken. Deze dimensie is het veranderende genotype van de thymocyt gedurende de differentiatie. In het algemeen lijkt het TcR-α/δ locus een betrouwbare en bruikbare marker voor T-cel differentiatie (Hoofdstuk 5), in het bijzonder voor het vaststellen van de keuze tussen de TcR-$\alpha\beta$ en de TcR-$\gamma\delta$ differentiatielijn. Ons onderzoek suggereert ook dat er speciale plaatsen in het DNA voorkomen die betrokken zijn bij de regulatie van TcR genherschikkingen. Identificatie van deze regulatoire elementen en de bijbehorende DNA bindende eiwitten zullen de toekomstige doelen zijn in T-cel differentiatieonderzoek.

ABBREVIATIONS

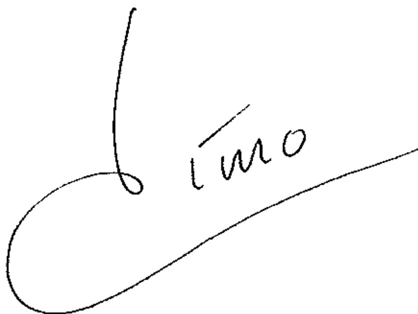
ALL	:	acute lymphoblastic leukemia
AML	:	acute myeloid leukemia
ANLL	:	acute non-lymphoblastic leukemia
(b)HLH motif	:	(basic) helix-loop-helix DNA binding motif
BM	:	bone marrow
bp	:	DNA base pairs
CD	:	cluster of differentiation/cluster of designation
C region	:	constant region
CNS	:	central nervous system
CSF	:	cerebrospinal fluid
CyCD3	:	cytoplasmic expression of CD3 antigen
D	:	deleted
DCLSG	:	Dutch Childhood Leukemia Study Group
D gene segment	:	diversity gene segment
G	:	germline
hprt gene	:	hypoxanthine-guanine phosphoribosyltransferase gene
IF	:	immunofluorescence
Ig	:	immunoglobulin
IgH	:	immunoglobulin heavy-chain
IgL	:	immunoglobulin light-chain
J gene segment	:	joining gene segment
kb	:	DNA kilobase pairs
kDa	:	kilo Dalton
Kde	:	kappa deleting element
McAb	:	monoclonal antibody
MHC	:	major histocompatibility complex
MNC	:	mononuclear cells
MRD	:	minimal residual disease
N-region	:	part of junctional region of randomly-inserted nucleotides
PAGE	:	polyacrylamide gel electrophoresis
PB	:	peripheral blood
PCR	:	polymerase chain reaction
precursor B-ALL	:	precursor B-cell acute lymphoblastic leukemia
P-region	:	junctional region nucleotides, which form a palindromic sequence together with the juxtaposed nucleotides of an untrimmed gene segment
R	:	rearranged
RAG	:	recombination activating gene
RSS	:	recombination signal sequence
RT	:	reverse transcription
SCID	:	severe combined immunodeficiency
sildb	:	5' *tal*-1 deletion breakpoint region located in the *sil* gene
SmIg	:	surface membrane Ig
tal-1 deletion	:	~90 kb deletion juxtaposing the 5' part of the *tal*-1 gene to the 5' part of the *sil* gene and thereby deleting all coding *sil* exons
taldb	:	3' *tal*-1 deletion breakpoint region located in the *tal*-1 gene
T-ALL	:	T-cell acute lymphoblastic leukemia
TcR	:	T-cell receptor
TcR-$\alpha\beta$	:	TcR, consisting of a TcR-α and a TcR-β chain
TcR-$\gamma\delta$	:	TcR, consisting of a TcR-γ and a TcR-δ chain
TdT	:	terminal deoxynucleotidyl transferase
TEA element	:	T early alpha element
V gene segment	:	variable gene segment

DANKWOORD

Door mijn promotiejaren heen hebben veel mensen mij ontelbare redenen gegeven om dankbaar te zijn. Dat ben ik vaak geweest en zal ik ook nog lang blijven. Maar oprechte dankbaarheid laat zich slecht in woorden vangen en is bovenal zeer persoonlijk. Daarom wil ik hier besluiten met de volgende bespiegeling:

Niets is sterker
Dan de stilte
Niets heeft zoveel kracht
Als het zwijgen van de nacht
Niets is sterker
Dan de stilte
Niets is sterker dan het woord
Dat niemand hoort

DE STILTE, Stef Bos



CURRICULUM VITAE

Timo Markus Breit

April 26th 1964	:	Born in Rotterdam, the Netherlands.
1976-1982	:	VWO at the Comprehensive school City-College Rotterdam, the Netherlands.
1982-1984	:	Propaedeutics Mechanical Engineering at the Technological University of Delft, The Netherlands.
1984-1985	:	Military service (Dutch Medical Corps).
1985-1990	:	Study Biology at the University of Leiden, the Netherlands.
- Sept. '88-May '89	:	1st research training: "Localisation of the functional regions of *Rhizobium* nodE-proteins involved in host-specificity" at the Department of Molecular Botany (supervisor: Dr. H.P. Spaink), University of Leiden, the Netherlands.
- June '89-June '90	:	2nd research training: "Combinatorial repertoire of $\gamma\delta$ T-cell receptors expressed by T-cell acute lymphoblastic leukemias" at the Department of Immunology (supervisor: Prof. Dr. J.J.M. van Dongen), Erasmus University Rotterdam, the Netherlands.
- June 1990	:	Master of Science in Medical Biology ("Doctorandus"), University of Leiden, the Netherlands.
Sept. '90-Nov. '94	:	Ph.D. research: "Rearrangement and expression of human T-cell receptor genes" at the Department of Immunology (promoter: Prof. Dr. J.J.M. van Dongen), Erasmus University Rotterdam, the Netherlands.
- Nov. 1991	:	Oxford higher certificate of proficiency in English, G.W. English Teaching Service, Leiden, the Netherlands.
- April-July '94	:	Working visit: "Determination and identification of basic helix-loop-helix proteins involved in lymphoid differentiation" at the Department of Biology (principal investigator: Prof. Dr. C. Murre), University of California, San Diego, USA.
Jan. 1995	:	Start of post-doctoral research at the Department of Clinical Immunology (principal investigator: Dr. T. Logtenberg), University Hospital Utrecht, The Netherlands.

PUBLICATIONS

1. Breit TM, Wolvers-Tettero ILM, Hählen K, Van Wering ER, Van Dongen JJM. Limited combinatorial repertoire of $\gamma\delta$ T cell receptors expressed by T cell acute lymphoblastic leukemias. Leukemia 1991;5:116-24.

2. Breit TM, Wovers-Tettero ILM, Hählen K, Van Wering ER, Van Dongen JJM. Extensive junctional diversity of $\gamma\delta$ T-cell receptors expressed by T-cell acute lymphoblastic leukemias: implications for the detection of minimal residual disease. Leukemia 1991;5:1076-1086 and Erratum 1992;6:169-170.

3. Van Dongen JJM, Breit TM, Adriaansen HJ, Beishuizen A, Hooijkaas H. Detection of minimal residual disease in acute leukemia by immunological marker analysis and polymerase chain reaction. Leukemia 1992;6S1:47-59.

4. Van Oostveen JW, Breit TM, Borst J, De Wolf JTM, Brandt RMP, Smit JW, Van Dongen JJM, Melief CJM. Polyclonal expansion of T-cell receptor $\gamma\delta^+$ T-lymphocytes associated with neutropenia and thrombocytopenia. Leukemia 1992;6:410-418.

5. Breit TM, Wolvers-Tettero ILM and Van Dongen JJM. Receptor diversity of human T-cell receptor $\gamma\delta$ expressing cells. Prog Histochem and Cytochem 1992;26:182-193.

6. Van Dongen JJM, Breit TM, Beishuizen A, Hooijkaas H. Detection of minimal residual disease in acute lymphoblastic leukemia by use of the PCR technique. Sangre 1992;37S3:140-145.

7. Van Dongen JJM, Breit TM, Adriaansen HJ, Beishuizen A, Hooijkaas H. Immunophenotypic and immunogenotypic detection of minimal residual disease in acute lymphoblastic leukemia. Recent Results Cancer Res 1993;131:157-183.

8. Breit TM, Mol EJ, Wolvers-Tettero ILM, Ludwig W-D, Van Wering ER, Van Dongen JJM. Site-specific deletions involving the *tal*-1 and *sil* genes are restricted to cells of the T-cell receptor $\alpha\beta$ lineage: T-cell receptor δ gene deletion mechanism affects multiple genes. J Exp Med 1993;177:965-977.

9. Breit TM, Beishuizen A, Ludwig W-D, Mol EJ, Adriaansen HJ, Van Wering ER, Van Dongen JJM. *tal*-1 deletions in T-cell acute lymphoblastic leukemia as PCR target for detection of minimal residual disease. Leukemia 1993;7:2004-2011.

10. Breit TM, Wolvers-Tettero ILM, Beishuizen A, Verhoeven M-AJ, Ludwig W-D, Van Wering ER, Van Dongen JJM. Southern blot patterns, frequencies and junctional diversity of T-cell receptor δ gene rearrangements in acute lymphoblastic leukemia. Blood 1993;82:3063-3074.

11. Beishuizen A, Verhoeven M-AJ, Mol EJ, Breit TM, Wolvers-Tettero ILM, Van Dongen JJM. Detection of immunoglobulin heavy chain gene rearrangements by Southern blot analysis: recommendations for optimal results. Leukemia 1993;7:2045-2053.

12. Uittenbogaart CH, Anisman DJ, Tary-Lehmann M, Vollger LW, Breit TM, Van Dongen JJM, Saxon A. The SCID mouse environment causes immunophenotypic changes of human immature T cell lines. Int J Cancer, in 1994;56:546-551.

13. Van Dongen JJM, Beishuizen A, Van Wering ER, Breit TM, Hählen K, Hooijkaas H. Detectie van minimal residual disease bij kinderen met acute lymfatische leukemie. In: Van Suijlekom-Smit LWA, ed. Nieuwe ontwikkelingen in diagnostiek en behandeling van kinderen. Cursusboek 22ᵉ kinderartsenweek, Sophia Kinderziekenhuis Rotterdam, 1994:11-16.

14. Breit TM, Wolvers-Tettero ILM, Van Dongen JJM. Unique selection determinant in polyclonal Vδ2-Jδ1 junctional regions of human peripheral γδ-lymphocytes. J Immunol 1994;152:2860-2864.

15. Breit TM, Wolvers-Tettero ILM, Van Dongen JJM. Phenotypic and genotypic characteristics of human early T-cell differentiation: the T-cell acute lymphoblastic leukemia model. Forum in Immunology: "Human early T-cell differentiation" in Res Immunol 1994; 145:139-143.

16. Breit TM, Van Dongen JJM. Human early T-cell differentiation: Discussion of 56th Forum in Immunology. Immunol Res 1994;145:155-156.

17. Breit TM, Van Dongen JJM. Unravelling human T-cell receptor junctional region sequences. Thymus 1994;22:177-199.

18. Breit TM, Wolvers-Tettero ILM, Bogers AJJC, De Krijger RR, Wladimiroff JW, Van Dongen JJM. Rearrangements of the human *TCRD* deleting elements. Immunogenet 1994;40:70-75.

19. Breit TM, Wolvers-Tettero ILM, Van Dongen. Lineage specific demethylation of *tal*-1 gene breakpoint region determines the frequency of *tal*-1 deletions in αβ lineage T-cells. Oncogene 1994;1847-1853.

20. Beishuizen A. Van Wering ER, Breit TM, Hählen K, Hooijkaas H, Van Dongen JJM. Molecular biology of acute lymphoblastic leukemia: Implications for detection of minimal residual disease. Haematol Blood Transfus, in press.

21. Beishuizen A, Verhoeven M-AJ, Hählen K, Van Wering ER, Breit TM, Van Dongen JJM. Cross-lineage T-cell receptor gene rearrangements in childhood precursor B-acute lymphoblastic leukemia. Submitted for publication.

22. Beishuizen A, Verhoeven M-AJ, Breit TM, Van Wering ER, Hählen K, Hooijkaas H, Van Dongen JJM. Heterogeneity in junctional regions of immunoglobulin kappa deleting element rearrangements in B-cell leukemias: a new target for detection of minimal residual disease in precursor B-acute lymphoblastic leukemia. Submitted for publication.

23. Breit TM, Wolvers-Tettero ILM, Van Gastel-Mol EJ, Hählen K, Van Wering ER, Van Dongen JJM. Human T-cells with an active V(D)J recombinase complex for TcR-δ gene deletion. Submitted for publication.

24. Breit TM, Wolvers-Tettero ILM, Van Gastel-Mol EJ, Blom B, Bogers AJJC, De Krijger RR, Spits H, Van Dongen JJM. Two pathways of sequential TcR-δ gene rearrangements in human thymocytes. Submitted for publication.