T CELL RECEPTOR-δ DELETION IN HUMAN T CELLS

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T CELL RECEPTOR-δ DELETION IN HUMAN T CELLS

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Onderzoek wordt niet in het luchtledige bedreven maar binnen een historisch gevoerde en gegroeide politieke en maatschappelijke samenhang waarin elke stap die men doet, ook elke wetenschappelijke stap, al of niet bewust evenwichtsbevorderende of evenwichtsverstorende effecten heeft. Kort gezegd: ook elke wetenschappelijke stap is een stap temidden van een vlechtwerk van conflicterende belangen en partijen, en al overziet een wetenschapper dat vlechtwerk niet, hij speelt zoals de geschiedenis bewijst, daarin terdege een rol, zelfs een fundamentele.

H.M. Kuitert

Aan mijn ouders

Voor Karin

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HUMAN T CELL DEVELOPMENT

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INTRODUCTION

The immune system

The immune system protects the body against pathogens such as bacteria, viruses, fungi, and parasites, when they pass the first line of body defence such as the skin or other epithelial and mucosal barriers.

After penetration into the body, micro-organisms encounter the second line of defence. This concerns the so-called aspecific immune system, which consists of phagocytes, such as macrophages and granulocytes, complement factors, and natural killer cells. Generally, support by the third line of defence is needed, i.e. the so-called specific immune system.

Specific immune system

The specific immune system consists of lymphocytes that are able to specifically recognize foreign molecules (antigens) by specific surface receptor molecules (antigen-specific receptors). Two types of lymphocytes exist: B lymphocytes and T lymphocytes. The antigen-specific receptor of B lymphocytes are immunoglobulin molecules, which are associated on the cell surface with CD79 molecules for signal transduction. In T lymphocytes the T cell receptor (TCR) and CD3 molecules have these functions, respectively.

When the immunoglobulin or TCR molecules of a particular B or T lymphocyte recognize the antigen against which the receptor is directed, lymphocyte activation and proliferation will follow. The B lymphocytes derived in this process can mature to plasma cells which secrete immunoglobulin molecules (antibodies) that are capable of recognizing soluble antigens. Activated T lymphocytes have a number of functions including: helping B lymphocytes to make antibodies (helper T lymphocyte); and recognizing and destroying cells infected with virus (cytotoxic T lymphocyte). After first contact with antigen (primary immune response) both B and T lymphocytes are capable of forming memory cells which mediate an efficient and rapid secondary immune response.

B and T lymphocytes are continuously generated in the human body from hematopoietic progenitor cells. Special micro-environments are needed for differentiation of the progenitor cells into mature lymphocytes: the **bone marrow** (BM) for B lymphocytes and the **thymus** for T lymphocytes (1). B and T cells are

subjected to selection processes during their differentiation to assure that B and T cell antigen-specific receptors react to the foreign antigens but not against autologous antigens (self antigens).

T lymphocytes

T cells differentiate and acquire their antigen-specific receptor in the thymus and mature into several subpopulations with different effector functions. Some subpopulations can be recognized via their cell surface molecules, such as CD4 molecules on T lymphocytes with helper functions and CD8 molecules on T lymphocytes with cytotoxic functions (2, 3). TCR molecules do not recognize soluble antigens but antigens that are bound in the groove of major histocompatibility (MHC) molecules on the surface of so-called antigen presenting cells. The TCR of $CD4^+$ T lymphocytes recognize antigens in the groove of MHC class I molecules, and the TCR of $CD8^+$ T lymphocytes recognize antigens in the groove of MHC class II molecules. These CD4 and CD8 molecules act as co-receptors in concert with the TCR-CD3 complex to transmit cell-activating signals through the cell membrane (4, 5).

Two forms of TCR molecules exist, which are disulphide-linked heterodimers consisting of α and ß, or γ and δ protein chains (6, 7). In healthy children and adults the majority of T lymphocytes in blood express the TCR-αß heterodimer, while only a minority expresses TCR-$\gamma\delta$ molecules (8). At present it is still unknown whether TCR-$\gamma\delta^+$ T lymphocytes recognize antigens presented by MHC molecules, or presented by other, nonpolymorphic, antigen presenting molecules. Therefore the exact function of TCR-$\gamma\delta^+$ T lymphocytes is still a subject of debate (reviewed in refs. 9-11).

All T lymphocyte subpopulations descent from a common precursor cell during T cell development. Most of the observations regarding T cell development have been made in mouse (1, 5, 12). In this chapter we will focus on the knowledge of human T cell development. Wherever relevant for understanding general T cell differentiation processes, information about murine T cell development will be reviewed.

IMMUNOPHENOTYPE OF T CELLS DURING DIFFERENTIATION

Early T cell progenitors generated in fetal liver or in BM migrate to the thymus (13). The human fetal thymus is colonized by hematopoietic stem cells at 8 weeks of gestation (14). These cells mature and differentiate through interactions with thymic stroma cells and the variety of cytokines they produce (1, 15).

The availability of monoclonal antibodies (McAb) reactive with functionally important and/or T cell lineage-specific molecules has increased our understanding of the developmental T cell biology (16). Application of these McAb for detailed immunophenotyping of BM and thymus cell samples allowed the recognition of different T cell subpopulations, the estimation of their relative frequencies, and the design of hypothetical schemes of human T cell differentiation, as illustrated in Figure 1 (8, 17-24).

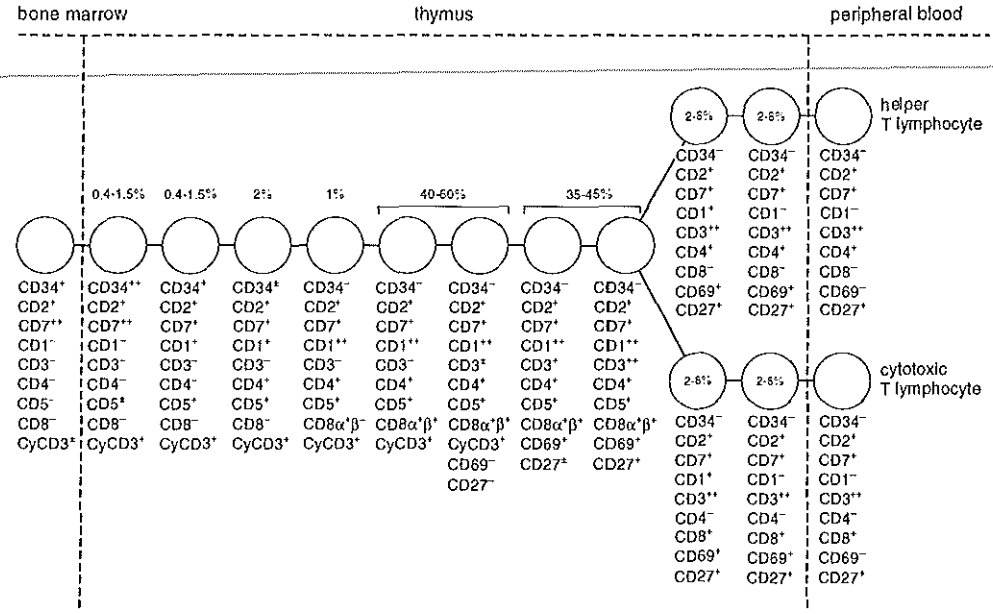

Figure 1. Hypothetical scheme of human T cell differentiation. The scheme is designed based on detailed immunophenotyping of BM, thymocytes, and peripheral blood (8, 17-24). The relative expression levels of the markers are indicated: ++, strong expression; +, intermediate expression; ± weak or partial expression; and −, no expression. The relative frequencies of the different thymocyte subpopulations (18) are indicated. See text for detailed explanation.

T cell differentiation takes place in the thymus, but some commitment towards the T cell lineage might already occur at the pre-thymic level in BM, because the immunophenotype of the putative T cell precursor in fetal BM was found to be similar to the earliest T cell precursor in the thymus (18). Although several attempts have been made to identify progenitor cells in BM that are already committed to one of the hematopoietic lineages (reviewed in refs. 13, 22), the problem of their precise identification has not yet been solved. It might well be that studies on the cell surface immunophenotype of these progenitor cells are too superficial and that detection of intracellular molecules (transcription factors and signalling molecules) is needed for precise identification.

The CD34 cell surface molecule appears to be an important marker for early T cell development. $CD34^+$ triple negative (TN; $CD3^-CD4^-CD8^-$) thymocytes develop into more mature thymocytes in human fetal thymic organ culture (FTOC) systems (19). In vitro cultured TN thymocytes can give rise to both TCR-$\alpha\beta^+$ and TCR-$\gamma\delta^+$ T cells (25, 26). Quantitatively the $CD34^+$ thymocytes represent 1-3% of the total thymocytes (18, 22). They can be subdivided by the expression of the CD1 cell surface marker: based on the higher density of the CD34 antigen on $CD34^+CD1^-$ TN thymocytes, it was suggested that these thymocytes are the most immature thymocytes followed by $CD34^+CD1^+$ TN thymocytes (18, 19).

Flow cytometric analysis of human thymocytes (18, 20), repopulation studies in SCID-hu mice (small fragments of human fetal thymus grafted under the left kidney capsule of SCID mice)(21), and human FTOC systems (19) have shown the existence

of an intermediate $CD3^-CD4^+CD8^-$ thymocyte subset before the double positive (DP; $CD4^+CD8^+$) $CD3^-CD4^+CD8^+$ and $CD3^+CD4^+CD8^+$ thymocyte stages. In the immature $CD3^-CD4^+CD8^-$ subset, the percentage of dividing cells is maximal (27). Finally, the $CD3^+CD4^+CD8^+$ thymocytes further differentiate into mature $CD4^+$ or $CD8^+$ single positive (SP) thymocytes (21, 28). Expression of CD69 and CD27 molecules is thought to be induced in the $CD3^+CD4^+CD8^+$ thymocyte stage during the early phase of positive selection (29, 30), whereby several differentiation stages can be defined by the relative expression of CD69, CD27, and CD1, as illustrated in Figure 1 (24). Down regulation of the CD1 antigen occurs before the thymocytes migrate from the thymus into the periphery (20).

TCR GENE REARRANGEMENT AND EXPRESSION DURING T CELL DEVELOPMENT

In the earliest phase of fetal thymus colonization, at 8.2 weeks of gestation, a small subset (5%) of thymocytes was stained with the ßF1 McAb, suggesting expression of TCR-ß protein chains. Furthermore, a few TCR-γ^+ cells were found to be present in these 8.2 weeks thymi, whereas no TCR-δ surface proteins were detectable (14). TCR-δ surface proteins could be detected in fetal thymus samples at 9.5 weeks of gestation (14).

Although four TCR chains exist (TCR-α, TCR-ß, TCR-γ, and TCR-δ), only TCR-$\gamma\delta$ or TCR-αß molecules are observed, except for the human T cell line DND41, which contains an unusual TCR-ßδ heterodimer (31, 32). The TCR protein chains consist of a variable domain and a constant domain. The variable domain is encoded by two, three, and sometimes four gene segments at a particular locus. The two protein chains of each TCR molecule are encoded by two different types of TCR genes: one gene contains variable (V), diversity (D), and joining (J) gene segments (TCR-δ and TCR-ß chains), whereas the other gene contains V and J gene segments (TCR-γ and TCR-α chains). The V, (D,) and J gene segments are joined via rearrangement processes.

The germline configuration of the four human TCR genes is depicted in Figure 2. The human TCR-α and TCR-ß genes contain large series of V and J gene segments, whereas the TCR-γ and TCR-δ genes contain a limited number of V and J gene segments (33, 34). The total potential receptor diversity does not only depend on the number of gene segments, but also on the junctional diversity; i.e. diversity due to nucleotide insertion (N-region) and deletion of nucleotides from the germline sequences of the gene segments at their joining sites. Because the junctional diversity of the TCR-δ genes is generally larger than of the TCR-α and TCR-ß genes, the total diversity of the TCR-$\gamma\delta$ and TCR-αß molecules is assumed to be comparable (7). The enormous diversity of TCR molecules is estimated to be larger than 10^{12} and has the potency of recognizing a very large number of foreign antigens.

Because of the limited number of available V(D)J gene segments in the TCR-γ and TCR-δ genes, several studies have been performed to investigate whether the gene segments are used in a preferential way in fetal and postnatal thymocytes (35, 36). In fetal thymi at 15-17 weeks of gestation, the Vδ2 gene segment is

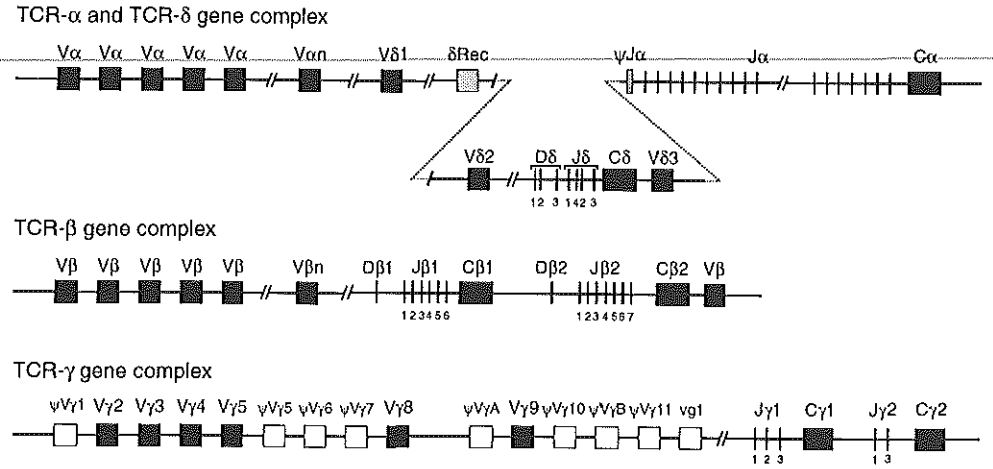

Figure 2. Schematic diagram of the germline configuration of the four human TCR genes. The TCR-α gene consists of many Vα gene segments (>50) and 61 Jα gene segments. The TCR-δ gene consists of approximately six Vδ gene segments, three Dδ gene segments, and four Jδ gene segments. The TCR-δ-deleting elements δRec and ψJα are also indicated. The TCR-β gene consists of many Vβ gene segments (>70), and two Dß-Jß gene segment clusters each consisting of one Dβ and six or seven Jβ gene segments, respectively. The TCR-γ gene consists of two clusters of a three and two Jγ gene segments, respectively, and a restricted number of six functional (solid boxes) and nine pseudo (open boxes) Vγ gene segments.

preferentially rearranged (Vδ2-Dδ3 and Vδ2-Dδ3-Jδ3) (35). The same preference was observed in human fetal liver (37). However, in postnatal thymocytes the Vδ1 gene segment is used preferentially (35). It was not possible to identify predominantly expressed Vγ gene segments throughout fetal thymic ontogeny (36).

Most data concerning the order of human TCR gene rearrangements are obtained from studies on T cell acute lymphoblastic leukemia (T-ALL), which are regarded to be malignant counterparts of cortical thymocytes (reviewed by Van Dongen et al. (8)). The TCR-δ genes rearrange early during T cell development, probably prior to other TCR genes; this assumption is based on a few CD3$^-$ T-ALL with rearranged TCR-δ genes and germline TCR-ß and TCR-γ genes (38). More recently, reverse transcriptase-polymerase chain reaction (RT-PCR) analysis performed by Ktorza et al. (39) showed the expression of full length TCR-δ transcripts in the CD34$^+$CD1$^-$ TN thymocyte subset, prior to the expression of full length TCR-ß transcripts in the CD34$^+$CD1$^+$ TN subset. The CD34$^+$CD1$^-$ TN subset only contained incomplete Dß-Jß-Cß transcripts.

Probably the TCR-γ genes rearrange before rearrangement of the TCR-ß genes, since a few CD3$^-$ T-ALL with rearranged TCR-δ and TCR-γ genes but germline TCR-ß genes have been reported (8). TCR-α gene rearrangements and transcripts are mainly found in CD3$^+$ T-ALL, whereas CD3$^-$ T-ALL have germline TCR-α genes. This suggests that TCR-α rearrangements occur late during T cell development and are probably immediately followed by TCR-$\alpha\beta$ expression on the cell surface.

In murine T cell development the TCR-ß gene is expressed at the cell surface together with a 33 kDa glycoprotein before expression of the TCR-αß heterodimer (40, 41). This so-called pre-Tα glycoprotein is postulated to play a crucial role in the

development of murine TCR-αß$^+$ T cells, but not in TCR-γδ$^+$ T cells (42). It is thought that the formation of the pre-T cell receptor complex results in expansion of the T cells before TCR-α rearrangement (43, 44). Recently, the human pre-Tα gene has been cloned (45), and RT-PCR analysis has shown that pre-Tα transcripts are present in the immature CD3$^-$CD4$^+$CD8$^-$ thymocyte subset, which, comparable to mice, also contains most of the dividing cells (27). Northern blot analyses using thymocyte subpopulations showed a strong expression of pre-Tα in the immature CD3$^-$CD4$^+$CD8$^-$ thymocyte subset and also in the more mature CD3$^-$CD4$^+$CD8$^+$ thymocyte subset, whereas the expression observed in earlier and later stages of development was much weaker (46).

Rearrangements of the TCR-α gene segments will delete the TCR-δ gene, because of the location of the TCR-δ gene between the Vα and Jα gene segments (47), therefore, rearrangements of the TCR-α gene segments will exclude TCR-δ gene expression. The here described order of TCR rearrangements is depicted in Figure 3.

The V(D)J gene rearrangement process itself remains a black box. However, several factors have been described that play an essential role in V(D)J recombination (reviewed by Lewis (48)). The expression of two of these essential genes, recombination activating gene (RAG-) 1 and RAG-2, seems to be lymphoid specific (49, 50). The other essential proteins were shown to be also involved in the more general processes of double-strand break repair (51-56). Another important protein for V(D)J recombination is the enzyme terminal deoxynucleotidyl transferase (TdT), which is responsible for the insertion of N-region nucleotides (57-59).

Knowledge of the expression pattern of these proteins can certainly add information to the understanding of V(D)J rearrangement during T cell development. RAG-1 and RAG-2 are co-expressed in both TCR-CD3$^-$ and TCR-CD3$^+$ thymocytes (60). More recently the expression pattern of RAG-1 and RAG-2 was examined in human thymocyte subpopulations using RT-PCR analysis. RAG-1 and RAG-2 are already expressed in the most immature CD34$^+$CD1$^-$ TN thymocyte subset (39), which is in line with the observed expression of full length TCR-δ transcripts and immature Dß-Jß transcripts in this subset (39). The RAG-1 gene was shown to be transcribed at high levels in DP T-ALL, but absent in more mature SP T cell leukemias (61). The enzyme TdT is also expressed in virtually all cortical thymocytes and T-ALL, while mature T cells and mature T cell leukemias are negative for TdT (8).

BIFURCATION OF THE TCR-αß AND TCR-γδ LINEAGES

Although the TCR-αß and TCR-γδ heterodimers are known for nine years now, the precise relationship between the progenitors of the TCR-αß and TCR-γδ T lymphocytes is unclear and has been subject of debate for several years. The majority of mature TCR-γδ$^+$ T lymphocytes are CD4$^-$CD8$^-$, while a minority expresses CD4 or CD8 molecules (8). In contrast, the vast majority of mature TCR-αß$^+$ T lymphocytes express CD4 or CD8 molecules. These TCR-αß$^+$ T lymphocytes originate from CD4$^-$CD8$^-$ thymocytes, raising the question whether TCR-αß and

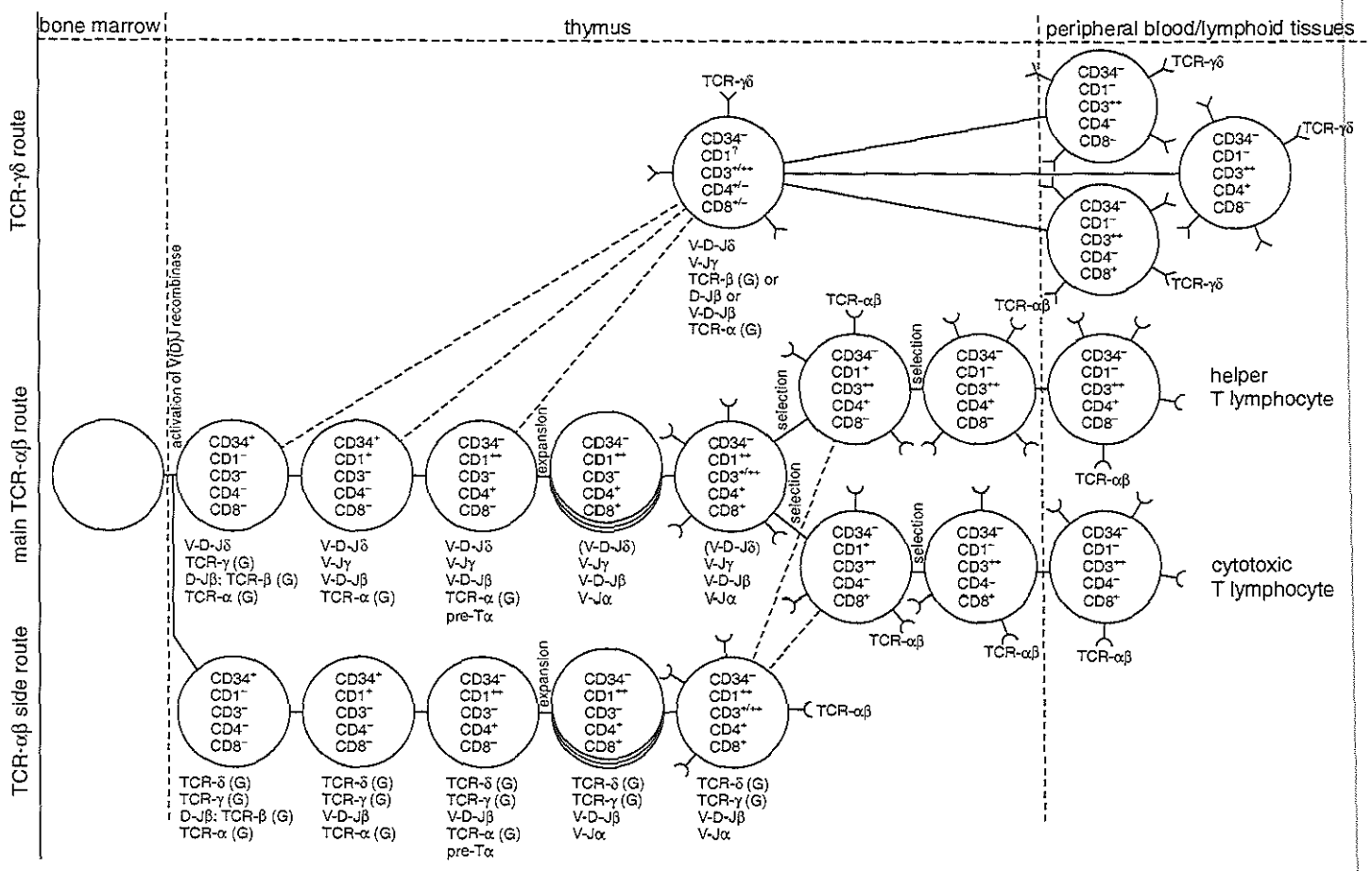

Figure 3. Hypothetical scheme of T cell lineage commitment. The immunophenotype and TCR gene rearrangements are depicted in and below the subpopulations, respectively. G: germline TCR sequence; pre-Tα: pre-T cell receptor-α glycoprotein. See text for detailed explanation.

TCR-γδ T lymphocytes are related or arise from two different lineages (62). TCR-αß- and TCR-γδ- thymocytes are thought to be derived from a common precursor. Two models have been proposed to explain the differentiation of TCR-αß and TCR-γδ thymocytes from this common progenitor (reviewed in ref. 63). The first model proposes that progenitor cells commit to the TCR-αß lineage or TCR-γδ lineage prior to rearrangement of any set of TCR gene segments. The single evidence for this model comes from a study of Winoto and Baltimore (62), who identified circular DNA excision products of Vα-Jα gene rearrangements with the TCR-δ gene in germline configuration. However, another study (64) has shown examples of rearranged TCR-δ genes in Vα-Jα excision circles. This finding fits into the second model proposing that progenitor T cells first attempt to rearrange TCR-γ and TCR-δ genes, and only attempt further TCR-α and TCR-ß rearrangements if they fail to successfully produce a functional TCR-γδ receptor (65). Further evidence for this the so-called sequential or stochastic model was obtained by studying TCR rearrangements in peripheral blood T lymphocytes and T-ALL. It appeared that 98% of normal TCR-αß$^+$ T lymphocytes have biallelic TCR-γ rearrangements, whereas the remaining 2% have one rearranged TCR-γ allele (66), and that TCR-γ genes are rearranged in all TCR-αß$^+$ T-ALL (8, 67).

Most of the data concerning the commitment of T cells to one of the lineages comes from studies on transgenic and mutant mice (reviewed in ref. 5). Some of these studies support the idea that lineage commitment occurs independently of TCR gene expression, because normal TCR-γδ T cell development was observed in TCR-ß and TCR-α knock-out mice (68), while disruption of the TCR-Cδ gene segment had little or no effect on the development of TCR-αß$^+$ T cells (69). Another interesting observation was that treatment of a murine FTOC system with CD81 McAb that block thymocyte-stroma cell interaction, inhibited TCR-αß, but not TCR-γδ T cell development (70). This suggests that both lineages can develop under different conditions in the thymus.

A recent study on the productivity of TCR-γδ rearrangements in TCR-αß$^+$ T cells further supports the stochastic model of lineage commitment. It was found that both TCR-γ and TCR-δ gene rearrangements are predominantly (80%) out-of-frame in TCR-αβ lineage T lymphocytes (71). This study also showed that TCR-β rearrangements are in-frame in TCR-γδ$^+$ T lymphocytes, which suggests that successful rearrangement of the TCR-β locus remains compatible with TCR-γδ or TCR-αβ T lymphocytes. Based on comparable data, Livak et al. (72) proposed an alternative model for the bifurcation of the TCR-αß and TCR-γδ lineages, the so-called competitive model for T cell lineage commitment. This competitive model has a number of elements in common with the stochastic model, but differs from it in assuming that thymocytes remain uncommitted and bipotential during and after completion of TCR-β rearrangements (72).

A compilation of the different models for T cell lineage commitment in combination with the immunophenotype and immunogenotype of the T cells during T cell development is depicted in Figure 3. The lower part of this figure represents the model of TCR-αß lineage commitment independent of TCR-γδ lineage commitment, in which germline TCR-δ genes can be found in Vα-Jα circular excision products (62). The middle and upper pathways in Figure 3 represent the competitive

model of lineage commitment (72). In this model, TCR-δ, TCR-γ, and TCR-ß can actively rearrange in the same uncommitted precursor cell. If in-frame rearranged TCR-γ and TCR-δ genes express a functional TCR-$\gamma\delta$ heterodimer, RAG expression will be down regulated, and the T cell is committed to the TCR-$\gamma\delta$ lineage. This can occur at any stage from immature $CD34^+CD1^-$ TN thymocytes, which can explain the presence of germline TCR-ß genes in some TCR-$\gamma\delta^+$ T-ALL (8), through $CD34^-CD1^+$ DP thymocytes. This latter stage can only be accomplished in (murine) T cells with in-frame TCR-ß rearrangements (5). In DP thymocytes, the pre-T cell receptor complex competes with the TCR-$\gamma\delta$ receptor, and the pre-T cell receptor might receive or induce signals which activate TCR-α rearrangement and other TCR-αß lineage commitment events.

REGULATION OF TCR GENE REARRANGEMENT

Little is known about the regulatory elements of T cell lineage commitment. Two of the factors that may play an important role are TCR gene enhancers and silencers. Human transcriptional enhancers of all TCR genes as well as transcription factors that likely play an essential role in the activation of these enhancers have been identified (73-81). The known human TCR enhancer binding proteins are being expressed in both TCR-$\alpha\beta^+$ and TCR-$\gamma\delta^+$ T cells (75, 78, 79). In the murine system evidence has been obtained for the existence of TCR-αß and TCR-$\gamma\delta$ lineage specific enhancers and silencers. Previous experiments of Ishida et al. (82) suggested that a transcriptional silencer in mice restricts TCR-γ gene expression to TCR-$\gamma\delta^+$ T cells. Winoto and coworkers identified two silencers in the TCR-α locus, which turn off the TCR-α enhancer (or TCR-α promoters) in TCR-$\gamma\delta^+$ but not in TCR-$\alpha\beta^+$ T cells (83, 84). Finally, Lauzurica and Krangel made several constructs of the human TCR-δ gene and showed that the Vδ-Dδ to Jδ rearrangement is controlled by the TCR-δ enhancer, whereas the Vδ to Dδ rearrangement is controlled by other elements. The TCR-δ enhancer positive constructs also showed that Vδ-Dδ-Jδ rearrangement is T cell specific but not TCR-$\alpha\beta$ or TCR-$\gamma\delta$ lineage specific (85). However, replacement of the TCR-δ enhancer with the TCR-α enhancer in the same construct showed restriction of Vδ-Dδ-Jδ rearrangements to the TCR-$\alpha\beta$ lineage (86). Further investigation on TCR enhancers and silencers will result in a better understanding of T cell lineage commitment and recombination processes during T cell development.

TCR-δ GENE DELETION

Because the TCR-δ and TCR-α gene segments are interspersed along chromosome 14q11, rearrangements of the TCR-α gene segments will delete the TCR-δ gene (47). Moreover, deletion of the TCR-δ gene will irreversibly commit the thymocytes to the TCR-αß lineage. In 1988 De Villartay et al. (87) identified two so-called TCR-δ-deleting elements, δRec and ψJα, that flank the major part of the TCR-δ gene segments. With the identification of these TCR-δ-deleting elements the question raised whether deletion of the TCR-δ gene defines an intermediate event in

the process of differentiation towards the TCR-αß lineage (38, 88). The observed high frequency of the non-functional δRec-ψJα rearrangement in human thymus cell samples suggests the importance of this rearrangement during T cell development (87, 89, 90). Previous experiments showed that the δRec-ψJα rearrangement occurs approximately 2,000-fold more frequently in TCR-$\alpha\beta^+$ T cells than in TCR-$\gamma\delta^+$ T cells, and that this rearrangement can precede subsequent Vα-Jα rearrangements (91, 92). Deletion of the TCR-δ locus prior to Vα-Jα rearrangement seems not to be obligatory in murine T cell differentiation (93).

One of the factors that is postulated to play a role in the regulation of the δRec-ψJα rearrangements is transcription of the so-called T-early-α (TEA) gene. This gene was first identified in immature TN T-ALL, as a 2-kilobase TCR-α-related transcript spliced to the Cα gene segment (94). The sterile TEA-Cα transcripts are present in human fetal thymocytes and the transcription declines during T cell differentiation (94). TEA was shown to be expressed in TCR-$\gamma\delta^+$ T-ALL, but not in TCR-$\alpha\beta^+$ T-ALL (38), and it was suggested that the activity of the TEA promoter could be directly linked to the tissue-restricted activity of the TCR-α enhancer (95). The TEA transcript starts directly upstream of the ψJα gene segment. Therefore TEA transcription may play a regulatory role in opening the TCR-Jα locus to obtain TCR-δ deletion, and subsequent Vα-Jα rearrangement (8, 38, 88, 95), but no definitive proof has been obtained yet for the exact role of the TEA gene. The function of the TEA gene stops after TCR-δ deletion, because the TEA gene is deleted by δRec-ψJα or Vα-Jα rearrangements.

The δRec gene segment rearranges preferentially to the ψJα gene segment and vice versa (87, 89, 90), but other rearrangements involving one of the two TCR-δ deleting elements have also been observed (33, 87, 96, 97), Although transcription of TEA can probably play an important role in the opening of the TCR-Jα locus, this does not fully explain why only the most upstream Jα gene segment (ψJα) is used preferentially. It is also unknown why the δRec gene segment is used most frequently, while it is located between different Vδ and Vα gene segments. Interestingly, the Vδ1 gene segment, which is preferentially used in postnatal thymocytes (35), is located upstream of the δRec gene segment. Therefore, rearrangements of the Vδ1 gene segment will delete the δRec gene segment, thereby excluding TCR-δ deletion via δRec-ψJα rearrangements.

It remains to be resolved whether germline or (non-)functional TCR-δ rearrangements are deleted prior to Vα-Jα rearrangements, according to the stochastic model of T cell commitment. Furthermore, there have to be some other important regulatory elements that play a role in preferential δRec-ψJα rearrangements and TCR-δ deletion.

AIM OF THE THESIS AND INTRODUCTION INTO THE EXPERIMENTAL WORK

The aim of this thesis was to gain more insight in the process of TCR-δ deletion via the TCR-δ-deleting elements. Therefore we tried to identify additional rearrangements of the TCR-δ-deleting elements (Chapters 2, 3, and 4), and we tried to identify putative DNA-binding proteins that might play a role in the preferential

δRec-ψJα rearrangements (Chapters 5 and 6).

Chapter 2 describes the identification of some additional preferential rearrangements of the δRec gene segment. Their junctional region sequences are compared to δRec-ψJα junctional region sequences. Furthermore, rearrangements of Vδ gene segments to ψJα are described and their relation to the δRec gene segment is discussed.

Chapter 3 compares the rearrangement patterns of Vδ1, δRec, Vδ2, and Vδ3 to Dδ, Jδ, and Jα gene segments, in order to identify the nature of the δRec gene segment with regard to the Vδ gene segments.

Chapter 4 provides a new experimental model to further investigate regulation of TCR-δ deletion, and δRec-ψJα rearrangements in particular.

Chapter 5 describes a DNA-binding protein in human thymocytes that recognizes the ψJα gene segment. The possible role of this protein in TCR-δ deletion is discussed.

The study in **Chapter 6** further characterizes the expression of the ψJα binding protein in T-ALL and thymocyte subpopulations. Its role with respect to T cell differentiation is discussed.

Chapter 7 integrates the obtained results into a new model for TCR-δ deletion during T cell development.

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CHAPTER 2

PREFERENTIAL REARRANGEMENTS OF THE T CELL RECEPTOR-δ-DELETING ELEMENTS IN HUMAN T CELLS

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PREFERENTIAL REARRANGEMENTS OF THE T CELL RECEPTOR-δ-DELETING ELEMENTS IN HUMAN T CELLS[1]

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ABSTRACT

The major part of the T cell receptor (TCR)-δ locus is flanked by the so-called TCR-δ-deleting elements δRec and ψJα, which preferentially rearrange to each other in human thymocytes. Based on our combined Southern blot and polymerase chain reaction analyses, we also identified the prominent δRec-Jα58 rearrangement and three other preferential δRec rearrangements.

The latter rearrangements concern δRec rearrangements to the Dδ3, Jδ1, and Jδ3 gene segments. These δRec rearrangements do not delete the complete TCR-δ locus and are homologous to Vδ-Jδ rearrangements, because the majority of their junctional regions contain Dδ gene segments. In contrast, the prominent δRec-ψJα and δRec-Jα58 rearrangements, representing ~68% and ~23% of all δRec rearrangements, respectively, are homologous to Vα-Jα rearrangements, because Dδ gene segments are absent in their junctional regions. Additional PCR analysis of Vδ-ψJα and Vδ-Jα58 coding and signal joints revealed that also these rearrangements resemble Vα-Jα rearrangements, except for Vδ2-ψJα and Vδ2-Jα58 rearrangements, which resemble Vδ-Jδ rearrangements.

Our data show that virtually all TCR-δ-deleting rearrangements are Vα-like, and the high frequency of these rearrangements in the human thymus suggests that most thymocytes use these rearrangements to further differentiate into the TCR-αß lineage. Based on the very low frequency of δRec and ψJα rearrangements in 4% of the T cell acute lymphoblastic leukemia patients (n=151) and 6% of the T cell lines (n=26), we hypothesize that rearranged TCR-δ-deleting elements exist for only an extremely short period during thymocyte differentiation, probably due to rapid subsequent Vα-Jα rearrangements.

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[1] Submitted for publication

INTRODUCTION

T cell receptor (TCR) molecules consist of two different glycoproteins (TCR-α and TCR-ß or TCR-γ and TCR-δ). The variable domain of each chain is encoded by a combination of variable (V), (diversity (D),) and joining (J) gene segments (1, 2). These gene segments are joined by rearrangement processes that are mediated via specific recombination signal sequences (RSS), present at rearranging sites of each gene segment (3, 4). RSS are classified according to the size of the spacer (12 or 23 base pairs (bp)), which is located between conserved heptamer and nonamer sequences. Only gene segments with differently sized spacers are able to rearrange to each other (12/23 rule) (3-5).

The human TCR-δ locus is located within the TCR-α locus, between the Vα and Jα gene segments (6) (Figure 1). This location of the TCR-δ gene excludes co-expression of a functional TCR-α and TCR-δ gene from the same allele. Therefore, deletion of the TCR-δ gene is thought to play an important role in TCR-$\gamma\delta$ versus TCR-αß lineage commitment (7-9). The major part of the TCR-δ locus is flanked by two gene segments (δRec and ψJα), which preferentially rearrange to each other during both human and murine thymocyte differentiation (7, 10, 11). Rearrangements of the δRec and ψJα gene segments, the so-called TCR-δ-deleting elements, are nonfunctional (7, 10). The TCR-δ-deleting δRec-ψJα rearrangement is thought to prepare the allele for subsequent TCR-α gene rearrangement (8). This is supported by a report of De Villartay et al. (9), who performed in vitro thymocyte differentiation experiments.

Based on the 12/23 rule, the δRec gene segment can potentially rearrange to Dδ, Jδ, and Jα gene segments. Indeed, δRec-Jδ1, δRec-ψJα, and δRec-Jα rearrangements

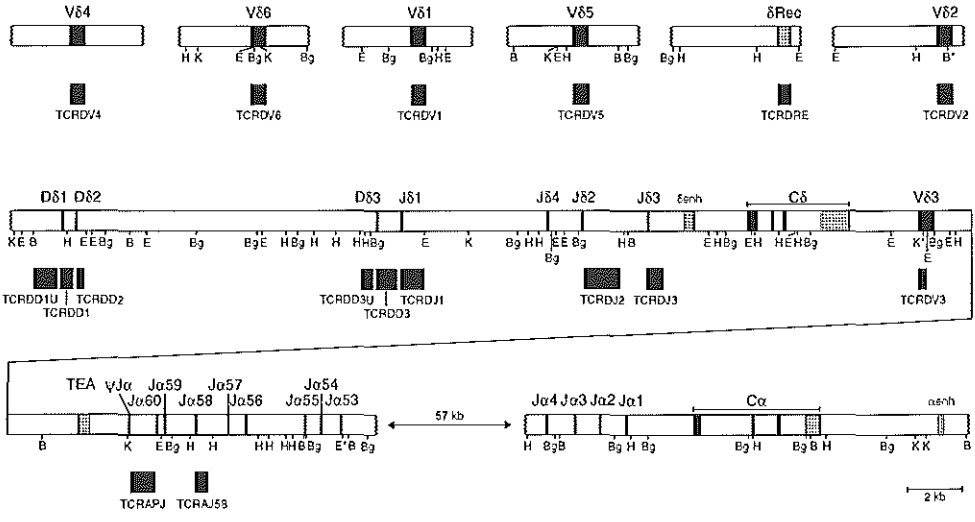

Figure 1. Restriction map of the complete human TCR-δ gene and a part of the human TCR-α gene. The relevant restriction sites are indicated: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; *, polymorphic restriction sites. Exons are indicated as solid boxes in the bars; dotted boxes in the bars represent non-coding gene segments. Solid boxes below the restriction map represent the probes used for Southern blot hybridization.

have been observed (7, 10-15). Only δRec-Jα rearrangements will delete the major part of the TCR-δ gene, but rearrangement of the δRec gene segment to the Jδ3 gene segment will also exclude functional TCR-δ gene transcripts (Figure 1). The genomic position of the human δRec gene segment as a major TCR-δ-deleting element is peculiar, because only two of the six known Vδ gene segments (Vδ2 and Vδ3) are located downstream from the δRec gene segment. Therefore, rearrangement of the frequently used Vδ1 gene segment (8, 16, 17), or the Vδ4, Vδ5, and Vδ6 gene segments will delete the δRec gene segment (Figure 1). Furthermore, it remains difficult to assign the δRec gene segment as a Vα-like or Vδ-like gene segment, because of lack of homology to any V gene segment (18).

The human ψJα gene segment is the most 5′ located Jα gene segment (Figure 1) (19). Based on the 12/23 rule, the ψJα gene segment can potentially rearrange to Vα, δRec, Vδ, and Dδ gene segments. In previous studies, Vα3.1-ψJα, δRec-ψJα, Vδ2-ψJα, and Dδ3-ψJα rearrangements have been observed (7, 10-12, 20, 21). All rearrangements to the ψJα gene segment will delete the TCR-δ gene, except for the Vδ3-ψJα inversional rearrangement (Figure 1).

In Southern blot analysis of thymocyte cell samples, the δRec-ψJα rearrangement appears as a strong band, indicating that it represents a dominant preferential rearrangement. Furthermore, we and others have shown the existence of other preferentially rearranged bands in Southern blot analysis (7, 10, 11). In this study we attempted to identify the additional preferential rearrangements of the δRec and ψJα gene segments, in order to gain more insight in their function in TCR-δ deletion.

MATERIALS AND METHODS

Cell samples

Thymocytes were obtained from a fetal thymus at 18 weeks of gestation and from three postnatal thymus samples of children undergoing cardiac surgery at the ages of 3 days, 10 months, and 15 years, respectively. The thymus samples were gently squeezed in an open filter chamber containing a nylon gauze filter with 100 μm openings (Nederlands productielaboratorium voor Bloedtransfusieapparatuur en InfusievloeistoffenB.V., Emmer-Compascuum, The Netherlands). The released thymocytes were washed twice in phosphate buffered saline (pH 7.8) supplemented with 0.5% (w/v) bovine serum albumin. Mononuclear cells (MNC) were isolated from 151 T cell acute lymphoblastic leukemia (T-ALL) patients at initial diagnosis, from peripheral blood (PB) of healthy volunteers, and from neonatal cord blood (NCB) by Ficoll paque (density 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. All human tissue and cell samples were obtained with the approval of the Medical Ethics Committee of the Erasmus University Rotterdam and University Hospital Rotterdam (Rotterdam, The Netherlands).

Twenty-six human continuous T cell lines were analyzed. The T cell lines were classified by their TCR immunophenotype; TCR^- cell lines: ARR, CCRF-CEM, CML-T1, COL-21, GH1, HSB-2, JVM-2, Karpas 299, MOLT-3, MOLT-4, MT-1, P12/ICHIKAWA, PF-382, RPMI-8402, SKW-3, SUP-T1, SUP-T3, and ST-4; TCR-$\gamma\delta^+$ cell lines: Loucy and PEER; and TCR-$\alpha\beta^+$ cell lines: HPB-ALL, HUT-78, JURKAT, Ke-37, KT-1, and MOLT-16 (22, 23). Cell line HeLa (adenocarcinoma) was used as a germline control in Southern blot analysis.

Southern blot analysis and quantitation

DNA was isolated from thymocytes, MNC, and T cell lines, digested with the restriction enzymes *Bgl*II, *Eco*RI, or *Hin*dIII, size fractionated in agarose gels (0.7%), and transferred to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) as previously described (2). TCR-δ rearrangements were studied by hybridization of the filters using our series of 16 TCR-δ gene DNA probes (Figure 1) (17), which were ^{32}P random oligonucleotide labeled. DNA probe TCRAJ58 (Jα58; 545 bp; Figure 1) was

obtained by cloning the purified polymerase chain reaction (PCR) amplification product of granulocyte DNA from a healthy volunteer, using specific oligonucleotide primers (Table I).

Hybridizations were analyzed with Fuji NIF-RX films (Fuji Photo Film Co., Tokyo, Japan), or with a phosphor-imager (PhosphoImager; Molecular Dynamics, Sunnyvale, CA) using ImageQuaNT analysis software.

PCR amplification analysis

PCR analysis was performed using 1 μg DNA, 12.5 pmol 5′ and 3′ primers, and 1 unit of *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) in 100 μl reaction volume. Oligonucleotide primers used are listed in Table I. The first PCR cycle was performed at 94°C for 3 min, 60°C for 1 min, and 72°C for 3 min in a thermal cycler (Perkin-Elmer Cetus), followed by denaturation, annealing and extension steps for another 39 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 3 min, respectively. Finally, an additional extension step of 72°C for 7 min was performed. Ten microliter of PCR amplification product was analyzed by electrophoresis in ethidium-bromide stained 1.2% agarose gels. The specificity of the amplified fragments was validated by their predicted size and most of these fragments were further identified with oligonucleotide hybridization experiments. Furthermore, DNA of cell line HeLa and water were used as negative controls in all experiments. All PCR primers were proven to be functional, because PCR analysis with coding joint and signal joint primers amplified germline fragments of the predicted size.

TABLE I. Oligonucleotide primers used in the construction of DNA probes or PCR amplification[a]

Primers for construction of DNA probes			Primers for PCR analysis of coding joints			Primers for PCR analysis of signal joints		
code	position (bp)[b]	length (bp)	code	position (bp)[b]	length (bp)	code	position (bp)[b]	length (bp)
Jα58p5′	+48	33	δRec-5′E	−204	33	δRecsj3′	+213	28
Jα58p3′	+526	35	Vδ1-5′XBg	−252	34	Vδ1sj3′	+84	24
Jα53p5′	+1	35	Vδ2-5′s	−242	32	Vδ2sj3′	+25	24
Jα53p3′	+178	33	Vδ3-5′s	−243	27	Vδ3sj3′	−247	23
δRecsj5′	+71	34	Vδ4-5′XBg	−240	33	Dδ1-5′s	−26	34
ψJαsj3′	−67	33	Vδ5-5′XBg	~ −238	33	Dδ1-3′XBg	+23	34
Jα58sj3′	−68	26	Vδ6-3′	−30	23	Dδ2-5′XBg	−11	33
			Dδ1-5′s	−34	34	Dδ2-3′s	+40	28
			Dδ1-3′XBg	+30	34	Dδ3-5′s	−30	31
			Dδ2-5′XBg	−20	33	Dδ3-3′XBg	+38	33
			Dδ2-3′s	+49	28	Jδ1sj5′	−139	24
			Dδ3-5′s	−43	31	Jδ2sj5′	−9	25
			Dδ3-3′XBg	+51	33	Jδ3sj5′	−130	23
			Jδ1-3′XBg	+157	31	ψJαsj5′	−127	27
			Jδ2-3′s	+64	32	Jα58sj5′	−99	34
			Jδ3-3′s	+88	32			
			ψJα-3′H	+105	34			
			Jα58-3′E	+40	33			

a. Only limited information is provided in this table, because most primers have been published (6, 10, 14, 17-19, 36-39). Additional oligonucleotide primer information can be obtained from the authors.

b. The position of the 3′ site of the oligonucleotide primers is indicated upstream (−) or downstream (+) relative to the cleavage site of the RSS.

Sequence analysis

A germline DNA fragment of 224 bp immediately downstream of the Jα53 gene segment, as well as a series of 34 δRec-Jα58 rearrangements were PCR amplified using specific oligonucleotide primers (listed in Table I) and cloned into pUC19 vector. Sequence reactions were performed with a T7-sequencing kit (Pharmacia), following the manufactor's instructions using ^{35}S radiolabeling, and run in a normal denaturing 8% polyacrylamide gel.

RESULTS

Southern blot patterns of the TCR-δ-deleting element δRec

DNA from fetal and postnatal thymocytes and DNA from MNC of NCB and adult PB was analyzed by Southern blotting for rearrangements of the TCR-δ-deleting element δRec, using the TCRDRE probe. Besides the germline band, several rearranged bands were visible (Figure 2, middle panel). The most prominent rearranged band in all cell samples represented δRec-ψJα rearrangements (11). We were interested whether we could identify the other preferential rearrangements.

Our series of 16 TCR-δ probes (Figure 1)(17) was used to detect and identify these preferential δRec rearrangements. In fetal thymocytes two rearranged bands with different density were detectable, besides the δRec-ψJα rearranged band (Figure 2, middle panel). The weaker rearranged band could be attributed to δRec-Dδ3 rearrangements, whereas the stronger rearranged band could not be identified with

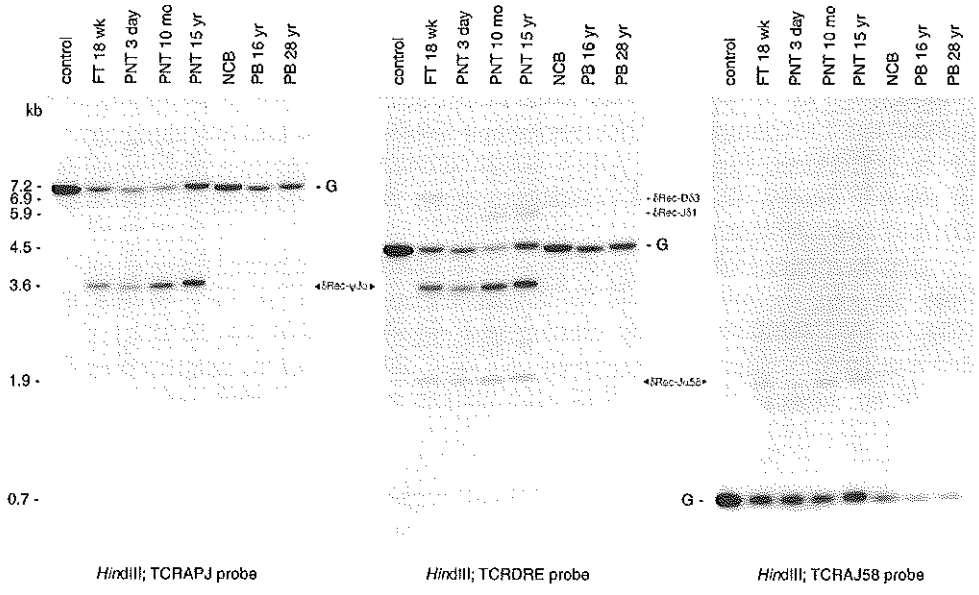

Figure 2. Southern blot analysis of preferential rearrangements of the δRec, ψJα, and Jα58 gene segments. Lane 1, control DNA (cell line HeLa); lane 2, fetal thymus (18 weeks); lane 3, postnatal thymus (3 days), lane 4, postnatal thymus (10 month); lane 5, postnatal thymus (15 years); lane 6, NCB; lane 7, PB (16 years); lane 8, PB (28 years). Filters with *Hin*dIII digested DNA were successively hybridized with the ψJα probe TCRAPJ (left panel), the δRec probe TCRDRE (middle panel), and the Jα58 probe TCRAJ58 (right panel). All identified rearranged bands and sizes are indicated. G indicates the germline band.

our Vδ, Dδ, or Jδ DNA probes. An additional faint rearranged band could be detected after long film exposure, and was identified as δRec-Jδ3 rearrangement (data not shown).

In addition to the δRec-ψJα rearranged band, we detected two rearranged bands with different density in postnatal thymocytes (Figure 2, middle panel). The weaker rearranged band could be attributed to δRec-Jδ1 rearrangements, whereas the stronger rearranged band was identical to the yet uncharacterized band in fetal thymocytes. Comparable to δRec-ψJα, this latter rearranged band was also visible in MNC of NCB and adult PB as a very faint rearranged band after long exposure times (data not shown).

Preferential δRec-Jα58 rearrangements

The second most prominent rearranged δRec band in fetal and postnatal thymocytes could not be identified with our TCR-δ DNA probes. Therefore we investigated whether this band contained δRec-Jα rearrangements. Southern blot analysis with three different restriction enzyme digests (*Bgl*II, *Eco*RI, and *Hin*dIII) was performed and the sizes of the restriction fragments of the unknown rearranged δRec bands were estimated. These estimated fragment sizes were compared with the calculated sizes of *Bgl*II, *Eco*RI, and *Hin*dIII restriction fragments of δRec-Jα rearrangements; i.e. calculated for all 61 TCR-Jα gene segments. These calculations were based on our δRec restriction map (17), and the TCR-Jα sequence data obtained by Koop et al. (19) (EMBL database accession no M94081).

The estimated δRec-Jα fragment sizes of the *Bgl*II and *Hin*dIII digests were identical to the calculated sizes of δRec-Jα58 rearrangements. However, the estimated size of the *Eco*RI digest was 7.7 kilobase pairs (kb) smaller than the calculated size. A similar 7.7 kb discrepancy was found in cell line HSB-2, which seemed to contain a δRec-Jα59 rearrangement. We performed Southern blot analysis using our Jα58 probe (TCRAJ58; Figure 1), which indeed allowed the detection and identification of the preferential δRec-Jα58 rearrangement on filters containing *Hin*dIII digested DNA (Figure 2, right panel).

Southern blot analysis of *Eco*RI digested DNA samples from 18 thymi and 5 cell lines showed the presence of an *Eco*RI polymorphism in 73% of the alleles (Figure 3A). Furthermore, by cloning and sequencing 224 bp of DNA, 7.7 kb downstream of the Jα58 gene segment, we were able to map the polymorphic *Eco*RI site 95 bp downstream of the RSS cleavage site of the Jα53 gene segment (Figure 3B).

Sequence analysis of δRec-Jα58 junctional regions

A total series of 34 δRec-Jα58 junctional region sequences was analyzed to determine their diversity and to investigate whether Dδ gene segments can occur in these junctional regions. The sequences of the various cell samples are presented in Figure 4. In fetal thymocytes, the mean number of inserted nucleotides was lower than in postnatal thymocytes (4.8 versus 6.3 nucleotides, respectively). The mean number of deleted nucleotides in fetal and postnatal thymocytes was comparable (7.5 and 7.9 nucleotides, respectively). P-region nucleotides were observed in 15% of the sequences (5/34).

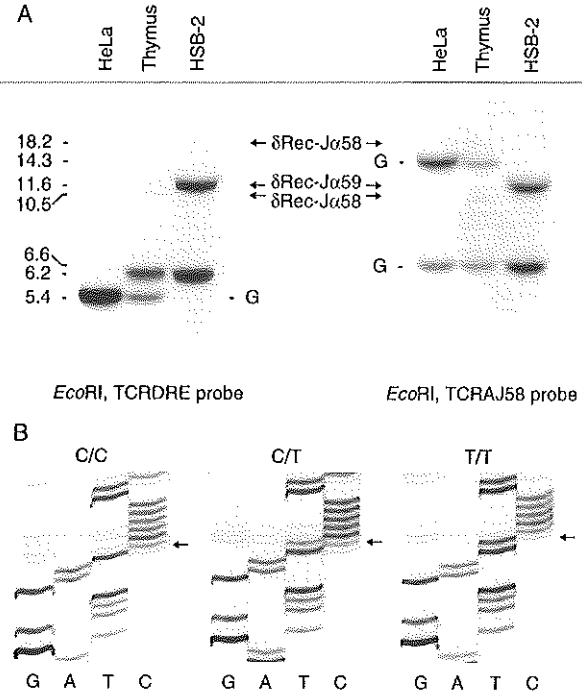

Figure 3. *Eco*RI polymorphism downstream of the Jα53 gene segment. **A.** Southern blot analysis for the detection of the polymorphic *Eco*RI restriction site in cell line HeLa, a thymocyte sample, and T cell line HSB-2. The *Eco*RI filter was successively hybridized with the δRec probe TCRDRE, and the Jα58 probe TCRAJ58. The HeLa cell line and the thymus sample are heterozygous for the *Eco*RI restriction site. Two δRec-Jα58 rearranged bands are detectable in the thymus cell sample. The HSB-2 cell line is homozygous for the *Eco*RI polymorphism, containing a 11.6 kb δRec-Jα59 rearranged band. **B.** *Eco*RI polymorphism at 95 bp downstream of the RSS cleavage site of the Jα53 gene segment. The nucleotide is either a C or a T (*Eco*RI site). The possible configurationsare presented: C/C, C/T, T/T.

We could identify putative Dδ-derived nucleotides in 47% of the junctional regions (underlined sequences in Figure 4). Although in some junctional regions (4/34) five putative Dδ3 derived nucleotides were present, no complete Dδ gene segments were observed.

PCR analysis of signal joints of rearrangements of the δRec gene segment

If δRec gene segments rearrange directly to the Jα58 gene segments, then circular excision products, containing Jα58-δRec signal joints, should be detectable by PCR analysis. We investigated the occurrence of signal joints of several δRec rearrangements. We were able to detect large amounts of PCR products of Jα58-δRec and ψJα-δRec signal joints in both fetal and postnatal thymocytes and these PCR products were also found in MNC of NCB and adult PB (Figure 5, Table II).

Dδ3-δRec signal joints were observed in fetal and postnatal thymocytes, but not in PB. Dδ1-δRec signal joints were not detectable in any of the tested cell samples, but Dδ2-δRec signal joints were observed in all cell samples (Figure 5, Table II). No Jδ3-δRec and Jδ2-δRec signal joints were observed in any sample tested. Although a weak PCR signal of Jδ1-δRec signal joints was observed in postnatal thymocytes, no Jδ1-δRec signal joints were detectable in fetal thymocytes and PB.

Ratio of the preferential δRec rearrangements

To obtain insight into the relative frequencies of the preferential δRec rearrangements, the relative density of the germline and rearranged bands was

FETAL THYMUS

δRec	junctional region	Jα58
GTGTGAGGAGCC		TTTAAGAAACCA
G		AAACCA
GTGTGAGGAGCC		AAGAAACCA
GTGTGAGGAGC		TTAAGAAACCA
GTG	CCG	AGAAACCA
GTGTGAGGAGC	AGG	GAAACCA
GTGTGAGGAGCC	TCG	GAAACCA
GTGTGAGGAGCC	*GGGG*	GAAACCA
GTGTGAGGAGCC	*GGGGG*	CC*
GTGTGAGG	GGGCTA	AAACCA
GTGTGAGGAGC	GGGGAG	ACC*
GTGTGAGGA	CCCTCTCT	AGAAACCA
GTGTGAGGAGCC	TCGCGTATGG	AACCA
GTGTGAGGAGCC	CTGGGTTCGGGGAG	GAAACC*

POSTNATAL THYMUS

δRec	junctional region	Jα58
GTGTGAGGAGCC		TTTAAGAAACCA
GTGTGAGGAGCC		TTAAGAAACCA
GTGTGAGGAGCC	CC	TTAAGAAACCA
GT	CGGG	GAAACCA
GTGTGAGG	TAGGGG	GAAACCA
GTGTGAGGAGCC	GGCTCT	GAAACCA
GTGTGAGGA	g*GAA*GA	AAACCA
-19	CCCTAAT	TTTAAGAAACCA
GTGTGAGGAGCC	GATGGGTAGGG	AACCA
	gAGTTGAGGTGGTGG	GAAACCA

PERIPHERAL BLOOD

δRec	junctional region	Jα58
GTGTGAGGAGCC		TTTAAGAAACCA
GTGTGA		AAACCA
GTGTGAGGAGCC	g	TAAGAAACCA
GTGTGAGGAG	GG	GAAACCA
GTGTGAGGAG	AG	AGAAACCA
GTGTGAGGAGCC	CGGG	CCA
GTGTGAGGAGCC	ggTGT	AGAAACCA
GTGTGAGGAGCC	*GGGG*TG	AGAAACCA
GTGTGAGGA	CCGGAGG	GAAACCA
GTGTGAGGAGC	TCCCGCTC	AAACCA
GTGTGAGGAGCC	ggGGGGGG	AACC*
GTGTGAGGAGCC	*GGGA*AAGAT	ACCA
GTGTGAGGAGCC	TCTTGCACG	TAAGAAACA
Dδ1	GAAATAGT	
Dδ2	CCTTCCTAC	
Dδ3	ACTGGGGGATACG	

Figure 4. Junctional region sequences of δRec-Jα58 rearrangements in fetal thymocytes, postnatal thymocytes, and adult PB. Germline sequences are double underlined, putative Dδ-derived nucleotides are single underlined, and lower characters represent P-region nucleotides. Nucleotides which might be assigned as either P-region nucleotides or Dδ derived nucleotides are underlined and indicated in italics. * indicates a junctional region with five nucleotides, which might be derived from the Dδ3 gene segment (see text). Germline Dδ gene segment sequences are also depicted.

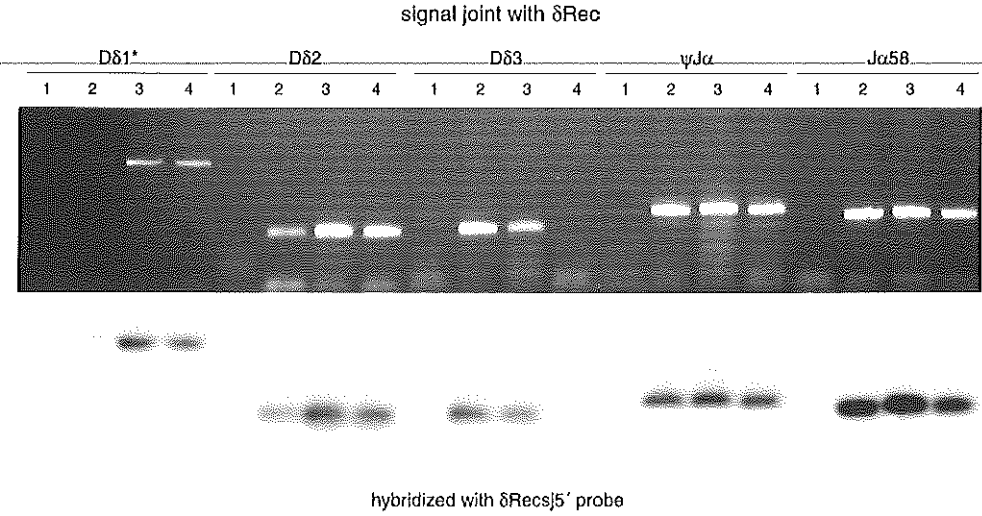

Figure 5. PCR analysis of signal joints of δRec-Dδ1, δRec-Dδ2, δRec-Dδ3, δRec-ψJα and δRec-Jα58 rearrangements. PCR products were analyzed by electrophoresis in ethidium bromide stained agarose gels followed by blotting and hybridization with the δRecsj5′ oligonucleotide probe. DNA was obtained from cell line HeLa (lane 1), fetal thymocytes (lane 2), postnatal thymocytes (lane 3), and adult PB (lane 4). * The observed bands represent Dδ2-δRec signal joints, which can be amplified from circular excision products because of the short distance between the Dδ1 and Dδ2 gene segments.

measured by use of a phosphor-imager. In both fetal and postnatal thymocytes the density of the δRec-ψJα rearranged band was nearly equal to the germline band, whereas the density of the δRec-ψJα rearranged band in MNC of NCB and adult PB was only ~1% of the germline band. In fetal thymocytes the ratio between δRec-ψJα and δRec-Dδ3 rearrangements was 10:1. In postnatal thymocytes the ratio between δRec-ψJα and δRec-Jδ1 rearrangements was approximately 8:1. The density of the δRec-Jα58 rearranged band was only three times less than the δRec-ψJα rearranged band in both fetal and postnatal thymocytes. We were not able to reliably determine the ratio of δRec-ψJα and δRec-Jα58 rearrangements in MNC of NCB or adult PB, because of the faintness of the rearranged bands.

TABLE II. PCR analysis of signal joints of δRec rearrangements[a]

signal joint	fetal thymus	postnatal thymus	peripheral blood
Dδ1-δRec	−	−	−
Dδ2-δRec	+	++	+
Dδ3-δRec	++	+	−
Jδ1-δRec	−	±	−
Jδ2-δRec	−	−	−
Jδ3-δRec	−	−	−
ψJα-δRec	++	++	+
Jα58-δRec	++	++	+

a. Symbols used for density of the PCR product: ++, strong band; +, moderate band; ±, faint band; −, no PCR product visible in agarose gel.

Southern blot patterns of the TCR-δ-deleting element ψJα

DNA from fetal and postnatal thymocytes and DNA from MNC of NCB and adult PB was analyzed for the presence of ψJα rearrangements by use of Southern blotting with the TCRAPJ probe. Besides the germline band and the δRec-ψJα rearranged band, multiple rearranged bands could be detected (11). We were unable to identify these rearranged bands using our series of six Vδ probes. Therefore, these rearranged bands probably represent other rearrangements, such as Vα-ψJα rearrangements.

PCR analysis of coding joints of Vδ-ψJα and Vδ-Jα58 rearrangements

Except for δRec-ψJα rearrangements, we were not able to identify preferential ψJα rearrangements by Southern blot analysis. To analyze coding joints of rearrangements of the six known Vδ gene segments and the three Dδ gene segments to the ψJα and Jα58 gene segments, PCR analysis was performed on DNA from fetal and postnatal thymocytes as well as on DNA from MNC of NCB and adult PB.

All Vδ gene segments were able to rearrange to ψJα and Jα58 in fetal and postnatal thymocytes and adult PB (Figure 6, Table III). In MNC of NCB only Vδ2-ψJα rearrangements were easily detectable.

Although Dδ1-ψJα and Dδ1-Jα58 rearrangements could not be detected, Dδ2-ψJα and Dδ2-Jα58 rearrangements were detected in postnatal thymocytes, and Dδ3-ψJα and Dδ3-Jα58 rearrangements were detectable in both fetal and postnatal thymocytes (Table III).

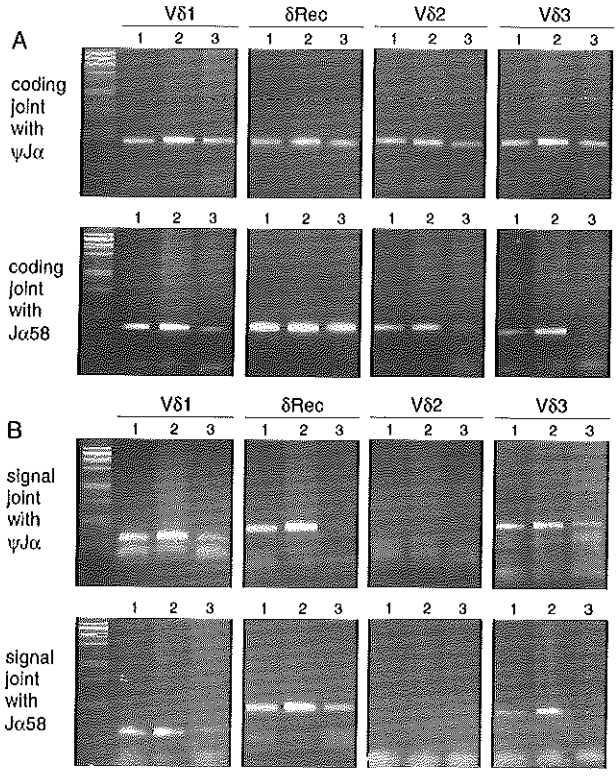

Figure 6. PCR analysis of coding joints (A) and signal joints (B) of Vδ and δRec rearrangements to the ψJα and Jα58 gene segments. DNA was obtained from fetal thymocytes (lane 1), postnatal thymocytes (lane 2), and adult PB (lane 3).

TABLE III. PCR analysis of coding and signal joints of rearrangements to the ψJα and Jα58 gene segments[a]

5′ gene segment (primer)	3′ gene segment (primer)							
	ψJα				Jα58			
	FT	PNT	NCB	PB	FT	PNT	NCB	PB
coding joint								
Vδ4	+	++	−	+	+	++	ND	+
Vδ6	++	++	±	++	±	+	ND	±
Vδ1	+	++	±	+	+	++	±	+
Vδ5	+	+	−	±	+	++	ND	++
δRec	+	++	++	+	++	++	++	++
Vδ2	+	+	+	+	+	+	±	±
Dδ1	−	−	−	−	−	−	−	−
Dδ2	−	+	±	−	−	±	ND	−
Dδ3	+	±	±	−	±	±	±	−
Vδ3	+	++	−	+	±	+	±	±
signal joint								
Vδ1	+	++	±	±	+	+	±	±
δRec	++	++	+	+	++	++	++	+
Vδ2	−	−	−	−	−	−	−	−
Dδ1	−	−	−	−	−	−	−	−
Dδ2	−	−	−	−	−	−	−	−
Dδ3	+	±	−	−	+	+	ND	−
Vδ3	+	+	±	±	±	+	−	−

a. Abbreviations used: FT, fetal thymocytes; PNT, postnatal thymocytes; NCB, neonatal cord blood; PB, adult peripheral blood; ND, not determined. Symbols used for density of the PCR product: ++, strong band; +, moderate band; ±, faint band; −, no PCR product visible in agarose gel.

As expected from our Southern blot analysis, rearrangements of δRec-ψJα and δRec-Jα58 were easily detectable in all thymus and blood cell samples tested.

PCR analysis of signal joints of Vδ-ψJα and Vδ-Jα58 rearrangements

The occurrence of signal joints of the most frequently used Vδ gene segments (Vδ1, Vδ2, and Vδ3) to ψJα and Jα58 gene segments was investigated. We were able to detect ψJα-Vδ1, ψJα-Vδ3, and Jα58-Vδ1 signal joints in all cell samples tested as well as Jα58-Vδ3 signal joints in fetal and postnatal thymocytes (Table III). We could not detect ψJα-Vδ2 or Jα58-Vδ2 signal joints with our PCR analyses, although coding joints of the latter rearrangements were detectable (Table III, Figure 6). Signal joints of ψJα-Dδ3 and Jα58-Dδ3 were observed in thymocytes, but signal joints using the Dδ1 and Dδ2 gene segments were not observed.

To further investigate the occurrence of ψJα-Vδ2 or Jα58-Vδ2 signal joints, we performed additional PCR-blotting-hybridization experiments. We observed weak hybridization signals of ψJα-Vδ2 signal joints in postnatal thymocytes and Jα58-Vδ2 signal joints in fetal, postnatal thymocytes, and adult PB after long exposure times (Figure 7).

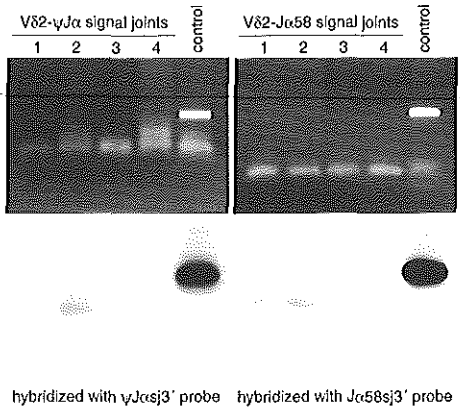

Figure 7. PCR analysis of signal joints of Vδ2-ψJα and Vδ2-Jα58 rearrangements. PCR products were analysed by electrophoresis in ethidium bromide stained agarose gels followed by blotting and hybridization with the ψJαsj3' and the Jα58sj3' oligonucleotide probes. DNA was obtained from fetal thymocytes (lane 1), postnatal thymocytes (lane 2), adult PB (lane 3), and cell line HeLa (lane 4).

Rearrangements of the δRec and ψJα gene segments in T-ALL and T cell lines

As T-ALL are assumed to originate from cortical thymocytes (2, 24), we also searched for rearrangements of the δRec and ψJα gene segments in 151 T-ALL with Southern blot analysis. We observed ten alleles containing a δRec rearrangement (Table IV), of which δRec-Jδ1 and δRec-ψJα were found on three alleles (T022, T149, T062) and two alleles (T044, T078), respectively (Figure 8). Two other rearranged bands were identified as δRec-Jα49 (T015) and δRec-Jα39 (T068) rearrangements (Figure 8). The other three rearrangements could not be identified. Besides the two T-ALL samples with a δRec-ψJα rearrangement, three additional unidentified rearrangements of the ψJα gene segment were observed. The majority (7/13) of the δRec and ψJα rearranged bands was found in immature TCR$^-$ T-ALL (Table IV). In three additional T-ALL patients (T001, T019, and T020) a weak δRec-ψJα rearranged band was visible, which we estimated to represent 5 to 10% of the cells.

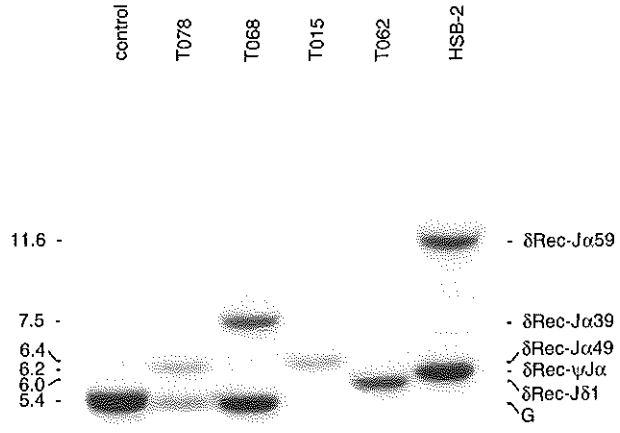

Figure 8. Southern blot analysis of rearrangements of the δRec gene segment in T-ALL and a T cell line. Lane 1, control DNA (cell line HeLa); lane 2, T-ALL T078; lane 3, T-ALL T068; lane 4, T-ALL T015; lane 5, T-ALL T062, lane 6, T cell line HSB-2. The filter containing *Eco*RI digested DNA was hybridized with the δRec probe TCRDRE.

TABLE IV. Allelic frequency of TCR-δ deletions and configuration of the TCR-δ-deleting elements δRec and ψJα in T-ALL and T cell line subgroups defined by TCR phenotype

		δRec[a]			ψJα[a]		
cell sample (number)	TCR-δ deletion (number of alleles)	G	R (number of alleles)	D	G	R (number of alleles)	D
T-ALL							
TCR$^-$	22%	53%	4%	43%	76%	2%	22%
(n=76)	(33)	(80)	(6)	(66)	(116)	(3)	(33)
TCR-γδ$^+$	2%	40%	2%	58%	98%	2%	0%
(n=30)	(1)	(24)	(1)	(35)	(59)	(1)	(0)
TCR-αβ$^+$	82%	16%	3%	81%	18%	1%	82%
(n=45)	(74)	(14)	(3)	(73)	(16)	(1)	(73)
T cell lines							
TCR$^-$	83%	8%	6%	86%	14%	6%	81%
(n=18)	(30)	(3)	(2)	(31)	(5)	(2)	(29)
TCR-γδ$^+$	25%	25%	0%	75%	75%	0%	25%
(n=2)	(1)	(1)	(0)	(3)	(3)	(0)	(1)
TCR-αβ$^+$	100%	0%	0%	100%	0%	0%	100%
(n=6)	(12)	(0)	(0)	(12)	(0)	(0)	(12)

a. Configuration of the gene segments: G, allele in germline configuration; R, rearranged allele; D, deleted allele.

Twenty-six human continuous T cell lines were analyzed by Southern blotting. We detected two rearranged bands with the TCRDRE probe in cell line HSB-2 and identified them as δRec-ψJα and δRec-Jα59 rearrangements (Figure 8). An additional cell line (ARR) was found to contain an unidentified rearrangement of the ψJα gene segment. As shown in Table IV, most T cell lines contain deletions of the δRec and/or ψJα gene segments on one or both alleles.

DISCUSSION

Deletion of the TCR-δ gene plays an important role in the bifurcation of the TCR-γδ and TCR-αß differentiation lineages (8). The high frequency of δRec-ψJα rearrangements in human thymocytes (7, 10, 11 and this study) strongly suggest that the major fraction of the thymocytes use this nonfunctional rearrangement to delete their TCR-δ genes during differentiation (9, 25, 26). Although the δRec-ψJα rearrangement seems to be the most prominent TCR-δ-deleting rearrangement in human thymocytes, other gene segments can preferentially rearrange to one of these so-called TCR-δ-deleting elements. Based on our extensive Southern blot analysis, we identified two types of preferential δRec rearrangements: rearrangements to TCR-δ gene segments (δRec-Dδ3, δRec-Jδ1, and δRec-Jδ3) and TCR-δ-deleting rearrangements to TCR-Jα gene segments (δRec-ψJα and δRec-Jα58).

The δRec gene segment is not homologous to any Vδ gene segment. However, we found that the occurrence of preferential δRec rearrangements to Dδ or Jδ gene segments parallels the occurrence of several Vδ rearrangements during human

ontogeny, i.e. δRec-Dδ3 and δRec-Jδ3 compared to Vδ2-Dδ3 and Vδ2-Jδ3 in fetal thymocytes (16, 27, 28), and δRec-Jδ1 compared to Vδ-Jδ1 in postnatal thymocytes (16, 27, 29). Rearrangement of the δRec gene segment to the Dδ3 or Jδ1 gene segments does not delete the complete TCR-δ gene, as other upstream Vδ gene segments can potentially rearrange to the downstream Jδ gene segments. The δRec-Jδ3 rearrangement on the other hand, will exclude functional TCR-δ gene rearrangements on that allele.

Several studies have shown that preferential Vδ2-Jδ3 rearrangements in fetal thymocytes contain Dδ3 gene segments (16, 27). To investigate the presence or absence of Dδ gene segments in preferential δRec-Jδ3 rearrangements, we analyzed signal joints. Signal joints, which generally reside in circular excision products, tell exactly which gene segments have rearranged to each other, because they remain stable, i.e. are unaffected by ongoing rearrangements. For the detection of signal joints, PCR primers were developed downstream of the RSS of the 5′ gene segment, and upstream of the RSS of the 3′ gene segment (Table I). This position of the primers excludes the detection of signal joints from δRec-Jδ3 rearrangements which contain Dδ gene segments. The absence of Jδ3-δRec signal joints in our PCR analysis suggests that the δRec gene segment preferably rearranges to the Jδ3 gene segment via Dδ gene segments. In contrast to the previously described Vδ1-Dδ2-Dδ3-Jδ1 preferential rearrangement in postnatal thymocytes (16, 27, 29), our signal joint analysis show that the δRec gene segment can directly rearrange to the Jδ1 gene segment in postnatal thymocytes.

Although our initial Southern blot analyses were hampered by a polymorphic *Eco*RI site downstream of the Jα53 gene segment, we could demonstrate that the δRec-Jα58 rearrangement is the second most prominent rearrangement of the δRec gene segment in human thymocytes. Unlike previously published data, which described the absence of distinct Jα58 rearranged bands in thymocytes (21), we were able to detect rearranged bands in fetal and postnatal thymocytes using our Jα58 DNA probe. Based on our densitometric studies, we estimate that the δRec-ψJα and δRec-Jα58 rearrangements represent ~68% and ~23%, respectively, of all detectable δRec rearrangements in human thymocytes. The nonfunctional δRec-Jα58 rearrangement also deletes the TCR-δ gene, which makes the Jα58 gene segment an important TCR-δ-deleting element.

The δRec-ψJα rearrangement appears to be comparable to Vα-Jα rearrangements, based on the absence of complete Dδ gene segments in their junctional region sequences (11). We show that δRec-Jα58 junctional sequences do also not contain complete Dδ gene segments. Nevertheless we observed five junctional region sequences with four putative Dδ3 derived nucleotides and four sequences with five putative Dδ3 derived nucleotides; most of these nucleotides (36/40) appeared to be G nucleotides. In four of these junctional regions two G nucleotides can also be regarded as P-region nucleotides (Figure 4). Furthermore, preferential utilization of G nucleotides and GG dinucleotides can be explained by the enzymatic activity of terminal deoxynucleotidyl transferase (30), implicating that we must be cautious to assign these G nucleotides to be Dδ3 derived.

To further investigate the occurrence of Dδ gene segments in preferential δRec-Jα58 and δRec-ψJα rearrangements, we analyzed signal joints. The presence of high

levels of Jα58-δRec and ψJα-δRec signal joints demonstrates that the δRec gene segment preferentially rearrange directly to the Jα58 and ψJα gene segments. Formally, this does not exclude that some of the δRec-ψJα and δRec-Jα58 rearrangements contain a Dδ gene segment.

The absence of Dδ derived nucleotides in δRec-ψJα and δRec-Jα58 rearrangements suggests that TCR-δ-deleting rearrangements to the ψJα and Jα58 gene segments are Vα-Jα like rearrangements. To investigate whether this also applies to Vδ-ψJα and Vδ-Jα58 rearrangements, we performed PCR analysis, because preferential Vδ-ψJα rearrangements are not detectable by Southern blot analysis, indicating that they probably occur in less than 5% of the cells. PCR analysis of coding joints demonstrated that both the ψJα and Jα58 gene segments can rearrange to the six known Vδ gene segments in thymocytes. All Vδ-ψJα and Vδ-Jα58 rearrangements will delete the TCR-δ gene, except for the Vδ3-ψJα and Vδ3-Jα58 inversional rearrangements. Although the Jα58 gene segment contains a stop codon three nucleotides downstream from the RSS heptamer, exonuclease activity during Vδ-Jα58 rearrangements can result in functional rearrangements, in contrast to Vδ-ψJα rearrangements (19). Indeed, Giachino et al. (21) showed that 80% of the Vδ2-Jα58 rearrangements in human thymocytes were functional. Several studies have shown the presence of Vδ-Jα-Cα transcripts in PB (31-34), and it was estimated that approximately 1% of PB lymphocytes contain Vδ-Jα-Cα transcripts (35). Therefore, we were not surprised to find Vδ-Jα58 rearrangements in PB. The occurrence of Vδ-ψJα rearrangements in PB lymphocytes indicates that they reside on the nonfunctional allele.

To further investigate the genesis of Vδ-ψJα and Vδ-Jα58 rearrangements, we evaluated the presence or absence of Dδ gene segments in these rearrangements via signal joint analysis. Both Vδ1 and Vδ3 rearrange directly to the ψJα and Jα58 gene segments, and are therefore homologous to Vα-Jα rearrangements. Interestingly, the lack of ψJα-Vδ2 and Jα58-Vδ2 signal joints in our PCR analyses and the faint hybridization signals of these signal joints suggests that the Vδ2 gene segment preferentially rearranges to the ψJα and Jα58 gene segment via a Dδ gene segment. This is supported by experiments of Giachino et al. (21), which showed Dδ derived nucleotides in 10 of 11 Vδ2-Jα58 rearrangements.

T-ALL and most available T cell lines are assumed to originate from cortical thymocytes (2, 24). Based on the abundance of δRec-ψJα and δRec-Jα58 rearrangements in thymocytes, we expected to find high frequencies of these rearrangements in T-ALL. In contrast only two T-ALL and one T cell line contained δRec-ψJα rearrangements, while δRec-Jα58 rearrangements were not observed in our series of 151 T-ALL and 26 T cell lines. An explanation for this discrepancy might be that these rearranged gene segments exist for an extremely short period during thymocyte differentiation, and are instantly replaced by Vα-Jα rearrangements. In that case δRec-ψJα and δRec-Jα58 rearrangements are part of circular excision products, which might still be detectable in the thymus, but which will disappear by dilution in dividing T-ALL cells and T cell lines. In three additional T-ALL patients we observed a weak δRec-ψJα rearranged band. This rearranged band could represent a small T-ALL subclone (5-10% of MNC) containing a δRec-ψJα rearrangement, or 'background' of δRec-ψJα rearrangements from normal blood cells. The latter

explanation is less likely, because normal T cells represent a minor fraction in blood of T-ALL patients at diagnosis.

We conclude from our study that preferential rearrangements of the δRec gene segment play a pivotal role during human T cell development, with δRec-ψJα and δRec-Jα58 being the most prominent δRec rearrangements. Preferential rearrangements of the δRec gene segment to Dδ3, Jδ1, and Jδ3 gene segments parallel and are homologous to Vδ-Jδ rearrangements during human ontogeny, whereas the most prominent δRec rearrangements to ψJα and Jα58 are homologous to Vα-Jα rearrangements. Only the latter δRec rearrangements are essential for deletion of the TCR-δ locus. Except for δRec-ψJα other ψJα rearrangements could not be identified by Southern blot analysis. Nevertheless we were able to detect Vδ-ψJα and Vδ-Jα58 TCR-δ deleting rearrangements by PCR analysis. Comparable to δRec-ψJα and δRec-Jα58 rearrangements, also Vδ-ψJα and Vδ-Jα58 rearrangements are homologous to Vα-Jα rearrangements, except for Vδ2-ψJα and Vδ2-Jα58 rearrangements which resemble Vδ-Jδ rearrangements, because the majority of their junctional regions contain Dδ gene segments. Our data show that virtually all TCR-δ deletional rearrangements are Vα-like rearrangements. Based on the very low frequency of δRec and ψJα rearrangements in 4% of the T-ALL patients (n=151) and 6% of the T cell lines (n=26), we hypothesize that rearranged TCR-δ-deleting elements exist for only an extremely short period during thymocyte differentiation. However the high frequency of these rearrangements during human ontogeny suggests that most human thymocytes use these rearrangements to further differentiate into the TCR-αß lineage. This supports the hypothesis that TCR-δ deletion is essential for subsequent Vα-Jα rearrangements.

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CHAPTER 3

T CELL RECEPTOR Vδ-Jα REARRANGEMENTS IN HUMAN T CELLS

CHAPTER 3

T CELL RECEPTOR Vδ-Jα REARRANGEMENTS IN HUMAN T CELLS
A comparative study of Vδ1, Vδ2, Vδ3, and δRec gene segments[1]

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BRIEF COMMUNICATIONS

The T cell receptor (TCR)-α and TCR-δ gene segments are interspersed on chromosome 14q11. The TCR-δ locus is located between the Vα and Jα gene segments (1). Therefore deletion of the TCR-δ gene plays an important role in the bifurcation of the TCR-αß versus TCR-γδ differentiation lineages (2-4).

Two non-functional TCR-δ-deleting gene segments (δRec and ψJα) have been identified which flank the major part of the TCR-δ gene (Figure 1A) (2). The high frequency of δRec-ψJα rearrangements in human thymocytes suggests that a major fraction of the thymocytes use this rearrangement to delete their TCR-δ genes. We recently showed that δRec-Jα58 rearrangements also occur at high frequency in human thymocytes, which makes the Jα58 gene segment an additionally important TCR-δ-deleting element (5).

Despite the identification of several TCR-δ-deleting elements, little is known about the precise regulation of TCR-δ deletion prior to Vα-Jα rearrangement. The position of the δRec gene segment as a major TCR-δ-deleting element is peculiar, because several Vδ gene segments (Vδ1, Vδ4, Vδ5, and Vδ6) are located upstream of the δRec gene segment (Figure 1A). Therefore, rearrangements of these Vδ gene segments will delete the δRec gene segment. On the other hand, Vδ-Jα rearrangements will delete the TCR-δ gene as well, and these rearrangements can indeed be detected in human thymocytes (5).

Several studies have shown the presence of Vδ-Jα-Cα transcripts in human thymocytes and peripheral blood (PB) (6-10). These studies suggest a difference in Jα gene segment usage between Vδ1 on the one hand, and Vδ2 and Vδ3 on the other hand. We recently analyzed Vδ-ψJα and Vδ-Jα58 rearrangements and found that the Vδ2 gene segment does not preferentially rearrange directly to the ψJα and Jα58 gene segments, but rearranges via Dδ gene segments, whereas the Vδ1, δRec, and Vδ3 gene segments can more easily rearrange directly to the ψJα and Jα58 gene segments

[1] Submitted for publication

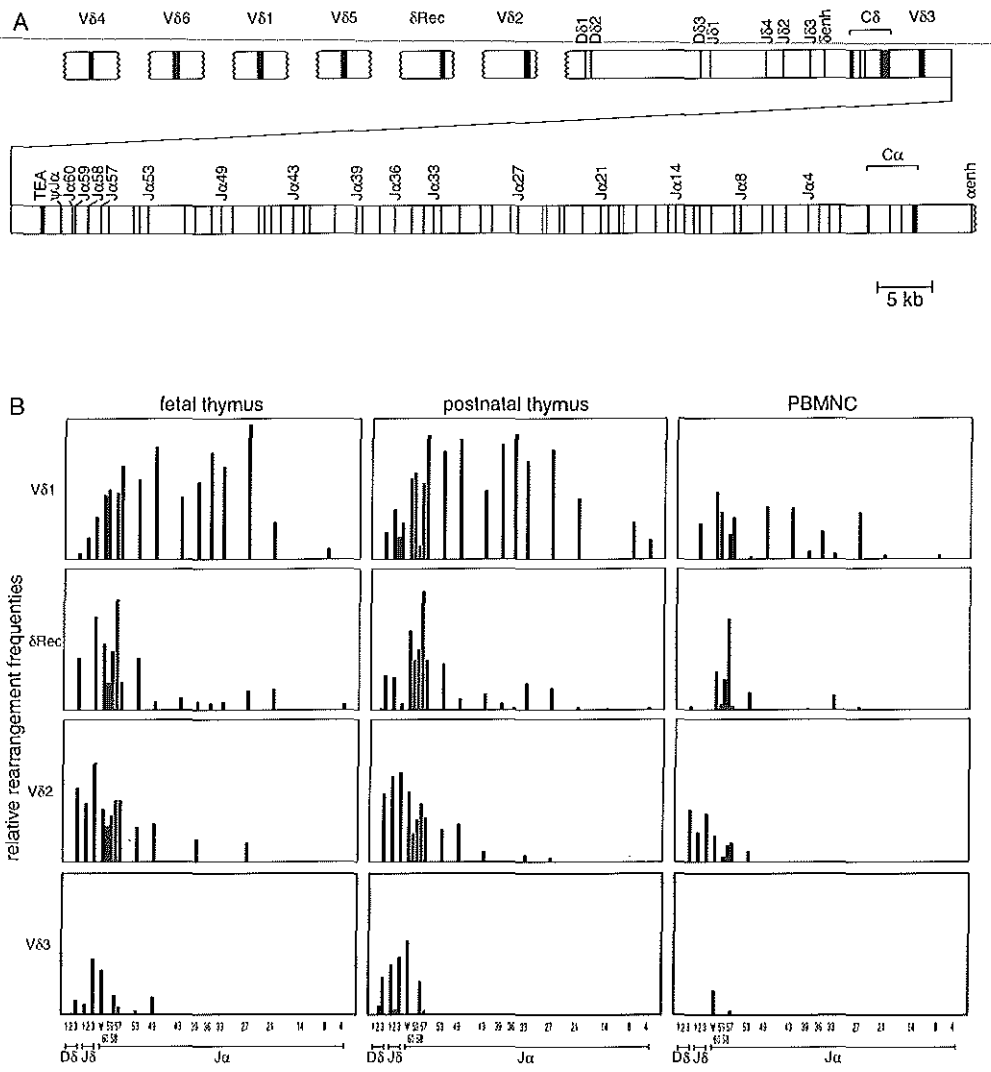

Figure 1. A. Map of the complete human TCR-δ gene and the complete human TCR-Jα to Cα region. The gene segments and enhancer (enh) regions are indicated as solid boxes. The map from Cδ to the TCRα enhancer was based on the sequence from koop et al. (25)(EMBL accession no. M94081). Only Jα gene segments used in this study are named. **B.** Comparison of relative rearrangement frequencies of Vδ-Jδ/Jα or δRec-Jδ/Jα in fetal thymus (left column), postnatal thymus (middle column) and PBMNC (right column). The upstream gene segments analyzed are indicated at the left, and the downstream J gene segments are indicated below each diagram. The bars represent the relative intensity of the signals of the PCR products. They therefore only represent a rough estimation of the rearrangement distribution between the cell samples.

(5). In order to further characterize the differences between these gene segments, rearrangements of Vδ1, δRec, Vδ2, and Vδ3 to three Dδ, three Jδ, and 16 (randomly chosen) Jα gene segments were analyzed (Figure 1A), using the polymerase chain reaction (PCR).

The cell samples consisted of a fetal thymus from 18 weeks of gestation, three

postnatal thymus samples of children undergoing cardiac surgery at the age of 9 days, 10 month, and 15 years, and mononuclear cells (MNC) isolated from PB of a healthy volunteer as described (5). DNA was extracted from the obtained cell samples as described (11). Of each DNA sample 1 μg was amplified in 100 μl PCR mixture using 12.5 pmol specific oligonucleotide primers (5, 12). DNA of cell line HeLa and water were used as negative controls in all experiments. Ten microliter of PCR amplification products was loaded on 1.2% ethidium-bromide agarose gels. The bands of the PCR products were visualized with UV light. A life-size photograph was made with a reflex-camera and used for scanning at a resolution of 100 dots per inch (Collorscanner 2, Highscreen, Würselen, Germany). Specificity of the amplified fragments was validated by their predicted size. The intensity of these bands was measured using computer software as described (13), to obtain a rough measure for the occurrence of the studied rearrangements. The measured values of the duplicate PCR experiments were always comparable, and the mean values of these experiments are presented in Figure 1B. We have no reason to assume that differences in PCR products were obtained by the use of inappropriate oligonucleotide primers, because most of the primers have been previously used for cloning of rearranged and germline gene segments (5, 12, 14, 15) and most of the obtained fragments were further identified with oligonucleotide hybridization experiments.

Due to the high sensitivity of the PCR amplification technique a lot of rearrangements showed a positive signal. The Vδ1 gene segment rearranges easily to the most upstream Jα gene segments (except for Jα59), and to Jα gene segments in the middle of the Jα locus, but rearrangements to gene segments downstream of Jα27 occur less frequently (Figure 1B). The main difference between rearrangements in fetal thymocytes and postnatal thymocytes is the amount of PCR products. In PBMNC, the amount of PCR products is less than in fetal and postnatal thymocytes. Still almost all tested Vδ1-Jα rearrangements were observed in PBMNC, which is in line with a previous study by Miossec et al. (6).

Interestingly, rearrangements of the δRec, Vδ2, and Vδ3 gene segments to Jα gene segments are very much alike. They have a high preference of using the most upstream Jα gene segments, and the amount of PCR products rapidly declines when Jα gene segments are used downstream of the Jα53 (in case of δRec) or Jα49 (in case of Vδ2 and Vδ3) gene segments (Figure 1B). The amount of PCR products of δRec and Vδ2 rearrangements is larger than of the Vδ3 gene segment in all samples tested. This might be explained by the fact that rearrangements of the Vδ3 gene segment are always inversional and are therefore probably more difficult (16). There is little difference between the amount of PCR products in fetal and postnatal thymocytes, but, as with the Vδ1 gene segment, less PCR amplification products are observed in PBMNC (Figure 1B). Although Vδ-Jα rearrangements can be found in PBMNC, this does not mean that Vδ-Jα-Cα transcripts are present in PBMNC. For instance, Vδ2-Jα-Cα transcripts are not observed in PBMNC (8), whereas Vδ1-Jα-Cα, and Vδ3-Jα-Cα transcripts are detectable in PBMNC (6, 7, 9).

Vδ1, Vδ2, and Vδ3 gene segments are the most frequently used Vδ gene segments in TCR-$\gamma\delta^+$ T cells (4, 9). Rearrangements of the Vδ1, Vδ2, and Vδ3 gene segments to the Dδ3 gene segment were easily detected in all cell samples tested, whereas rearrangements of the Vδ gene segments to the Dδ1 gene segment were not

observed and rearrangements to the Dδ2 gene segment were hardly detectable (Figure 1B). The latter can be explained by the fact that we cannot detect Vδ-Dδ2 coding joints which are included in Vδ-Dδ2-Dδ3(-Jδ) rearrangements due to the position of the PCR primers. We observed rearrangements of the Vδ gene segments to Jδ gene segments, although rearrangements to the Jδ2 gene segment were hardly detectable in most cell samples. The δRec gene segment is also able to rearrange to the Dδ3 gene segment in all cell samples tested, and to the Jδ1 and Jδ3 gene segments in postnatal thymocytes. This is in line with our recent observation that preferential rearrangements of Vδ gene segments to Dδ and Jδ gene segments during human ontogeny, parallel δRec rearrangements to these gene segments (5).

Based on the amount of PCR amplification products, we observed a difference in Vδ-Jδ rearrangements compared to Vδ-Jα rearrangements between the Vδ gene segments. The amount of Vδ2-Dδ and Vδ2-Jδ PCR products is slightly higher than the amount of Vδ2-Jα PCR products, whereas Vδ1-Dδ and Vδ1-Jδ rearrangements seem to occur less frequent than Vδ1-Jα rearrangements in both thymocytes and PBMNC. The δRec gene segment seems to resemble the pattern of the Vδ1 gene segment, whereas the (low) amount of PCR products of the Vδ3 gene segment to the Dδ, Jδ, and Jα gene segments were comparable.

Our data show that rearrangement of the Vδ1, δRec, Vδ2, and Vδ3 gene segments to several Jα gene segments is easily detectable with PCR analysis in fetal and postnatal thymocytes and PBMNC. Based on our previous Southern blot studies, the δRec gene segment is used most frequently for TCR-δ deletion (5, 17). Nevertheless Vδ-Jα rearrangements also seem to play a role in TCR-δ deletion, except for Vδ3-Jα rearrangements which will not delete the TCR-δ gene. The δRec and Vδ2 gene segments delete the TCR-δ gene preferably by rearrangement to the most upstream Jα gene segments, whereas the Vδ1 gene segment frequently rearranges to more downstream Jα gene segments. If the latter rearrangements are used for TCR-δ deletion then less Jα gene segments are available for subsequent Vα-Jα rearrangements, which results in a smaller potential TCR-α diversity. However, we cannot exclude the possibility that rearrangements of Vδ1 to more downstream Jα gene segments are already "replacement" rearrangements, that occurred after TCR-δ deletional rearrangements of δRec or Vδ2 to the upstream Jα gene segments.

We conclude that the different Vδ gene segments and the δRec gene segment play different roles in T cell development. The Vδ1 gene segment is preferentially used in TCR-δ gene rearrangements in postnatal thymocytes (18, 19). The Vδ1 gene segment can also be used to delete the TCR-δ gene by rearrangements to most Jα gene segments. Moreover, the Vδ1 gene segment is also easily used as a Vα-like gene segment in Vδ1-Jα-Cα transcripts (6, 20-22).

The Vδ2 gene segment is preferentially used in TCR-δ gene rearrangements in fetal thymocytes (18, 23), and the majority of TCR-$\gamma\delta^+$ T-lymphocytes express a $V\delta2^+$ TCR (24). The Vδ2 gene segment can also be used for TCR-δ deletion, by rearrangement to the most upstream Jα gene segments. The occurrence of Vδ2-Jα-Cα transcripts in thymocytes, but not in PBMNC, and the presence of Dδ gene segments in these transcripts (5, 8) suggest that this gene segment is the only true Vδ gene segment.

The Vδ3 gene segment is the third most used Vδ gene segment in PBMNC (9).

Rearrangement of the Vδ3 gene segment will not delete the TCR-δ gene. Nevertheless, Vα-Jα like Vδ3-Jα-Cα transcripts can be detected in PBMNC (9).

Finally, the δRec gene segment seems pre-eminently suitable for TCR-δ deletion, because all non-functional δRec rearrangements will force the T cell further into the TCR-αß differentiation lineage. The TCR-δ deleting δRec-Jα rearrangements preferably use ψJα and Jα58 or other 5′ located Jα gene segments; these rearrangements preserve an extensive TCR-α combinatorial diversity, because most Jα gene segments are kept available for subsequent Vα-Jα rearrangements.

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CHAPTER 4

HUMAN T CELL LEUKEMIAS WITH CONTINUOUS V(D)J RECOMBINASE ACTIVITY FOR T CELL RECEPTOR-δ GENE DELETION

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HUMAN T CELL LEUKEMIAS WITH CONTINUOUS V(D)J RECOMBINASE ACTIVITY FOR T CELL RECEPTOR-δ GENE DELETION[1]

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ABSTRACT

The so-called T cell receptor (TCR)-δ-deleting elements, δRec and ψJα, 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 subsequent TCR-α rearrangement. This intermediate rearrangement is thought to be caused by a specific V(D)J recombinase complex.

In our studies on TCR-δ deletion mechanisms, we identified several T cell acute lymphoblastic leukemias (T-ALL) with continuous activity of the δRec-ψJα rearrangement process. Extensive Southern blot, polymerase chain reaction, and sequencing analyses on the coding joints as well as the signal joints of the δRec-ψJα rearrangements in these patients, allowed us to prove that this continuous rearrangement activity occurred in the leukemic cells and that these cells therefore represent a polyclonal subpopulation of the otherwise monoclonal T-ALL. In additional studies, we also identified a T cell line (DND41) with continuous activity of the δRec-ψJα rearrangement process.

These T-ALL and the T cell line can serve as experimental model in further studies on the elements of the V(D)J recombinase complex which are specific for TCR-δ gene deletion.

INTRODUCTION

T cell acute lymphoblastic leukemias (T-ALL) are assumed to originate from differentiating cortical thymocytes (1-3). This is suggested by the presence of the enzyme terminal deoxynucleotidyl transferase (TdT) in T-ALL cells, because cortical

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thymocytes are the only T cells expressing TdT (4). During the recombination processes in early T cell differentiation, the enzyme TdT randomly inserts nucleotides in the junctional regions, which connect the variable (V), (diversity (D)) and joining (J) gene segments of the T cell receptor (TCR) (5, 6). In principle, a T-ALL is a clonal cell population in which all cells originate from a single malignantly transformed thymocyte. Therefore, all leukemic cells in a T-ALL have identical monoclonal TCR gene rearrangements (2, 4). This is also the case for T cell lines which are derived from T-ALL cells.

Comparable to cortical thymocytes, T-ALL cells are able to express TCR molecules in combination with the signal transducing CD3 complex (2-4). Because a complete TCR exists of α/β chains or γ/δ chains, T-ALL cells can feature at least three 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 majority of the TCR-δ gene is located in between the Vα and Jα gene segments, which excludes the possibility of simultaneous TCR-δ and TCR-α gene rearrangements on the same allele (8, 10). Secondly, there are two human TCR-δ gene deleting elements, δRec and ψJα, which delete the intermediate TCR-δ gene by rearrangement to each other (Figure 1). This deleting δRec-ψJα rearrangement is a dominant process in cortical thymocytes (7, 9, 11, 12). As for now, the hierarchical model of gene rearrangements in the human

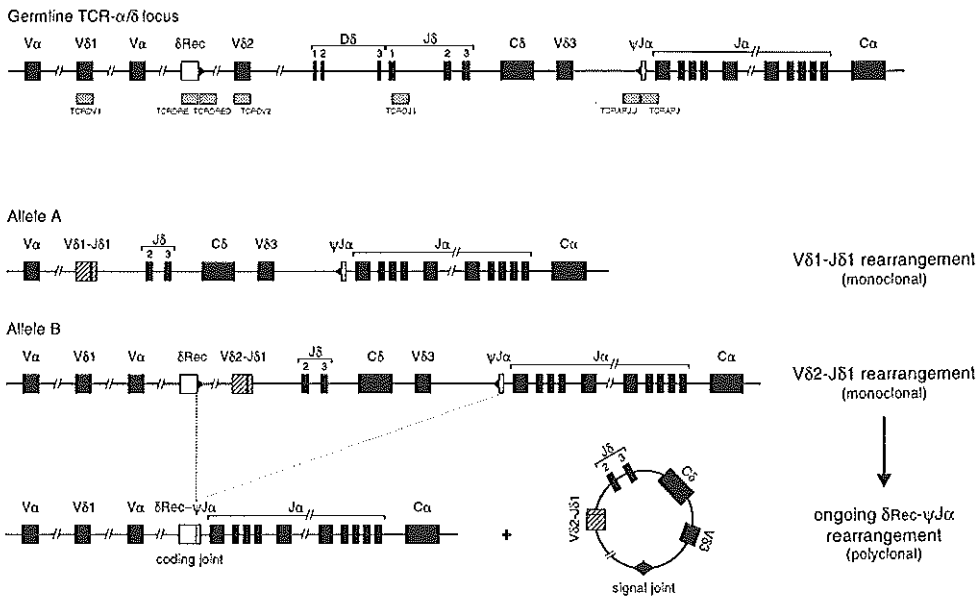

Figure 1. Schematic representation of ongoing δRec-ψJα rearrangements in leukemic cells of patient T019. Top: the germline TCR-α/δ locus. The open bars represent the various TCR-δ and ψJα probes. Bottom: allele A contains a monoclonal Vδ1-Jδ1 rearrangement and allele B contains a monoclonal Vδ2-Jδ1 rearrangement. 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.

TCR-α/δ locus starts with TCR-δ gene 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).

Indication for the existence of a specific TCR-δ gene deletion recombinase complex that mediates rearrangement of δRec to ψJα was found in the observation that T-ALL subgroups defined by their TCR-δ gene configuration showed skewed distribution of a specific chromosome aberration: the so-called *tal*-1 deletion (13-15). Because the heptamer-nonamer recombination signal sequences (RSS) and fusion regions of the *tal*-1 deletion breakpoints strongly resemble 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 T-ALL that originated from cells which were already committed to the $\alpha\beta$ lineage, ie. thymocytes that have two non-functional TCR-δ gene rearrangements or a TCR-δ gene deletion on one or both alleles (13, 16, 17). Also, we recently discovered a DNA binding protein (PJA-BP) that recognizes a 46 base pair (bp) binding site inside the ψJα gene segment and seems to be involved in the specific deletion of the TCR-δ gene (18). This hypothesis is supported by the expression pattern of PJA-BP which is primarily restricted to T cells with deletion of TCR-δ genes on both alleles (18).

In our studies on the TCR-δ gene deletion recombinase complex, we identified a CD3$^-$ T-ALL, with out-of-frame, complete (V-J) TCR-δ gene rearrangements on both alleles and with a minor subpopulation containing δRec-ψJα rearrangements. Extensive polymerase chain reaction (PCR), Southern blot, and sequencing analysis enabled us to prove that this subpopulation was polyclonal and was caused by ongoing δRec-ψJα rearrangements in the otherwise monoclonal T-ALL cells. The results of this T-ALL were compared with other T-ALL and T cell lines and showed that some of them also contain continuous V(D)J recombinase activity for TCR-δ gene deletion.

MATERIAL AND METHODS

Cell samples

Mononuclear cells (MNC) and/or granulocytes were isolated from peripheral blood (PB) or bone marrow (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 thymus samples were gently squeezed in an open filter chamber containing a nylon gauze filter with 100 μm openings (Nederlands Productielaboratorium voor Bloedtransfusieapparatuur en Infusievloeistoffen B.V., Emmer-Compascuum, The Netherlands). The released thymocytes were washed twice in phosphate buffered saline (pH 7.8) supplemented with 0.5% (w/v) bovine serum albumin. All human cell samples were obtained with the approval of the Committee of Medical Ethics of the Erasmus University Rotterdam and University Hospital Rotterdam, Rotterdam, The Netherlands. T cell lines CEM, DND41, GH1, HPB-ALL, HPB, HSB-2, HUT-78, JURKAT, MOLT-4, MOLT-16, PEER, and RPMI-8402; B-cell lines 697, BV173, ROS-5, ROS-17, ROS-27, RS4;11, and SMS-SB; myeloid cell lines K562 and Lama; and the non-hematopoietic cell line HeLa were used (19, 20).

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 monoclonal antibodies (McAbs), BMA031 (anti-TCR-$\alpha\beta$), 11F2 (anti-TCR-$\gamma\delta$), 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 immunofluorescencestainings were performed as described (21) 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 $\psi J\alpha$ gene segment (TCRAPJU), and in the coding region of the recombination activating genes (RAG1 and RAG2) were obtained by cloning the purified PCR amplification products of granulocyte DNA from a healthy volunteer using specific oligonucleotide primer sets (Table I). pUC19 was used as cloning vector (22). All oligonucleotideprimers were synthesized according to published sequence data (9, 23-25) on a 392 DNA synthesizer (Applied Biosystems, Forster City, CA) with the solid-phase phosphotriester method and used without further purification.

Table I. Oligonucleotide primers used in PCR and sequencing analysis of δRec-$\psi J\alpha$ rearrangements or in the construction of DNA probes.

Application		Cloning sites	Code	Position[a]	Sequence[b] 5′ 3′	Reference[c]
DNA probes (size)						
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	25
		*Hin*dIII	ψJαUp3′	−40 bp	TGGGaAgCTTTACAAAAACCAGAGGTGTCAGC	9
RAG1	(1071 bp)	*Bam*HI	RAG1p5′	+35 bp[d]	GCCTggaTCCCACCCACCTTGGGACTCAG	23
		*Hin*dIII	RAG1p3′	+1059 bp[d]	TTGCaagCTTTTGCTGGACATTTCACCATCAGG	23
RAG2	(1632 bp)	*Eco*RI	RAG2p5′	−25 bp[d]	AGTCATTTTATTTTAgAATTCTTTCAGACA	24
		*Hin*dIII	RAG2p3′	+1588 bp[d]	ACACCTGAATCTGAAAaGCTTTTGCA	24
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]	CACTCCTGTG CACGGTGATGC	9
coding joint probe		–	δRec-3′	−45 bp	GCAACATCACTCTGTGTCTAGC	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 oligonucleotideprimer is indicated upstream (−) or downstream (+) relative to the ATG codon.
e. The δRec-ψJα signal joint probe contains the head to head fused heptamers of the δRec and ψJα gene segments.

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) (26). 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) (Langerak et al., unpublished results).

Northern blot analysis

Total RNA was isolated using the method as described by Chomczynski and Sacchi (27). 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-labelled probes for the RAG genes: RAG1 and RAG2 probes, and for the GAPDH gene: GAPDH probe (28).

PCR amplification analysis

PCR was essentially performed as described previously (13). A 1 μg sample of DNA, 12.5 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 I and Verschuren et al. (Manuscript submitted). The PCR reaction mixture was incubated at 94°C for 3 min, at 60°C for 1 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 29-39 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. Vδ-Jδ and Vδ-Jα gene rearrangements in cell line DND41 were compared to Vδ-Jδ and Vδ-Jα gene rearrangements in postnatal thymocytes and PBMNC, by measuring the intensity of the PCR bands on agarose gels using computer software as described (29).

Sequencing analysis

The polyclonal δRec-ψJα PCR products were cloned into a pUC19 vector as described previously (12). For the direct sequencing analysis, one μl of the original PCR product, 12.5 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 25-30 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 (30). 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 are listed in Table I. All sequence reactions were performed with the T7-sequencing kit (Pharmacia) following the manufacture's instructions using ^{35}S radiolabeling, and run in normal denaturing 8% polyacrylamide sequence gels.

RESULTS

δRec-ψJα coding joint analysis

To identify monoclonal 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 2A). Hybridization of the PCR products with the δRec-3′ oligonucleotide probe confirmed their identity

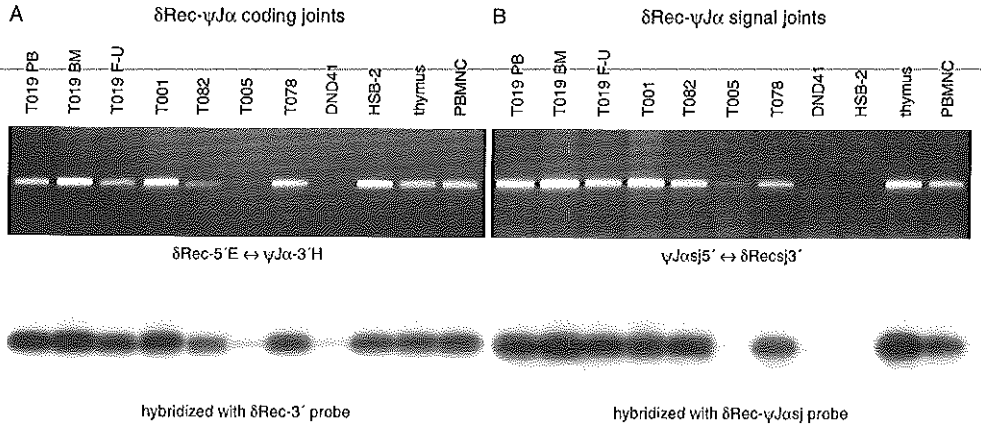

Figure 2. PCR analysis of coding joints (**A**) and signal joints (**B**) of δRec-ψJα rearrangements. Indicated are the PCR product analyzed by electrophoresis in ethidium-bromide-stained agarose gels, subsequent blotting and identification by oligonucleotide probe hybridization.

(Figure 2A). All thymic cell samples, as well as the PBMNC and the majority of 139 tested T-ALL were positive (Table II). Of all 21 cell lines tested, only T cell line HSB-2 was strongly positive, because one allele contains a δRec-ψJα rearrangement (Table II) (7). However, a second T cell line (DND41) appeared to be weakly positive (Table II). 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 II) (31, 32).

To confirm the results of the PCR analysis, DNA of the cell samples was analyzed by Southern blotting. The advantage of Southern blotting over the PCR technique is that it does not amplify the DNA and will therefore allow a realistic estimation of the relative frequency of the δRec-ψJα rearrangements. However, the disadvantage is the low sensitivity, which is reflected by a detection limit of 1-5%. 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 (polyclonal rearrangement), and in the T-ALL and T cell line with a δRec-ψJα rearrangement on one allele (monoclonal rearrangement), e.g. patient T078 and cell line HSB-2 (Figure 3 and Table II). Weak bands were also visible in PBMNC of all healthy volunteers, as well as in some T-ALL. Except for cell line HSB-2, all tested cell lines, including DND41 were negative. 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 PBMNC or whether these rearrangements originated from the T-ALL cells.

δRec-ψJα signal joint analysis

To determine the origin of the δRec-ψJα rearrangements, we investigated the circular excision products that are formed as signal joint by-products of this particular rearrangement (Figure 1). PCR amplification using the δRecsj3´ and ψJαsj5´ primers gave δRec-ψJα signal joint PCR products, which identity was confirmed by hybridization with the δRec-ψJαsj oligonucleotide probe (Figure 2B). All thymic cell samples

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

	TCR/CD3 phenotype	CD4/CD8	δRec germline[b] polymorphism	Rearrangement TCR-α/δ	frame[c]	TCR-γ	frame[c]	TCR-β	RNA expression RAG-1	RAG-2	δRec-ψJα rearrangement PCR coding joint	PCR signal joint	Southern blot rearrangement	Southern blot excision products	Direct sequencing junctional regions	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	NA	Jβ2	NA	NA	+	+	±	±	polyclonal	T
			T	Vδ2-Jδ1	−	G	○	G								
T082	$CD3^-$	NA	NA	Vδ1-Jδ1	−	Vγ3-Jγ2.3	NA	Jβ1	+	+	+	+	+	+	polyclonal	T
			T	Vδ2-Jδ1	−	Vγ8-Jγ2.3	NA	Jβ2								
T005	TCR-$\gamma\delta^+$	$4^+/8^-$	NA	Vδ1-Jδ1	+	Vγ2-Jγ1.1	+	Jβ1	±	±	±	±	−	−	polyclonal	T
			T	Vδ2-Jδ1	−	Vγ2-Jγ1.3	+	G								
T078	$CD3^-$	$4^+/8^+$	NA	Vδ3-Jδ2	−	Vγ3−Jγ1.3	NA	Jβ1	±	±	+	±	+[e]	±	monoclonal	C
			C	δRec-ψJα	○	Vγ2/4−Jγ2.3	NA	G								
T cell line																
DND41	TCR-$\beta\delta^+$	$4^+/8^-$	NA	Vδ1-Jδ1	+	Vγ5-Jγ2.3	−	Jβ1	±	±	±	±	−	−	polyclonal	T
			T	V?-Jδ1	NA	Vγ8-Jγ2.3	−	Jβ2								
HSB-2	$CD3^-$	$4^-/8^\pm$	T	δRec-ψJα	○	Vγ9-Jγ2.3	NA	Jβ1	−	−	+	−	+[e]	−	monoclonal	T
			NA	δRec-Jα59	○	Vγ10-Jγ2.3	−	Jβ1								
controls[f]																
thymus	$CD3^-$/TCR-$\alpha\beta^+$/$\gamma\delta^+$	○	○	Vδ-Jδ	±	Vγ-Jγ1	±	Jβ1	+	+	+	+	+	+	polyclonal	○
			○	Vα-Jα	±	Vγ-Jγ2	±	Jβ2								
PBMNC	TCR-$\alpha\beta^+/\gamma\delta^+$	○	○	Vδ-Jα	±	Vγ-Jγ1	±	Jβ1	−	−	+	+	±	±	polyclonal	○
			○	Vα-Jα	±	Vγ-Jγ2	±	Jβ2								
HeLa	$CD3^-$	$4^-/8^-$	○	G	○	G	○	G	−	−	−	−	−	−	○	○
			○	G	○	G	○	G								

a. Abbreviations used; NA, not available; ○, not applicable.
b. This polymorphism is present in the δRec germline sequence at position − 87 relative to the RSS.
c. Frame; −, rearrangement is out-of-frame; +, rearrangement is in-frame.
d. For the δRec polymorphism cf Figures 6 and 7.
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.

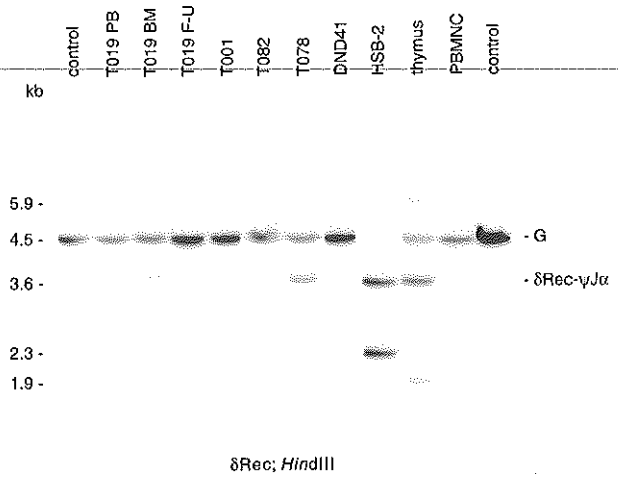

Figure 3. Southern blot analysis of δRec-ψJα rearrangements. A Southern blot filter with *Hind*III digests of DNA from various cell samples hybridized with the ^{32}P labelled TCRDRE (δRec) probe. The positions of the germline (G) band and preferential δRec-ψJα rearrangement are indicated (26).

were strongly positive, whereas the results in the tested T-ALL varied from strongly positive to negative (Figure 2B and Table II). Surprisingly, the control PBMNC were also positive, meaning that δRec-ψJα excision products do occur in normal PBMNC. So, we could not distinguish "background" signal joints from signal joints of T-ALL cells, therefore it remained impossible to determine the origin of the δRec-ψJα rearrangements in the T-ALL samples. The tested cell lines were all negative, except for cell line DND41, in which, after long exposure times (data not shown), a weakly positive signal proved - by lack of background - that this T cell line actively rearranges δRec to ψJα.

If the δRec-ψJα excision products found by PCR in normal blood 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 also showed a weak band (Figure 4 and Table II). In addition, a very faint band was visible in control PBMNC, whereas no excision product could be identified in all cell lines tested (data not shown). 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 combined positive and negative results we selected five T-ALL and two T cell lines which we analyzed further for their ability to actively perform δRec-ψJα rearrangements (Table II). The most important phenotypic and genotypic characteristics of these T-ALL and T cell lines are summarized in Table II.

δRec-ψJα junctional regions

To further prove that the continuous δRec-ψJα rearrangements in the T-ALL cell samples originate from leukemic cells and thus define a polyclonal subpopulation

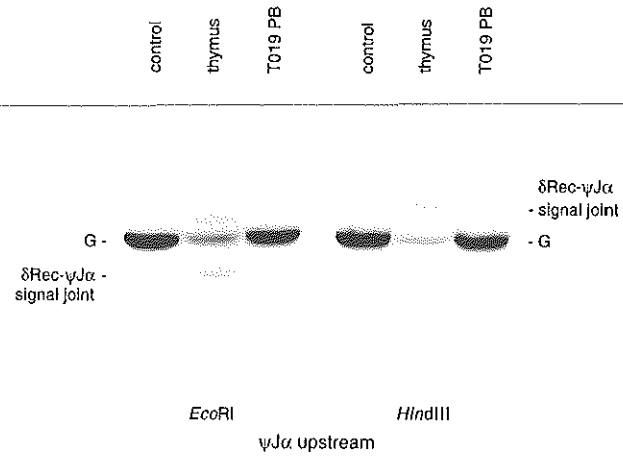

Figure 4. Southern blot analysis of δRec-ψJα circular excision products. A Southern blot filter with *Eco*RI or *Hin*dIII digests of DNA from cell line HeLa, thymocytes, and patient T019 hybridized with the TCRAPJU (ψJα upstream) probe. The positions of the germline (G) band and δRec-ψJα signal joint are indicated.

within the monoclonal malignancy, we investigated the junctional regions of these rearrangements.

Direct sequencing analysis of PCR products from the δRec-ψJα coding joint can ascertain the clonal 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 T078 and T cell line HSB-2, both of which contain a monoclonal δRec-ψJα rearrangement on one allele (Table II).

Cloning and sequencing of the PCR products revealed the actual junctional regions of 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 (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 PBMNC in BM at the time this patient was in complete remission (Figure 6).

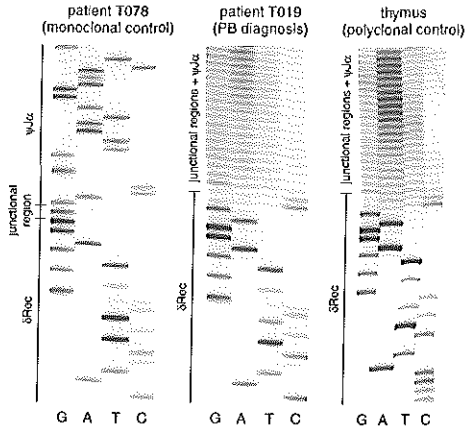

Figure 5. Direct sequencing analysis of δRec-ψJα rearrangement PCR products. The δRec-ψJα rearrangements of patient T019 are compared with a monoclonal control (patient T078) and a polyclonal control (thymus).

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	ACGGAc	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 HSB-2 (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 DND41

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 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 87 bp upstream of the RSS heptamer.

Allele-specific δRec rearrangements

If the δRec-ψJα rearrangements originate from T-ALL cells, they can only use the δRec gene segment that is still present in the leukemic cells. Four out 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 δRec gene segment available, is present on the allele with the Vδ2-Jδ1 rearrangement. In some individuals it is possible to discriminate between δRec gene segments of the different alleles on basis of a polymorphism present 87 bp upstream relative to the RSS heptamer of this gene segment, which contains either a T or a C nucleotide (Figure 7 and Table II). In patient T019 we were able to prove that only one δRec allele was used in the polyclonal δRec-ψJα rearrangements, because this patient contained on one allele a C and on the other a T nucleotide at this position (Figure 7). All δRec-ψJα

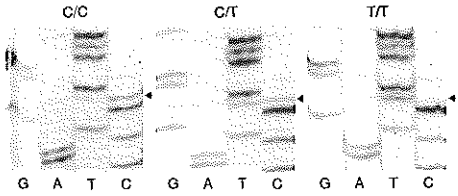

Figure 7. The polymorphism in the δRec gene segment at position -87 bp from the RSS. This nucleotide is either a T or a C and there are therefore three possible configurations: C/C, C/T, and T/T.

rearrangements in the T-ALL cells contained the δRec gene segment with the T nucleotide, whereas in the normal BM cells of this patient, precisely half of these rearrangements contained a T and half a C nucleotide (Figure 6 and Table II). Sequencing of the δRec germline DNA sequence in the leukemic cells revealed a T nucleotide, whereas in the δRec germline DNA sequence of normal PBMNC cells both nucleotides 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 δRec present on the same allele as the Vδ2-Jδ1 rearrangement. Thus, the T-ALL cells were actively deleting the Vδ2-Jδ1 rearrangement by δRec-ψJα recombination (Figure 1).

Comparable results were obtained in the T-ALL cell sample derived from BM of patient T001 (Figure 6 and Table II). However, in leukemic cells derived from PB of this patient a mixture of T and C nucleotides was observed in the δRec-ψJα rearrangements. This phenomenon is in line with a high tumor load of the BM sample (88%) versus a low tumor load of the PB sample (45%). Taken together, it can be concluded that in the BM sample of patient T001, the δRec-ψJα rearrangements come from T-ALL cells, whereas in the PB sample at least a considerable portion originates from "background" PBMNC.

In patient T082 and also in patient T005 with TCR-γδ$^+$ T-ALL no normal cells were available to determine the presence of the T/C polymorphism. So, the exclusive presence of T nucleotides in the δRec-ψJα rearrangements (Figure 6) formally does not exclude "background" rearrangements derived from normal PBMNC, because these patients might contain a T nucleotide on both alleles.

TCR-α/δ gene rearrangements in T cell line DND41

To investigate whether other rearrangements can also occur in leukemic T cells with ongoing δRec-ψJα rearrangements, we performed PCR analysis using Vδ and δRec primers versus Jδ and Jα primers. Because of the "background" problem caused by rearrangements in normal PBMNC that are present in the T-ALL samples, these PCR analyses were performed exclusively on cell line DND41. The results are depicted in Figure 8. We compared the relative PCR product intensities of the analyzed rearrangements to each other and to those present in PBMNC and postnatal thymocytes from healthy donors. The most obvious observation was the absence of rearrangements involving either Vδ2, Jδ2, or Jδ3 gene segments in cell line DND41 (Figure 8), although Southern blot analysis proved the presence of these gene segments on at least one allele (data not shown). In normal PBMNC and thymocytes these rearrangements do occur (Figure 8). Rearrangements of the Vδ1, Vδ3, and δRec to Jα gene segments did occur, with a skewed distribution to the most 5' located Jα gene segments. Noticeable, the ψJα gene segment is the most frequently rearranged Jα gene segment, not only in combination with δRec, but also with Vδ1 and Vδ3. The Jα gene segment usage in DND41 seemed to be comparable to that observed in PBMNC (Figure 8). Cell line DND41 appears to have an active

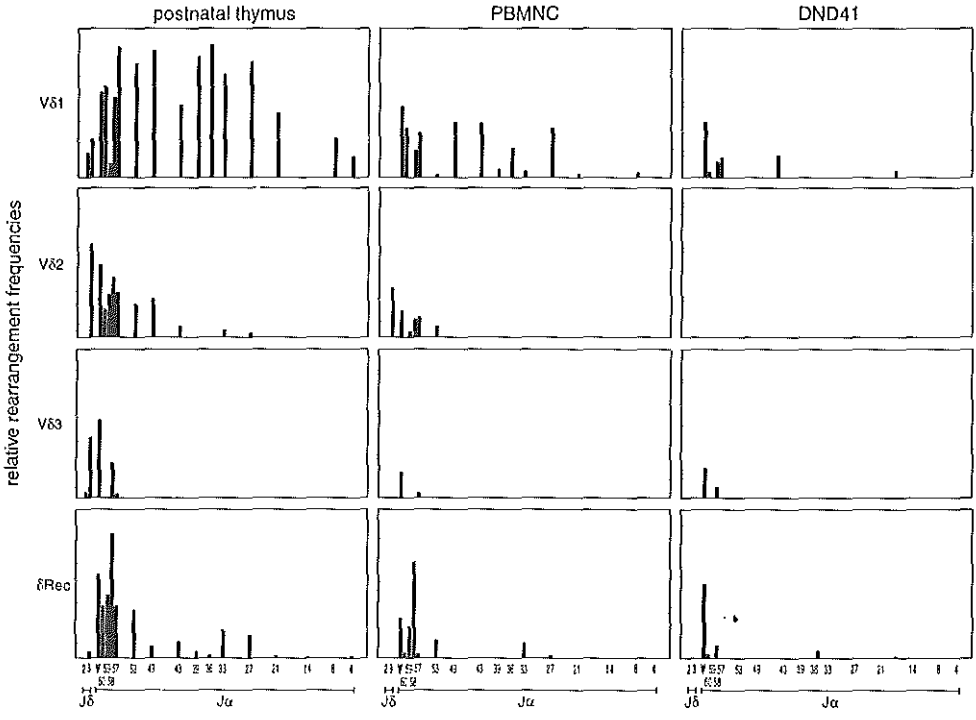

Figure 8. PCR analysis of ongoing rearrangements in T cell line DND41. Comparison of relative rearrangement frequencies of Vδ-Jδ/Jα or δRec-Jδ/Jα in postnatal thymocytes (left column) and normal PBMNC (middle column) compared to cell line DND41 (right column). The upstream gene segments analyzed are indicated at the left, and the downstream J gene segments are indicated below each diagram. The bars represent the relative intensity of the signals of the PCR products. They therefore only represent a rough estimation of the rearrangement distribution between the cell samples.

recombinase complex that does not mediate 'conventional' TCR-δ gene rearrangements, but primarily the intermediate TCR-δ-deleting rearrangements and the mixed Vδ-Jα gene rearrangements.

Expression of RAG genes

The products of the RAG genes are essential for V(D)J recombination and are therefore also assumed to be involved in the recombinase complex that mediates TCR-δ gene deletion. To prove RAG expression in the T-ALL samples and the T cell line with ongoing δRec-ψJα rearrangements, we designed RAG-1 and RAG-2 probes for Northern blot analysis. Although not quantified, it appeared that all T-ALL tested as well as T cell line DND41 transcribe both RAG genes as is the case in thymic cell samples (Figure 9 and Table II). Control PBMNC, cell line HeLa and cell line HSB-2 did not show RAG gene transcription (Figure 9 and Table II).

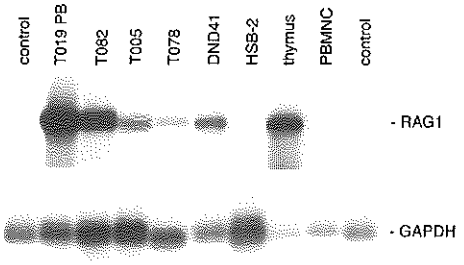

Figure 9. Northern blot analysis of the RAG expression in several cell samples. A Northern blot filter was successively hybridized with the RAG1 and GAPDH probes.

DISCUSSION

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 in which this particular rearrangement occurs in a total thymic cell sample indicates that the majority of thymocytes use the δRec-ψJα rearrangement to delete their TCR-δ genes during differentiation (7, 9, 11, 33, 34).

There is evidence that the δRec-ψJα rearrangement is caused by a V(D)J recombinase complex, which contains 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 (7, 11, 33, 34). Inversely, although the ψJα gene segments can rearrange to various gene segments, 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 (Verschuren and Blom,

unpublished results), meaning that different factors play a role in (the regulation of) this unique rearrangement. One of these factors could be the protein PJA-BP that binds to the $\psi J\alpha$ gene segment presumably during later stages of TCR gene rearrangement. Furthermore, our studies on the *tal*-1 deletions 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 the active recombinase complex is present. To avoid complicated cell-sorting experiments with differentiating thymocytes to obtain sufficient cells for our experimental purposes, we decided to search for a monoclonal cell population with an active TCR-δ gene deleting recombinase.

By screening a large number of T-ALL and cell lines using PCR, Southern blotting, and direct sequencing analysis we were able to identify several T-ALL and one T cell line (DND41) with small subpopulations containing δRec-$\psi J\alpha$ rearrangements. To investigate whether these rearrangements are derived from the leukemic cells or from "background" PBMNC, not only the coding joints, but also the excision products of the δRec-$\psi J\alpha$ rearrangements were analyzed. An unexpected problem we encountered was the presence of circular excision products of the δRec-$\psi J\alpha$ rearrangements in normal PBMNC. To discriminate "background" rearrangements of normal cells and T-ALL-derived rearrangements, we applied several Southern blot and PCR-sequencing methods. Frequently occurring circular excision products in T-ALL cells and polyclonal δRec-$\psi J\alpha$ junctional region sequences provided evidence that several T-ALL and T cell line DND41 contain small polyclonal subclones. A polymorphism in the δRec gene segment proved convincingly that these cells were actively rearranging δRec to $\psi J\alpha$ on only one allele, thereby generating a polyclonal subpopulation. This is a remarkable observation, because leukemic cells in ALL patients generally are monoclonal or oligoclonal in some cases (35, 36), and cell lines are considered to be fully monoclonal.

It is especially surprising to find a polyclonal subpopulation in a cell line, which is most probably not the result of DNA contamination, because repeatedly isolation of DNA from DND41 cells gave similar results. The presence of a polyclonal subpopulation in this cell line means that continuous rearrangements can not induce and support clonal outgrowth. What might occur is that the TCR-δ gene deleting rearrangement is immediately followed by a TCR-α rearrangement, which in turn deletes the δRec-$\psi J\alpha$ rearrangement and may lead to TCR-$\alpha\beta$ expression. These TCR-$\alpha\beta^+$ cells may have a lower growth capacity and therefore never result in 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 the presence of a minor TCR-$\alpha\beta^+/TdT^+$ subpopulation (14%), which is normally not found in BM or PB (21). This confirmed the continuous rearrangement capacity of these T-ALL cells. This is in line with the observation that in cell line DND41 other rearrangements occur involving the Jα gene segments, whereas TCR-δ rearrangements do not take place. Altogether, it seems that cell line DND41 is in the intermediate phase between TCR-δ rearrangement and TCR-α rearrangement. Because the rearrangements occur in T-ALL cells that are outside the thymus, or in an in vitro cultured T cell line, this suggests that for this particular

rearrangement to take place, the thymic environment and all other thymic extracellular signals are not absolutely needed.

It was remarkable that all three T-ALL that showed the most intense ongoing δRec-ψJα rearrangement, were TCR$^-$CD3$^-$ and contained a Vδ1-Jδ1 and a Vδ2-Jδ1 rearrangement, both being out-of-frame. However, the characteristics of T cell line DND41 were different compared to these T-ALL, because this cell line expressed an unusual TCR-βδ heterodimer (31, 32). DND41 cells contain a functional Vδ1-Jδ1 on one allele and a TCR-δ gene rearrangement of an unknown gene segment (or RSS) 5.2 kb downstream of the Vδ2 gene segment to the Jδ1 gene segment on the other allele (Verschuren, unpublished results). No TCR-γδ heterodimer can be formed in this cell line because of the absence of in-frame TCR-γ rearrangements (Table II). Thus far, the DND41 cell line is the only T cell reported that expresses a TCR-βδ, and it does not seem likely that this cell line represents an intermediate stage in normal thymocyte differentiation. Our data suggests that ongoing δRec-ψJα gene rearrangements predominantly occur in T cells that cannot express a functional TCR-γδ, due to biallelic out-of-frame TCR-δ and/or TCR-γ rearrangements.

We can conclude that some T-ALL and T cell lines contain an active TCR-δ gene deleting recombinase complex. These monoclonal cell populations are invaluable for experiments on identification of regulating elements involved in the deletion of the TCR-δ gene.

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CHAPTER 5

A DNA BINDING PROTEIN IN HUMAN THYMOCYTES RECOGNIZES THE T CELL RECEPTOR-δ-DELETING ELEMENT $\psi J\alpha$

CHAPTER 5

A DNA BINDING PROTEIN IN HUMAN THYMOCYTES RECOGNIZES THE T CELL RECEPTOR-δ-DELETING ELEMENT ψJα[1]

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ABSTRACT

The T cell receptor (TCR) is a heterodimeric molecule composed of either γδ or αß chains. The differentiation mechanisms that force thymocytes into the γδ or αß lineage are poorly understood, but rearrangement processes in the TCR-δ/α locus are likely to play an important role. The TCR-δ gene complex is flanked by the TCR-δ-deleting elements δRec and ψJα, which are assumed to delete the TCR-δ gene before Vα-Jα rearrangement.The nonproductive δRec-ψJα recombination occurs at high frequency in both fetal and postnatal immature thymocytes.

To find DNA-binding proteins involved in the δRec-ψJα preferential rearrangement, we performed electrophoretic mobility shift assays using the recombination signal sequence of ψJα with additional upstream and downstream sequences. We observed a 180 kilodaltons DNA binding protein in nuclear extracts from human thymocytes that recognized a 46 base pairs binding site on the ψJα gene segment, containing the core motif GTTAATAGG. This ψJα binding protein, which we call PJA-BP, was also detected in immature $CD3^-$ T cell lines with TCR-δ genes deleted on both alleles, in a TCR-$\alpha\beta^+$ cell line, and in two of four myeloid cell lines. This protein was absent in a TCR-$\gamma\delta^+$ T cell line, in non-hematopoietic cell lines, and in all but one B cell lines tested. Although we could detect binding activity of the PJA-BP to some other Jα gene segments, we postulate that binding of PJA-BP to the ψJα gene segment is one of the factors involved in the preferential δRec-ψJα gene rearrangement process.

INTRODUCTION

The T cell receptor (TCR) plays an important role in the differentiation of prothymocytes to mature thymocytes. Two forms of the TCR exist, consisting of a

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heterodimer of α and ß or γ and δ glycoproteins (1, 2). Each chain is encoded by a gene complex of variable (V), diversity (D), joining (J), and constant (C) gene segments. The TCR-δ gene is located within the TCR-α gene complex between the Vα and Jα gene segments (Figure 1) (3). Therefore Vα to Jα rearrangement will delete the TCR-δ gene, which makes the TCR-α/δ locus very important in the bifurcation of the TCR-αß and TCR-$\gamma\delta$ lineages (4).

Two models have been proposed to explain the differentiation of TCR-αß and TCR-$\gamma\delta$ T cells from a common progenitor (4, 5). In the first model, the TCR-δ gene is deleted after failing to successfully rearrange TCR-δ and/or TCR-γ genes (6, 7), whereas in the second model, the TCR-δ gene is deleted before rearrangement, because the T cells are committed to one of the two lineages (8).

In 1988 De Villartay et al. (9) identified the so-called TCR-δ-deleting elements δRec and ψJα, which flank the TCR-δ gene. The nonfunctional rearrangement of δRec to ψJα is assumed to delete the TCR-δ gene before Vα-Jα rearrangement (Figure 1). Although TCR-δ deletion before Vα-Jα rearrangement is not obligatory (10), the δRec-ψJα rearrangement is predominantly present in human and mouse thymocytes throughout ontogeny (9, 11, 12). In addition, several other rearrangements involving δRec or ψJα have been observed, such as δRec-Jδ1, δRec-JαI, and δRec-JαII (13-15) as well as Dδ3-ψJα, Vα3.1-ψJα and Vδ2-ψJα (9, 16, 17). Previously, we have shown that the δRec-ψJα rearrangement is highly homologous to Vα-Jα rearrangements (12). However, little is known about the mechanisms regulating this preferential rearrangement.

V(D)J-gene rearrangement processes are mediated by recombination enzymes that recognize specific recombination signal sequences (RSS). These RSS consist of a conserved palindromic heptamer and nonamer, separated by spacer regions of 12 or 23 base pairs (bp) that are less conserved (18). Several proteins (recombination enzymes) have been identified which might play a role in V(D)J recombination (for review see Ref. 19).

The recombination activation genes RAG-1 and RAG-2 (18) are essential for V(D)J recombination. They were shown to be involved in the generation of double strand breaks and the formation of hairpins at RSS (20, 21). Another involved protein complex is the DNA-dependent protein kinase, which consists of three polypeptide components: Ku-70, Ku-80 and a 350 kilodaltons (kDa) catalytic subunit (p350). The

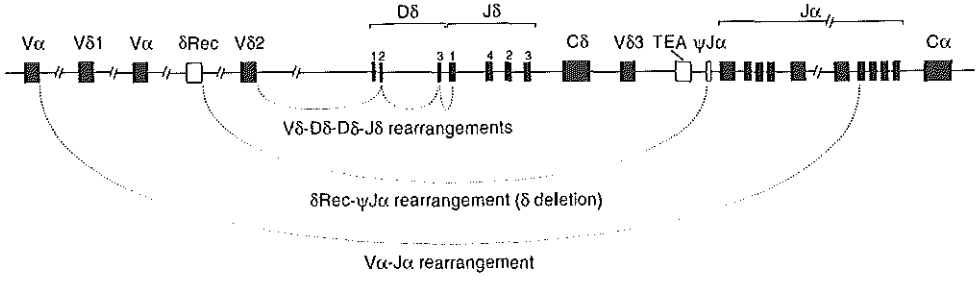

Figure 1. Schematic representation of the human TCR-α/δ locus. The position of the TCR-δ gene within the TCR-α gene complex is indicated as well as the TCR-δ-deleting elements (δRec and ψJα) and the TEA genetic element. The dotted lines indicate the possible consecutive gene rearrangements: Vδ-Dδ-Dδ-Jδ, δRec-ψJα, and Vα-Jα.

DNA binding proteins Ku-70 and Ku-80 have been proven to be essential components in both double strand break repair and V(D)J recombination (22-24), whereas the p350 protein is encoded by the *scid* gene (25, 26). Many attempts have been made to identify DNA binding proteins capable of binding to the conserved RSS heptamer and nonamer sequences (27-31). All (DNA binding) proteins mentioned are thought to play a general role in V(D)J recombination because of their presence in both B and T cells, but they cannot account for the lineage-restricted V(D)J rearrangements in B and T cells or for the occurrence of preferential rearrangements.

Here we describe a DNA binding protein in human thymocytes, that recognizes the ψJα gene segment. This protein might play a role in the preferential δRec-ψJα rearrangement, and therefore it might be involved in the TCR-γδ/TCR-αß lineage commitment.

MATERIALS AND METHODS

Cell samples

Thymocytes were obtained from 14 postnatal thymus samples of children undergoing cardiac surgery at the age of 22 days to 2 years. Two additional thymus samples were obtained from older patients at the ages of 4 and 20 years respectively. The thymus samples were minced with scissors in RPMI 1640 medium and were flushed through a nylon gauze filter with 100 μm openings. All human thymus samples were obtained with the approval of the Medical Ethics Committee of the Erasmus University Rotterdam/University Hospital Rotterdam (Rotterdam, The Netherlands).

Nineteen human continuous cell lines were used to study the PJA-BP expression pattern: seven T cell lines (HSB, P12, CEM, MOLT-4, DND41, PEER, and HPB-ALL) (32); six B cell lines (RVT, P30/Ohkubo, NALM-1, Daudi, ROS-15, and U266) (33, 34); four myeloid cell lines (KG-1a, K562, HEL, and U937) (35); and 2 non-hematopoietic cell lines (HeLa (adenocarcinoma) and HEP-2 (laryngeal carcinoma)).

Nuclear extracts

Crude nuclear extracts were prepared from thymocytes (25 x 10^6), HeLa cells (2.5 x 10^6) or other cell lines (10 x 10^6), according to the methods of Schreiber et al. (36) and Olnes and Kurl (37) with minor modifications. Cells were washed twice with 25 mM Tris, 0.14 mM NaCl, and 2.7 mM KCl; pH 7.4, and cell pellets were resuspended in 400 μl of ice-cold buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF (Sigma Chemical Co., St. Louis, MO)). The cells were allowed to swell on ice for 15 min, after which 0.6% (vol/vol) Nonidet P-40 (Sigma Chemical Co.) was added followed by vigorously vortexing for 10 s. Nuclei were pelleted for 30 s at 10.000 x g in a microcentrifuge and pellets were washed twice with 100 μl of ice-cold buffer A to remove residual Nonidet P-40. The pellets were resuspended in 30 to 50 μl of ice-cold buffer B (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF) and vigorously rocked at 4°C for 15 min. The lysates were microcentrifuged for 5 min at 10.000 x g and the supernatants were aliquoted and stored at -80°C for a maximum of 1 month. The protein concentrations were usually 1 to 3 mg/ml as determined by the method of Bradford (38).

DNA probes

Eight ψJα DNA probes, seven Jα DNA probes, and one δRec DNA probe were obtained by cloning purified polymerase chain reaction (PCR) amplification products of granulocyte DNA from a healthy volunteer, using the cloning vector pUC19 as previously described (39). All oligonucleotide primers (listed in Table I) were synthesized according to published sequences of the ψJα, Jα, and δRec regions (11, 40) (EMBL databank accession no. M94081).

Oligonucleotides δRECcod (ATGCATAGGCACCTCGACCCCGT, located 8 bp upstream of the δRec heptamer RSS) and δRECsj5′ (Table I) were used to obtain PCR amplification products for electrophoretic mobility shift assay (EMSA) competition experiments.

For EMSA experiments, cesium chloride purified plasmid containing one of the DNA probes was

TABLE I. Oligonucleotide primers used in construction of ψJα, Jα, and δRec DNA probes for EMSA

Probe Name	Size[a]	Cloning Sites	Primer Code	Position[b]	Sequence[c]
ψJα probes					
TCRAPJ-RSS	155	*Sma*I	ψJαsj3′	− 67	TGTGaaGcttAGAGGGGTGCCTCTGTCAACAAA
	171[d]	*Sma*I	ψJα5′	+ 43	CTTGCTGAGTTTCATGATTCCTC
TCRAPJ-C1	61	*Sma*I	ψJαU1	+ 23	TGTGGGatCCGGGTTAATAGGAAACTG
	79[d]	*Sma*I	ψJα5′	+ 43	
TCRAPJ-C2	48	*Bam*HI	ψJαU1	+ 23	
		*Eco*RI	ψJαD2	+ 35	GCTGgaaTTCATGATTCCTCTAGTGTTG
TCRAPJ-C3	41	*Bam*HI	ψJαU1	+ 23	
		*Eco*RI	ψJαD1	+ 25	TTCAgaATTCCTCTAGTGTTGGCTCCAAATG
TCRAPJ-C4	43	*Hin*dIII	ψJαU2	+ 31	GTACaaGcTTAATAGGAAACTGACATTTGG
		*Eco*RI	ψJαD2	+ 35	
TCRAPJ-C5	40	*Hin*dIII	ψJαU3	+ 33	CCGGaagctTAGGAAACTGACATTTGGAG
		*Eco*RI	ψJαD2	+ 35	
TCRAPJ-C6	36	*Hin*dIII	ψJαU4	+ 35	GGTTAAgcttAAACTGACATTTGGAGCC
		*Eco*RI	ψJαD2	+ 35	
TCRAPJ-C7	33	*Bam*HI	ψJαU5	+ 38	AATAGGAtcCTGACATTTGGAGCCAAC
		*Eco*RI	ψJαD2	+ 35	
Jα probes					
TCRAJ60	58	*Bam*HI	Jα60/5′	+ 22	tgtgggatccTGAAGATCACCTAGATGCTCAACT
		*Eco*RI	Jα60/3′	+ 35	tgtggaattCAGGCTCACAATTAACTCAGTC
TCRAJ57	65	*Bam*HI	Jα57/5′	+ 23	tgtgggatccTAACTCAGGGCGGATCTGAAAAG
		*Eco*RI	Jα57/3′	+ 40	tgtggaattcATGGGTTTACTGTCAGTTTCGTTC
TCRAJ49	58	*Bam*HI	Jα49/5′	+ 25	tgtgggatccGAACACCGGTAACCAGTTCTATTTT
		*Eco*RI	Jα49/3′	+ 33	tgtggaattcTTGGAATGACCGTCAAACTTGTCC
TCRAJ39	65	*Bam*HI	Jα39/5′	+ 25	tgtgggatccTGAATAATAATGCAGGCAACATGCT
		*Eco*RI	Jα39/3′	+ 40	tgtggaattcGGGGTTTGACCATTAACCTTGTTC
TCRAJ36	61	*Bam*HI	Jα36/5′	+ 23	tgtgggatccTCAAACTGGGGCAAACAACCTCT
		*Eco*RI	Jα36/3′	+ 37	tgtggaattcAGGGAATAACGGTGAGTCTCGTT
TCRAJ33	58	*Bam*HI	Jα33/5′	+ 25	tgtgggatccTGGATAGCAACTATCAGTTAATCTG
		*Eco*RI	Jα33/3′	+ 34	tgtggaattCTGGCTTTATAATTAGCTTGGTCC
TCRAJ8	62	*Bam*HI	Jα8/5′	+ 24	tgtgggatccTGAACACAGGCTTTCAGAAACTTG
		*Eco*RI	Jα8/3′	+ 39	tgtggaattcTTGGACTGACCAGAAGTCGGGT
δRec probe					
TCRDRE-RSS	160	*Sma*I	δREC3′	− 45	GCAACATCACTCTTGTGTCTAGC
	178[d]	*Sma*I	δRECsj5′	+ 71	GCTGAAGCTTTTGCAACTCGTGAGAACGGTGAAT

a. Size of double strand DNA fragments after restriction endonuclease digestion.
b. The position of the 3′ side of the oligonucleotide primer is indicated upsteam (-) or downstream (+) relative to the cleave site of the RSS.
c. The sequences in lower case characters represent the aspecific nucleotides, which generate restriction sites.
d. Size of double strand DNA fragments of *Sma*I cloned PCR products after digestion with *Bam*HI or *Eco*RI (Pharmacia), including aspecific pUC19 vector sequences.

digested using *Bam*HI or *Hin*dIII restriction endonucleases (Pharmacia, Uppsala, Sweden) and incubated for 1 hr at 37°C with calf intestinal alkaline phosphatase (CIAP; 1.7 U; Pharmacia). After extraction and precipitation, the DNA was resuspended in H_2O. Subsequently, 1.5 μg was labeled for 30 min at 37°C with 0.74 MBq [γ-^{32}P]dATP (ICN Biomedicals, Costa Mesa, CA, USA) and 10 U T4 polynucleotide kinase (Pharmacia) in 10 μl of reaction mixture containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 0.1 mM spermidine, 0.1 mM EDTA and 5mM DTT. After heat inactivation, the second appropriate restriction endonuclease was added: *Eco*RI, *Hpa*II (Pharmacia); *Mae*I, *Dde*I (Boehringer Mannheim, Mannheim, Germany), *Nla*III (Minotech, Heraklio, Greece) or *Nla*IV (New England Biolabs, Beverly, MA). After incubation for 1 hr at 37°C, the samples were purified by 8% polyacrylamide gel electrophoresis (PAGE; Biorad Laboratories, Hercules, CA) for 1.5 hr at 150 V at room temperature in 0.5x TBE (1x TBE= 890 mM Tris, 890 mM boric acid, and 20 mM EDTA; pH 8.0). The labeled probes were isolated from gel

slices by overnight elution in elution buffer (0.5 M ammonium acetate, 0.1% SDS and 1 mM EDTA) and precipitation. Pellets were resuspended in water at a concentration of 7500 cpm/μl and stored at 4°C for a maximum of 2 weeks.

EMSA

Two micrograms of crude nuclear extract was incubated with 7500 cpm labeled DNA for 30 min at room temperature in 20 μl of reaction mixture containing 10 mM HEPES (pH 7.9), 60 mM KCl, 4% (vol/vol) glycerol, 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 1mM DTT, supplemented with 0.1 to 2 μg unlabeled competitor poly (dI:dC)-(dI:dC) DNA (Pharmacia). The optimal amount of poly (dI:dC)-(dI:dC) was determined empirically. Samples were loaded on a 4% polyacrylamide gel (PAG) containing 0.25x TBE. Electrophoresis was carried out for 1.5 hr at 150 V at room temperature, and gels were dehydrated for 15 min in 10% methanol and 10% glacial acetic acid, dried under vacuum, and exposed to a Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY) or Fuji NIF-RX film (Fuji Photo Film Co., Tokyo, Japan).

To define the protein nature of the retarded bands, crude nuclear extracts were heated for 5 min at 90°C or treated with 20 μg of proteinase K (Merck, Darmstadt, Germany) at room temperature for 15 min.

For EMSA competition experiments nuclear extracts were first incubated for 10 min at room temperature with various concentrations of unlabeled competitor DNA (TCRAPJ-C1 or TCRDRE-C1), then the labeled DNA probe was added, and incubation proceeded for another 30 min at room temperature.

Missing contact analysis

Plasmid containing TCRAPJ-C1, TCRAJ39, or TCRAJ33 was digested with *Hin*dIII, treated with CIAP, labeled with [γ-^{32}P]dATP, and digested with *Eco*RI as described above. A separate sample of the same plasmid was first digested with *Eco*RI, treated with CIAP, labeled and then digested with *Hin*dIII. 10 microliters of labeled probe (100,000 cpm/μl) was incubated for 20 min with 25 μl of formic acid. The reaction was stopped by adding 200 μl of stop buffer (0.3 M sodium acetate, 0.1 mM EDTA and 25 μg/ml tRNA, pH 7.0) and the DNA was precipitated with 750 μl of ice-cold ethanol. Pellets were resuspended in 10 μl of water and used in an EMSA as described above using 5 μg crude nuclear extract from thymocytes. After electrophoresis the gel was exposed for 3 hr to a Kodak X-OMAT film, after which both the retarded band as well as the free probe were isolated from gel slices by overnight elution in elution buffer followed by precipitation. DNA pellets were resuspended in 200 μl of 10 mM Tris-HCl and 1 mM EDTA, pH 7.6) supplemented with 0.1 M NaCl and purified by passage through DE52 columns (Whatman, Maidstone, U.K.) as previously described (41). After precipitation, pellets were resuspended in 70 μl of 10% piperidine (Aldrich-Chemie, Steinheim, Germany) and incubated at 90°C for 30 min. Samples were then passed on Sephadex G-50 spin columns as previously described (41) and again precipitated. Finally, pellets were resuspended in formamide loading buffer (80% deionized formamide, 10 mM EDTA, 10 mM NaOH, 0.025% xylene cyanol FF (Biorad Laboratories) and 0.025% bromphenol-blue (Biorad Laboratories)), loaded and run on a 10% denaturing PAG (7 M urea) containing 1x TBE. The gel was dried under vacuum and exposed to Kodak X-OMAT film.

DNA protein UV-cross-linking

Plasmid (1.5 μg) containing the TCRAPJ-C1 probe was digested with *Bam*HI, treated with CIAP, and resuspended in 25 μl *Eco*RI restriction enzyme dilution buffer (Pharmacia). Six nanograms of M13 primer was added and the reaction mixture was heated for 5 min at 90°C, incubated for 10 min at 60°C, and slowly cooled down to room temperature for primer annealing. Primer extension was performed by incubating 50 μl reaction mixture containing 7.4 MBq [α-^{32}P]dATP (ICN), 0.05 mM dCTP, 0.05 mM dGTP, 0.005 mM dATP (Pharmacia), 0.05 mM 5-bromo-2′-dUTP (Sigma Chemical Co.), 1 mM DTT, and 10 U Klenow polymerase (Pharmacia) for 90 min at room temperature. After heat inactivation, the DNA was digested with *Eco*RI (1 hr, 37°C) and purified by 8% PAGE. Labeled DNA probe (120,000 cpm) was used in an EMSA containing 5 μg of thymocyte nuclear extract and 2.5 μg poly (dI:dC)-(dI:dC). After electrophoresis, the gel was irradiated with UV light of 245 nm in an UV Stratalinker 2400 (Stratagene, La Jolla, CA) on ice for various intervals (1 to 30 min). Subsequently, the gel was exposed for 3 hr to a Kodak X-OMAT film. The retarded DNA-protein band and the free probe were excised from the gel; eluted overnight in 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, and 0.1% SDS at room temperature; and precipitated with 4 volumes of ice-cold acetone. Proteins were separated by SDS-10% PAGE according to the method of Laemmli (42) and visualized by autoradiography.

RESULTS

Detection of a specific PJA-BP in crude nuclear extracts from human thymocytes

In order to identify DNA binding proteins that could be involved in δRec-ψJα preferential rearrangements, we cloned δRec and ψJα germline sequences containing RSS with additional upstream and downstream sequences (TCRDRE-RSS and TCRAPJ-RSS). EMSA experiments with the TCRAPJ-RSS probe using crude nuclear extracts from human thymocytes resulted in two faint retarded bands with intermediate and high electrophoretic mobilities, and one strong retarded band with low electrophoretic mobility (Figure 2B). EMSA experiments with the same probe using crude nuclear extracts from HeLa cells showed that only the strong, low electrophoretic mobility, retarded band was absent (Figure 2B). This retarded band was also not visible when the TCRDRE-RSS probe was used in EMSA experiments (data not shown).

The TCRAPJ-RSS DNA fragment was digested with restriction endonuclease *Hpa*II, resulting in two fragments (TCRAPJ-RSS/*Hpa*II5′ and TCRAPJ-RSS/*Hpa*II3′) as depicted in Figure 2A. EMSA experiments showed that binding activity of the PJA-BP in crude nuclear extracts from human thymocytes was restricted to the 3′ side of the TCRAPJ-RSS probe containing the ψJα gene segment (Figure 2A and 2B). Again, crude nuclear extracts from HeLa cells were negative (Figure 2B). Because this protein recognizes the ψJα gene segment, we call it the ψJα-binding protein (PJA-BP).

After cloning the ψJα gene segment (TCRAPJ-C1; Figure 2A), we demonstrated that heating the crude nuclear extracts from thymocytes or preincubating the nuclear extracts with proteinase K completely destroyed DNA-binding activity (Figure 3A), confirming the protein nature of the PJA-BP.

To confirm the DNA specificity of the PJA-BP, competitive EMSA experiments were performed. We were able to compete binding of the PJA-BP to the TCRAPJ-C1 probe by using 100 ng of unlabeled TCRAPJ-C1 probe, but not by using 100 ng of TCRDRE-C1 probe (Figure 3B).

Determination of the minimal DNA-binding sequence of the PJA-BP

To determine the minimal sequence to which the PJA-BP binds, we constructed a series of deletion mutants from the ψJα gene segment. We obtained seven 3′ deletion constructs by digesting the TCRAPJ-C1 fragment with restriction endonucleases *Dde*I, *Nla*III, *Mae*I, and *Nla*IV or by cloning PCR amplified products of ψJα fragments (TCRAPJ-C2 and TCRAPJ-C3). The position and length of these constructs are depicted in Figure 2A. EMSA experiments with crude nuclear extracts from thymocytes using these different deletion constructs showed strong binding of the PJA-BP with TCRAPJ-C1/*Dde*I and TCRAPJ-C2, and weak binding with TCRAPJ-C1/*Nla*III (Figure 2C; right panel). We were not able to detect binding of the PJA-BP to smaller 3′ deletion constructs (Figure 2C; right panel).

Four 5′ deletion constructs (TCRAPJ-C4, TCRAPJ-C5, TCRAPJ-C6, and TCRAPJ-C7; Figure 2A) were obtained by cloning PCR amplification products of the ψJα gene segment using the minimal 3′ DNA-binding sequences as determined by the 3′ deletion construct TCRAPJ-C2. Another three 5′ deletion constructs were

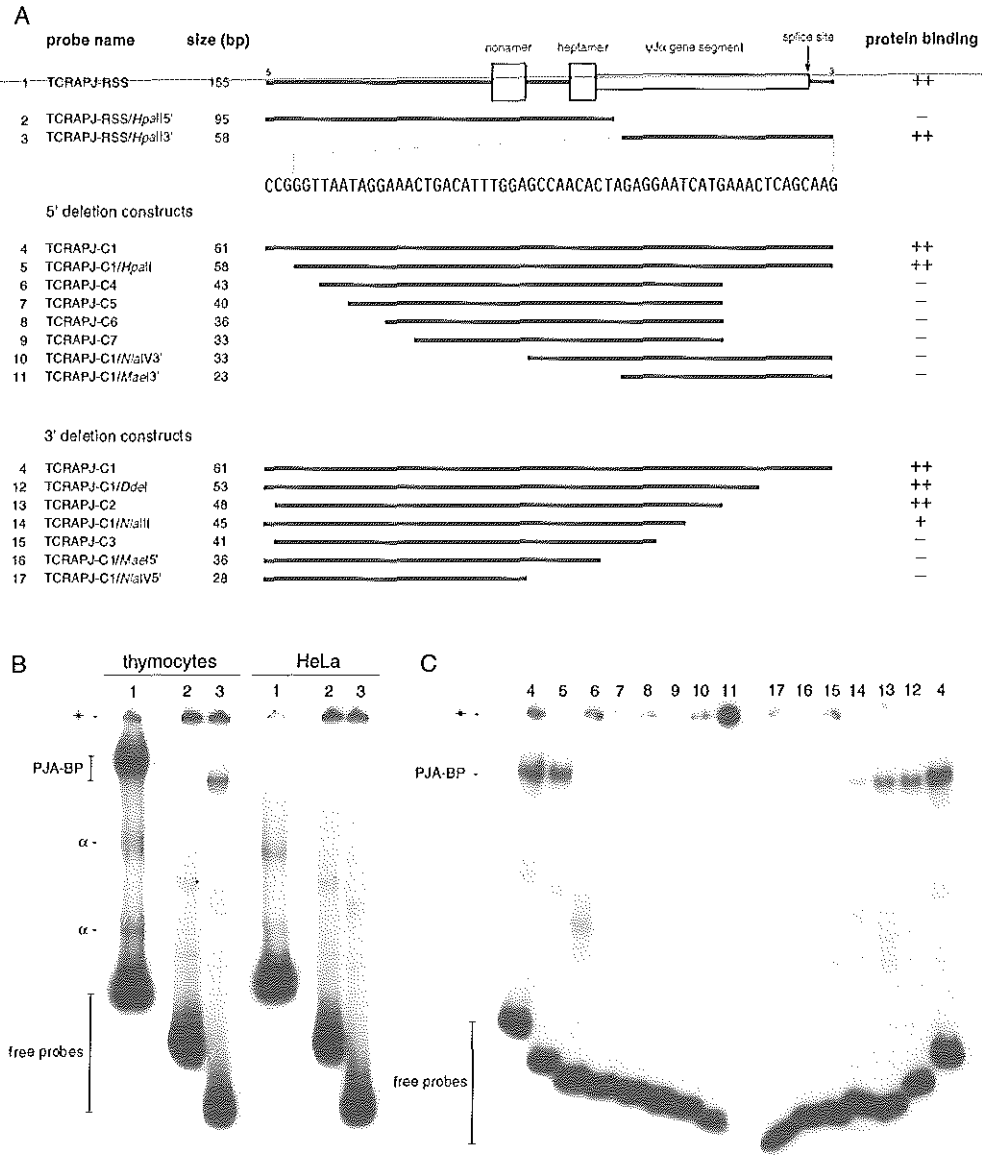

Figure 2. Detection and mapping of the minimal binding sequence of the PJA-BP. A) Outline of the ψJα DNA constructs used in EMSA experiments (see Materials and Methods). Size of the ψJα DNA constructs include ψJα-specific double strand bp only. B) EMSA of ψJα DNA probes TCRAPJ-RSS (1) or TCRAPJ-RSS digested with restriction endonuclease *Hpa*II, TCRAPJ-RSS/*Hpa*II5′ (2) and TCRAPJ-RSS/*Hpa*II3′ (3), with crude nuclear extracts from thymocytes or HeLa cells. C) EMSA of ψJα 5′ deletion constructs (left panel) and ψJα 3′ deletion constructs (right panel) with crude nuclear extracts from thymocytes. The positions of the free probes, the specific protein complex (PJA-BP), and the nonspecific bands (α) are indicated. * indicates residual activity retained in the PAG slots. Numbering of the lanes in B and C is according to the construct numbering outlined in A.

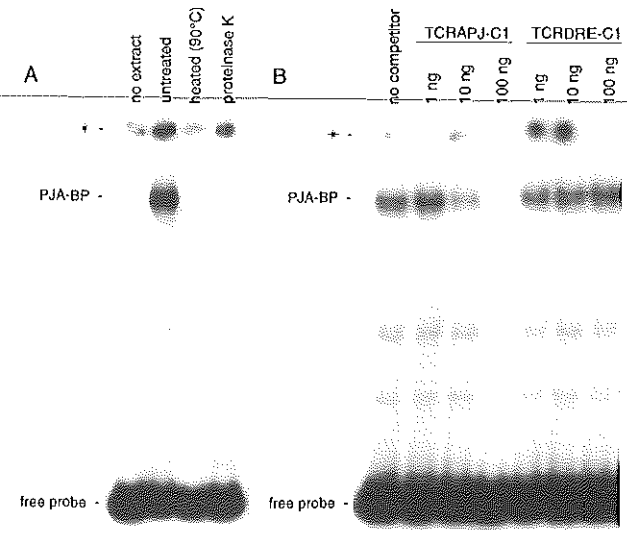

Figure 3. Inactivation and sequence specificity of the $\psi J\alpha$ binding activity of the PJA-BP. A) EMSA of probe TCRAPJ-C1 with (lanes 2,3,and 4) or without (lane 1) crude nuclear extracts from thymocytes. Nuclear extracts were heated for 5 min at 90°C or preincubated with proteinase K for 15 min at room temperature. B) EMSA of approximately 1 ng of labeled TCRAPJ-C1 probe with 1,10 or 100 ng of unlabeled TCRAPJ-C1 or TCRDRE-C1 as competitor DNA. *indicates residual activity retained in the PAG slots.

obtained by digesting probe TCRAPJ-C1 with *Hpa*II, *Nla*IV or *Mae*I. Although PJA-BP recognized the TCRAPJ-C1/*Hpa*II fragment (shown in Figure 2B), deletion of another 3 bp completely abolished binding of the PJA-BP (Figure 2C; left panel). Therefore we conclude that the minimal DNA-binding sequence for proper binding of the PJA-BP consists of a 46 bp fragment of the $\psi J\alpha$ gene segment starting 8 bp downstream of the $\psi J\alpha$ RSS heptamer.

Most of the known DNA binding proteins i.e. transcription factors recognize only 5 to 10 bp sequences (43, 44), whereas the recognition site of the PJA-BP spans 46 bp. To identify the exact sequences involved in DNA binding we performed missing contact analysis (45) using both strands of the TCRAPJ-C1 probe. The G+A sequence reaction was applied to identify the nucleotides involved in the binding of the PJA-BP. Figure 4A illustrates several contact sites (marked by vertical bars) on both the coding and the noncoding strands of the TCRAPJ-C1 probe. The DNA binding site for the PJA-BP starts 9 bp downstream of the $\psi J\alpha$ RSS heptamer and consists of a sequence of 9 bp (GTTAATAGG; Figure 4B). We also observed a very weak second binding site, consisting of 3 bp (TCA), starting 49 bp downstream of the $\psi J\alpha$ RSS heptamer (Figure 4). However, detection of this binding site was inconsistent. The data obtained by the missing contact analysis were in line with those obtained from the deletion studies.

Binding of the PJA-BP to other Jα gene segments

The $\psi J\alpha$ gene segment, also called TCRAJ61 (40), is the most 5′ $J\alpha$ gene segment. We compared the sequences of the 61 different human $J\alpha$ gene segments, for homology between the PJA-BP binding site of the $\psi J\alpha$ gene segment and other $J\alpha$ gene segments. The 9 bp GTTAATAGG binding site of the PJA-BP was not found in any other $J\alpha$ gene segment, although homology (7/9 bp) was observed in four $J\alpha$ gene segments ($J\alpha$60, $J\alpha$54, $J\alpha$39, and $J\alpha$33). Because the homologous sequence of the $J\alpha$54 gene segment overlaps with the conserved RSS heptamer sequence, which might be bound by other recombination enzymes (see the introduction), cloning was

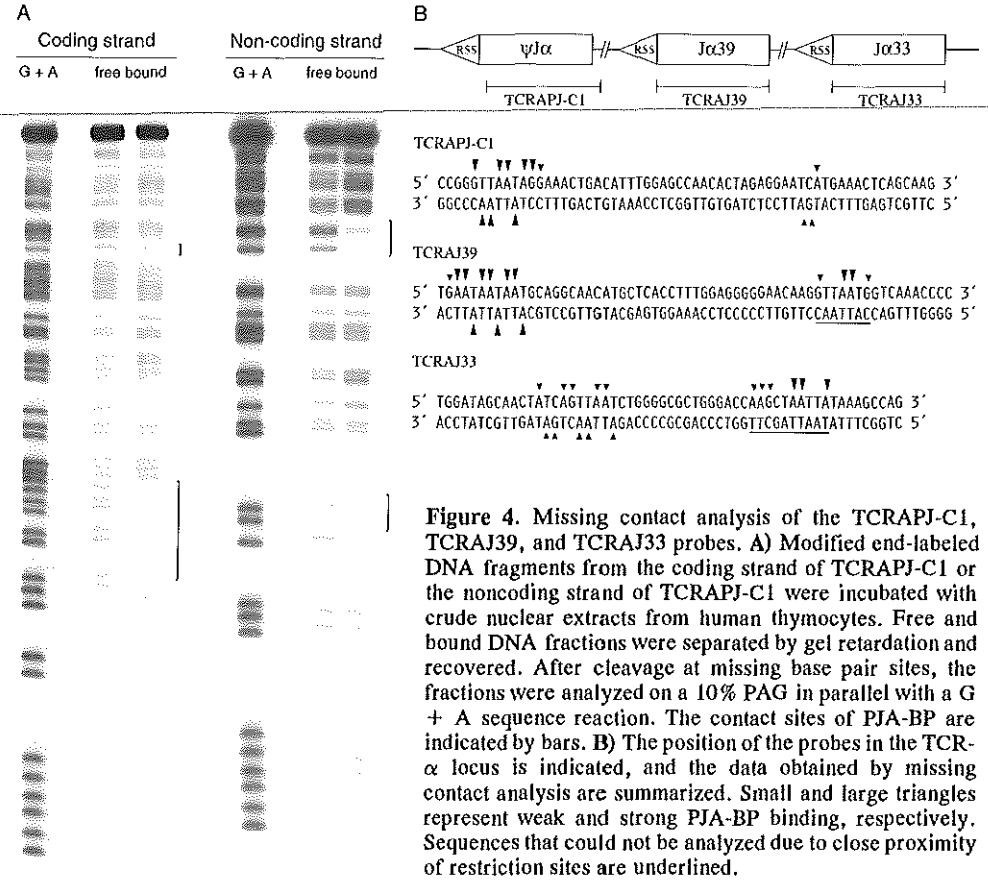

Figure 4. Missing contact analysis of the TCRAPJ-C1, TCRAJ39, and TCRAJ33 probes. **A)** Modified end-labeled DNA fragments from the coding strand of TCRAPJ-C1 or the noncoding strand of TCRAPJ-C1 were incubated with crude nuclear extracts from human thymocytes. Free and bound DNA fractions were separated by gel retardation and recovered. After cleavage at missing base pair sites, the fractions were analyzed on a 10% PAG in parallel with a G + A sequence reaction. The contact sites of PJA-BP are indicated by bars. **B)** The position of the probes in the TCR-α locus is indicated, and the data obtained by missing contact analysis are summarized. Small and large triangles represent weak and strong PJA-BP binding, respectively. Sequences that could not be analyzed due to close proximity of restriction sites are underlined.

restricted to the other three Jα gene segments (TCRAJ60, TCRAJ39, and TCRAJ33). EMSA experiments with the TCRAJ60 probe showed no retarded band of similar size as the PJA-BP, whereas the probes TCRAJ39 and TCRAJ33 showed strong binding of the PJA-BP (Figure 5). Competitive EMSA experiments, using unlabeled TCRAPJ-C1, indicated differential binding affinity of PJA-BP to these TCRAJ probes: TCRJA33 > TCRJA39 > TCRAPJ-C1 (data not shown).

To determine which sequences were recognized on the TCRAJ39 and TCRAJ33 gene segments, we performed missing contact analysis experiments. These experiments showed that PJA-BP recognizes the sequences GAATAATAAT and GTTAATG on TCRAJ39, and the sequences ATCAGTTAAT and AAGCTAATTA on TCRAJ33 (Figure 4B). Interestingly, the binding sequences on TCRAPJ-C1, TCRAJ39 and TCRAJ33 contain one or two TAAT/ATTA motifs.

We compared the 61 Jα gene segments for the presence of the TAAT/ATTA motif; 11 Jα gene segments contain the TAAT sequence and eight Jα gene segments contain the complementary ATTA sequence (Table II). EMSA experiments with randomly cloned Jα gene segments without a TAAT/ATTA motif (TCRAJ-57, TCRAJ49, TCRAJ36, and TCRAJ8) showed no retarded bands of similar size as the PJA-BP (Figure 5). We therefore conclude that the TAAT/ATTA sequence is the core motif for PJA-BP binding.

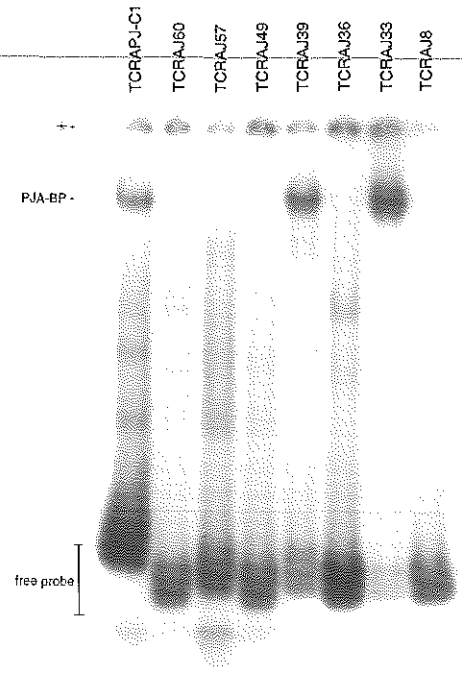

Figure 5. Binding of the PJA-BP to Jα sequences. EMSA experiment using crude nuclear extracts from human thymocytes with probes TCRAPJ-C1 (ψJα), TCRAJ60 (Jα60), TCRAJ57 (Jα57), TCRAJ49 (Jα49), TCRAJ39 (Jα39), TCRAJ36 (Jα36), TCRAJ33 (Jα33), and TCRAJ8 (Jα8). ↓ indicates residual activity retained in the PAG slots.

TABLE II. Sequence homology between the PJA-BP binding sequence of the ψJα gene segment and other Jα gene segments containing the TAAT/ATTA core motif

Gene segment	position[a]	PJA-BP binding sequence
ψJα	+9	GTTAATAGG
Jα60	+41	------T-T
Jα55	+10	-G----GCT
Jα54	−2	TG----TCA
Jα52	−1	-C----GCT
Jα48	+20	------TTC[b]
Jα45	+50	TC----CAT
Jα42	+1	CA----TCA[b]
JαJ39	+3	AA-----AT
	+6	AA----GCA
	+47	------G-T
Jα38	−2	TG----GCT
Jα37	+22	AC----CTT
Jα34	+39	TG----CT-[b]
Jα33	+17	------CT-
	+41	-C----TAT
	+42	TA----TA-[b]
Jα32	+1	CA----TCA[b]
Jα19	+45	CA----GTC
Jα14	+33	-A----CTT[b]
Jα3	+22	-A----CTT
Jα2	+54	TA----GAA[b]
Jα1	+8	-G-----CT[b]

a. Position of the sequence upstream (−) or downstream (+) relative to the cleave site of the RSS.
b. Homology of the noncoding strand of Jα gene segments with the coding strand of the ψJα gene segment.

Expression of the PJA-BP in human hematopoietic cell lines

To investigate whether PJA-BP expression was differentiation stage and/or lineage specific, we performed EMSA experiments using crude nuclear extracts from 17 different human continuous hematopoietic cell lines (representative examples are given in Figure 6). Expression of the PJA-BP was determined in seven T cell lines representing differentiation stages from immature to more mature T cells (32). We were able to detect a faint retarded band in cell lines CEM ($CD3^-$), MOLT-4 ($CD3^{+/-}$) and DND41 (TCR-$\beta\delta^+$) and a strong retarded band in cell line HPB-ALL (TCR-$\alpha\beta^+$). The other $CD3^-$ immature T cell lines HSB and P12 and the TCR-$\gamma\delta^+$ T cell line PEER were negative for PJA-BP.

We were not able to detect PJA-BP expression in nuclear extracts from the immature B cell lines RVT and NALM-1, the Burkitt cell line Daudi, the mature B cell line ROS-15, and the plasma cell line U266. Cell line P30/Ohkubo (a very immature B cell line with germline TCR-δ genes) showed a faint retarded band of similar size as the PJA-BP.

PJA-BP expression is not T cell restricted, because we could detect retarded

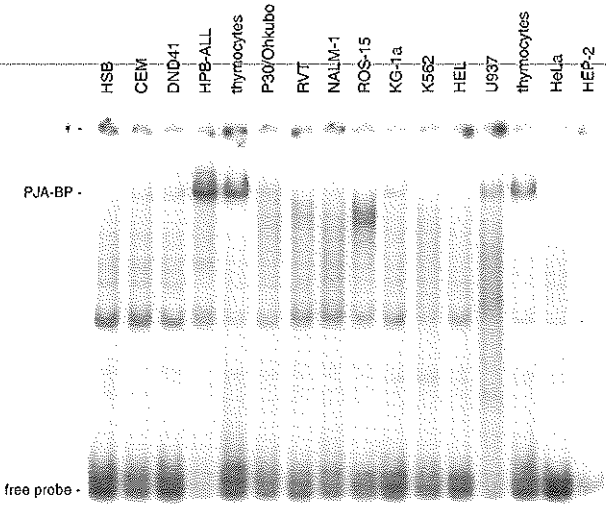

Figure 6. PJA-BP expression in human continuous hematopoietic cell lines. EMSA of probe TCRAPJ-C1 with crude nuclear extracts from immature CD3⁻ T cell lines (HSB, and CEM), more mature T cell lines (DND41 and HPB-ALL), immature B cell lines (P30/Ohkubo, RVT, and NALM-1), mature B cell line (ROS-15), myeloid cell lines (KG-1a, K562, HEL, and U937), and non-hematopoietic cell lines (HeLa and HEP-2). *indicates residual activity retained in the PAG slots.

bands of similar size in two of four myeloid cell lines. The cell lines KG-1a (early myeloblast) and U937 (monoblast) were positive, whereas the cell lines K562 and HEL (both myeloblast/erythroblast) were negative.

Determination of the molecular weight of the PJA-BP

Finally we tried to determine the molecular weight of the PJA-BP with UV cross-linking experiments. The TCRAPJ-C1 probe was used in an EMSA experiment with crude nuclear extracts from thymocytes. The gel was subjected to UV light to covalently link DNA-protein interactions. After 1 min of UV cross-linking, we were able to detect a faint band of approximately 210 kDa. This band was only visible in the lanes containing the PJA-BP bound DNA fraction and became more intense after longer UV incubation times (Figure 7). No other bands appeared, even after 30 min of UV cross-linking. To determine whether the observed band consisted of a protein, we used proteinase K in one of the experiments; this resulted in total loss of the 210 kDa band (Figure 7). After subtracting the molecular weight of the free probe, the molecular weight of the PJA-BP was estimated to be approximately 180 kDa. We assume that the PJA-BP consists of only one protein, but we cannot fully exclude the possibility that the PJA-BP represents a protein complex.

DISCUSSION

In this report we describe a DNA binding protein of approximately 180 kDa, expressed in nuclei of thymocytes and capable of binding the ψJα gene segment. We call this protein PJA-BP. From missing contact analysis, we conclude that the PJA-BP recognizes the sequence GTTAATAGG on the ψJα gene segment, but also needs an additional sequence three DNA helix turns downstream for proper binding.

The 9 bp GTTAATAGG binding site of the PJA-BP was not found in any other Jα gene segment, but four Jα gene segments showed homology for 7 of 9 bp (Jα60,

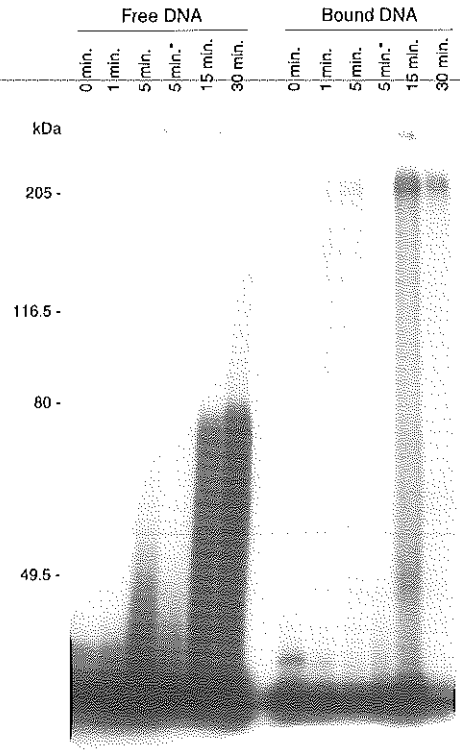

Figure 7. Molecular size of the PJA-BP estimated after UV cross-linking to TCRAPJ-C1. DNA fragment TCRAPJ-C1 was incubated with crude nuclear extracts from human thymocytes. Free and bound DNA fractions were recovered after UV cross-linking for 0, 1, 5, 15, or 30 min respectively, followed by SDS-PAGE. The asterisks indicate lanes (5 min*), in which protein activity was tested after incubating the recovered gel slices with proteinase K. The molecular mass standards are indicated (kDa).

Jα54, Jα39, and Jα33). Using EMSA experiments and missing contact analysis, we showed that the PJA-BP recognizes the TAAT/ATTA core motif on ψJα, Jα39, and Jα33. The TAAT/ATTA core motif is generally recognized by homeodomain proteins, which are suggested to regulate the development of higher organisms (46). There is evidence that homeodomain proteins are also important in hematopoietic cell proliferation and lineage commitment (reviewed in Ref. 47). The higher binding affinity of the PJA-BP to TCRAJ33 and TCRAJ39 compared with that to TCRAPJ-C1 can be explained by the presence of multiple TAAT/ATTA core motifs in the former two probes. Interestingly, the TAAT/ATTA motif in ψJα is conserved between human and mouse, and two of three TAAT/ATTA motifs of both Jα39 and Jα33 are also conserved (40). Although the Jα60 gene segment contains a TAAT motif, we could not detect binding of the PJA-BP; probably because the probe did not contain binding sequences three DNA helix turns downstream of the TAAT motif.

We were able to detect the PJA-BP in all thymus samples tested regardless of age (data not shown). Also the δRec-ψJα rearrangement is present as a dominant preferential rearrangement in human thymus cell samples throughout ontogeny (9, 11, 12). Two out of four immature $CD3^-$ T cell lines showed weak expression of the PJA-BP. Interestingly these two positive cell lines have both TCR-δ genes deleted, whereas the other two immature T cell lines have one rearranged TCR-δ allele (CEM) or a δRec-Jα59 rearrangement on one allele (HSB) (our unpublished observations). The PJA-BP was also expressed in the TCR-$\alpha\beta^+$ cell line HPB-ALL (both TCR-δ genes deleted), but not in the TCR-$\gamma\delta^+$ T cell line PEER. PJA-BP expression might

be correlated with TCR-δ deletion and consecutive Vα-Jα rearrangements.

We could not detect PJA-BP expression in five of six B cell lines, but the immature B cell line P30/Ohkubo was weakly positive. This cell line was obtained from a patient with a T cell acute lymphoblastic leukemia (ALL) (33) and is the only published cell line with transcription of the T-early-α (TEA) element which is assumed to be responsible for opening the TCR-Jα locus to the V(D)J recombinase (48). It would be interesting to determine whether high expression of PJA-BP in this cell line actively induces TCR-δ deletion. Previous experiments showed the presence of cross-lineage TCR-δ deletion in precursor B-ALL, but no cross-lineage δRec-ψJα rearrangements were found (39). Two of four myeloid cell lines were weakly positive for the PJA-BP, of which cell line U937 has a cross-lineage Vδ2-Dδ3 rearrangement (our unpublished observations), and cell line KG-1a also has some T cell characteristics (49).

Different factors can play a role in the process of V(D)J recombination, including 1) the expression of general and lineage specific recombination proteins, such as RAG-1, RAG-2, DNA-dependent protein kinase and perhaps PJA-BP; 2) the presence and sequence of the RSS; 3) locus accessibility; and 4) the distance between the rearranging genes (reviewed in Ref. 19).

1) RAG-1 and RAG-2 are essential for V(D)J recombination (18). RAG-1 may play a role in both the initiation of V(D)J recombination as well as the formation of coding junctions (21, 50). Recently, it was shown that a RAG-1 mutant needs particular dinucleotides adjacent to the RSS heptamer for efficient recombination (51). The enzyme terminal deoxynucleotidyl transferase (TdT) is responsible for the template-independent addition of nucleotides at gene segment junctions, but the exact mechanism is not understood (52, 53). PJA-BP is not probably the same molecule as RAG-1 or TdT, because our EMSA experiments showed no retarded band with lower electrophoretic mobility (supershift) after adding polyclonal anti-RAG-1 or anti-TdT (unpublished observations).

Binding of the PJA-BP to the ψJα gene segment might play a role in the process of TCR-δ deletion, but PJA-BP might also play a role in ongoing Vα-Jα gene rearrangements. The preferential partner of the ψJα gene segment in thymocytes is the δRec gene segment, but no comparable T cell specific DNA binding protein could be detected that recognized the δRec gene segment. If binding of the PJA-BP to the TAAT core motif of ψJα were important for δRec-ψJα rearrangements, then, based on our in vitro data, 19 different Jα gene segments (including ψJα) can theoretically rearrange to the δRec gene segment. However, we have to be careful to extrapolate the results of in vitro experiments to functions in vivo. We are currently investigating whether other (preferential) δRec-Jα gene rearrangements can be detected in thymus cell samples. We assume that not all Jα gene segments containing the TAAT/ATTA core motif (Table II) are likely to be involved in rearrangement to the δRec gene segment.

2) The three bases of the RSS heptamer sequence closest to the recombination cleavage site and the sixth and seventh nucleotides of the RSS nonamer sequence are critical for V(D)J recombination (54). Jα60, Jα19, Jα2, and Jα1 gene segments, do not contain all essential nucleotides (40) and are therefore probably used less frequently.

3) It has been hypothesized that transcription of the TEA element could be responsible for opening the TCR-Jα locus to the V(D)J recombinase system (48, 55, 56). It has not yet been determined whether the complete TCR-Jα locus or only the 5′ part of the TCR-Jα gene region becomes accessible by TEA transcription. The ψJα gene segment might be the first accessible Jα gene segment because of its location immediately downstream of TEA (Figure 1).

4) The distance and the proximity of the gene segments have been proposed to be involved in V(D)J recombination as well. Three studies showed that Jα gene rearrangements during T cell ontogeny are nonrandom (57-59). They conclude that the most 5′ Jα gene segments are preferentially used in immature thymocyte populations. According to the above-mentioned locus accessibility and distance effects, only the first few 5′ Jα gene segments might easily rearrange to the δRec gene segment. The ψJα and Jα60 gene segments are the only 5′ gene segments containing the PJA-BP binding motif, and because Jα60 does not contain the appropriate RSS, the ψJα gene segment is the most likely candidate for rearrangement to the δRec gene segment. During further differentiation, the PJA-BP might play an additional role in rearrangements of Vα gene segments to more downstream Jα gene segments (e.g., Jα39 and Jα33).

Based on our in vitro data, we postulate that the PJA-BP might be one of the factors involved in the preferential recombination of δRec to ψJα. However, in vivo characterization of the PJA-BP during ontogeny as well as characterization of DNA binding proteins capable of binding the preferential partner gene segment of ψJα (i.e., δRec) await further investigation.

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CHAPTER 6

PJA-BP EXPRESSION AND T CELL RECEPTOR-δ DELETION DURING HUMAN T CELL DIFFERENTIATION

CHAPTER 6

PJA-BP EXPRESSION AND T CELL RECEPTOR-δ DELETION DURING HUMAN T CELL DIFFERENTIATION[1]

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ABSTRACT

Recombination of δRec to ψJα will delete the T cell receptor (TCR)-δ gene, which is thought to play an important role in the bifurcation of the TCR-αß versus TCR-γδ lineages. We recently detected a DNA-binding protein in human thymocytes, the so-called PJA-BP, which might be one of the factors involved in the regulation of this preferential δRec-ψJα rearrangement. In this regard we investigated PJA-BP expression and its correlation with TCR-δ deletion in human thymocytes.

Our electrophoretic mobility shift assay experiments showed that the PJA-BP is evolutionary conserved in human, simian, and murine thymocytes. Using a large series of human hematopoietic malignancies (n=30), we showed that PJA-BP expression is probably thymocyte specific, and might be restricted to thymocytes committed to the TCR-αß lineage.

Analysis of seven well-defined human thymocyte subpopulations showed that preferential δRec-ψJα rearrangements as well as PJA-BP expression can be detected from the $CD34^-/CD1^+/CD3^-/CD4^+/CD8\alpha^+\beta^-$ thymocyte cell stage onwards. These experiments suggest that expression of the PJA-BP in human thymocytes starts simultaneously with preferential δRec-ψJα rearrangement, which supports our hypothesis that the PJA-BP is one of the factors involved in the preferential recombination of δRec to ψJα.

INTRODUCTION

The T cell receptor (TCR)-δ gene complex is flanked by the TCR-δ-deleting elements δRec and ψJα (1, 2), which are assumed to delete the TCR-δ gene before Vα-Jα rearrangement (3). The nonproductive δRec-ψJα recombination occurs at high

[1] Submitted for publication

frequency in both human and murine thymocytes (1, 2, 4). However, little is known about the cell stage at which this rearrangement is initiated in human T cell differentiation, or the mechanisms that regulate this preferential rearrangement.

We recently detected a DNA-binding protein of approximately 180 kilodaltons, the so-called $\psi J\alpha$-binding protein (PJA-BP), which recognizes the $\psi J\alpha$ gene segment (5). Preliminary results on the expression pattern of the PJA-BP in a few human continuous cell lines suggested that PJA-BP expression might be correlated with TCR-δ deletion or consecutive Vα-Jα rearrangements. Based on our previous data, we postulated that the PJA-BP might be one of the factors involved in the preferential recombination of δRec to $\psi J\alpha$ (5).

In this study we characterized the expression pattern of the PJA-BP in different species and in human hematopoietic malignancies. Furthermore, we investigated the correlation between PJA-BP expression and TCR-δ deletion in a series of seven well-defined human thymocyte subpopulations.

MATERIALS AND METHODS

Cell samples

Human thymocytes were obtained from postnatal thymus samples of children undergoing cardiac surgery at the age of 22 days to 2 years, as described previously (5). All human thymus samples were obtained with the approval of the Medical Ethics Committee of the Erasmus University Rotterdam/University Hospital Rotterdam, Rotterdam, The Netherlands.

Murine thymocytes were obtained from seven weeks old Balb/C mice and 10-14 weeks old C57BL/6 mice. Simian thymocytes were obtained from an adult Macaque.

Patient cell samples were obtained from 30 patients with a hematopoietic malignancy: 10 T cell acute lymphoblastic leukemias (ALL), including six TCR$^-$/CD3$^-$ T-ALL, two TCR-$\alpha\beta^+$ T-ALL, and two TCR-$\gamma\delta^+$ T-ALL; five mature T cell leukemias, including three TCR-$\alpha\beta^+$ T cell large granular lymphocyte leukemias (T-LGL) and two TCR-$\alpha\beta^+$ Sézary syndromes; five precursor-B-ALL; five B cell chronic lymphocytic leukemias (B-CLL); and five acute myeloid leukemias (AML). Periheral blood mononuclear cells (PBMNC) were isolated from the patients and a healthy volunteer by Ficoll-paque (density: 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation.

Preparation of thymocyte subpopulations.

To isolate the most immature thymocyte subpopulations, Lymphoprep (density 1.077 g/ml; Nycomed Pharma, Oslo, Norway) density centrifugation was performed on the thymocyte suspension. The CD34$^+$ population was then enriched using the QBEND/10 kit (Miltenyi Biotec Inc., Auburn, CA) following manufacturer's instructions. Subsequently, the CD34$^+$/CD1$^-$ subpopulation (fraction 1) and the CD34$^+$/CD1$^+$ subpopulation (fraction 2) were sorted with the FACStar plus (Becton Dickinson, San Jose, CA) using CD34 (HPCA-2) and CD1a (T6-RD1) antibodies. The CD8$^-$ subpopulation (fraction 4) was obtained by depletion of glycophorin$^+$, CD8$^+$, CD27$^+$, CD69$^+$, CD19$^+$, and CD3$^+$ cells by incubation with the 10F7 MN, RPA-T8, CLB-3A12, Leu-23, CLB CD19, and SPV-T3b antibodies, respectively, followed by depletion with magnetic beads coated with sheep anti-mouse immunoglobulins (Dynal, Oslo, Norway). Part of this subpopulation was further sorted for the CD3$^-$/CD4$^+$/CD8α^- phenotype (fraction 3) with the FACStar plus (Becton Dickinson). Purity of the cell population was >97%.

Two additional subpopulations were obtained by depletion of CD8β^+, CD27$^+$, and CD69$^+$ cells (fraction 5), or CD27$^+$ and CD69$^+$ cells (fraction 6) using the 2ST8-5H7, CLB-3A12, and Leu-23 antibodies and magnetic beads (Dynal). Bead-coated thymic cells of the latter fraction, which contained the more mature CD27$^+$/CD69$^+$ thymocytes (fraction 7), were obtained by magnetic separation.

Electrophoretic mobility shift assay (EMSA)

Two μg of crude nuclear extract was incubated for 30 min at room temperature with the labeled human DNA probes TCRAPJ-C1 (Figure 1A) as described previously (5). Furthermore, EMSA

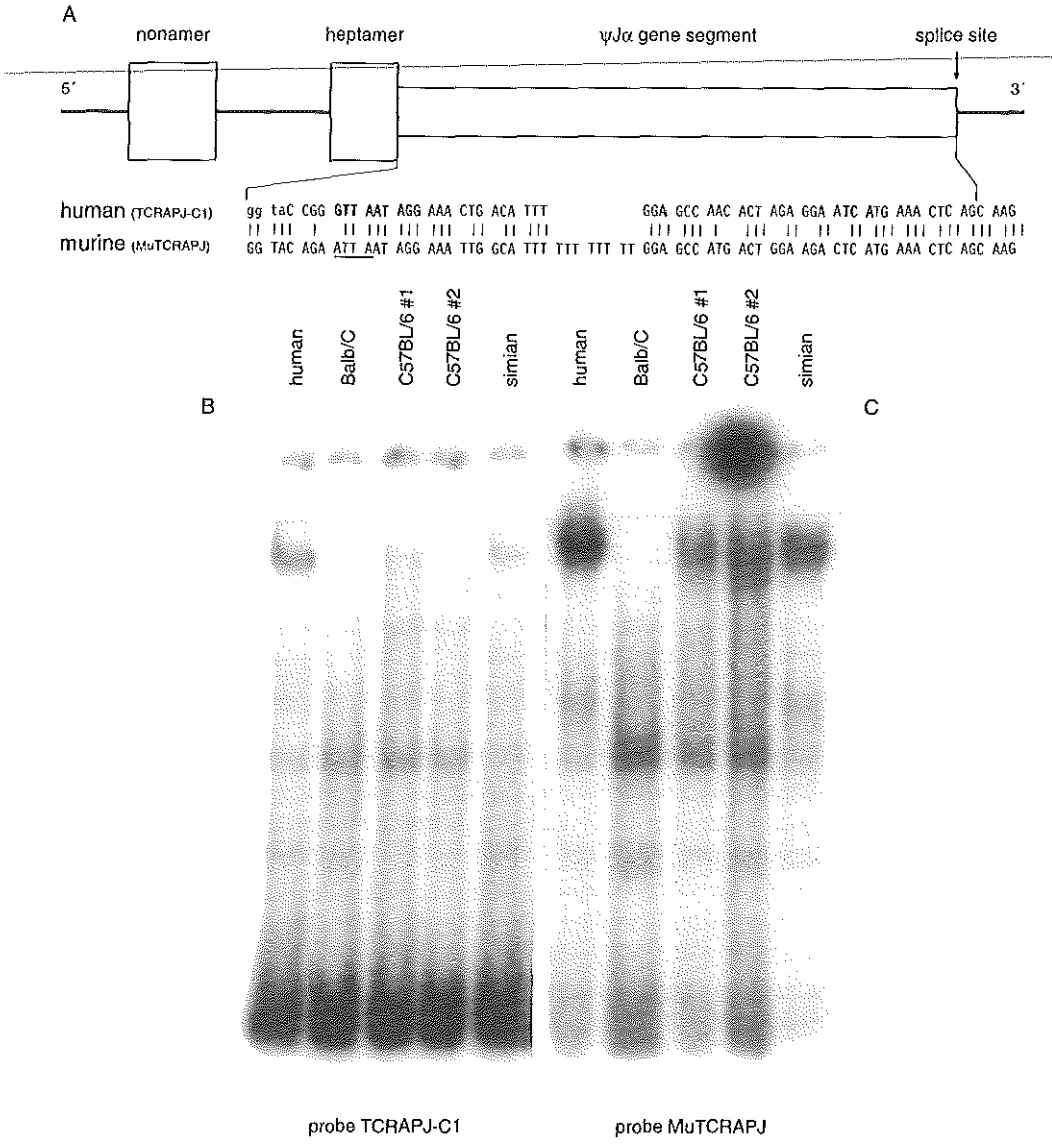


Figure 1. PJA-BP expression in human, simian, and murine thymocytes. A) Homology between the human and murine ψJα gene segments (2, 6, 10, 11). The presented ψJα sequences start immediately downstream from the heptamer of the recombination signal sequence. The human (TCRAPJ-C1) and murine (MuTCRAPJ) DNA probes used in this study are indicated; the ψJα sequences in lower case characters are not present in the TCRAPJ-C1 probe. The human PJA-BP binding site is depicted in bold, and the additional ATTA core domain of the murine ψJα gene segment is underlined. EMSA of the human DNA probe TCRAPJ-C1 (B) or the murine DNA probe MuTCRAPJ (C) using crude nuclear extracts from human thymocytes (lane 1), murine thymocytes of a Balb/C mouse (lane 2) and C57BL/6 mice (lanes 3 and 4) and simian thymocytes (lane 5).

experiments were performed with an additional DNA probe (MuTCRAPJ; Figure 1A), containing the murine ψJα gene segment (6, 7).

Southern blot analysis

DNA was isolated from thymocyte subpopulations, digested with the restriction enzyme *Eco*RI, size fractionated in agarose gels (0.7%), and transferred to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) as previously described (8). Filters were hybridized with a ^{32}P random oligonucleotide labeled TCRDRE probe (9).

RESULTS AND DISCUSSION

Expression of the PJA-BP in simian and murine thymocytes

We recently detected a DNA-binding protein in human thymocytes which recognizes the $\psi J\alpha$ gene segment (5). We were interested to see whether this protein is evolutionary conserved. Initial EMSA experiments using the human TCRAPJ-C1 DNA probe showed PJA-BP expression in nuclear extracts from simian thymocytes and in two out of three nuclear extracts from murine thymocytes (Figure 1B). Because the negative sample was obtained from a Balb/C mouse, whereas the positive samples were obtained from C57BL/6 mice, additional experiments with several murine nuclear extracts from these mouse strains were performed. We found a variable expression of PJA-BP in these cell samples irrespective of the mouse strain used (data not shown). We formally cannot exclude that proteolysis caused the variable PJA-BP expression in murine thymocyte nuclear extracts, but we never observed a variable expression of PJA-BP in our large series of human thymocyte nuclear extracts.

The human $\psi J\alpha$ gene segment shows 80% homology with the murine $\psi J\alpha$ gene segment (2). The major difference between the human and murine gene segments is the presence of a variable number of T nucleotides in the murine $\psi J\alpha$ gene segment, which is not strain dependent (2, 6, 10, 11) (Figure 1A). However, these nucleotides are not located in the major DNA-binding site of the PJA-BP (5).

We cloned and sequenced the murine $\psi J\alpha$ DNA probe MuTCRAPJ (Figure 1A). EMSA experiments using this DNA probe showed that the PJA-BP from human, simian, and murine nuclear extracts can bind to the murine $\psi J\alpha$ DNA probe, except for the Balb/C cell sample tested (Figure 1C). PJA-BP binding to the murine DNA probe appeared to be stronger than to the human DNA probe in all nuclear extracts tested (Figures 1B and 1C). This can be explained by the presence of an additional ATTA core domain, necessary for PJA-BP binding (5), in the murine $\psi J\alpha$ sequence (Figure 1A).

Our EMSA experiments show that PJA-BP is evolutionary conserved, although the expression in murine thymocytes is variable.

Expression of the PJA-BP in human hematopoietic malignancies

Our previous EMSA experiments with nuclear extracts from human hematopoietic cell lines showed that PJA-BP expression might be correlated with TCR-δ deletion and subsequent Vα-Jα rearrangements (5). In order to further investigate the correlation between TCR-δ deletion and the expression pattern of the PJA-BP, we performed EMSA experiments using crude nuclear extracts from 30 different human hematopoietic malignancies (representative examples are given in Figure 2).

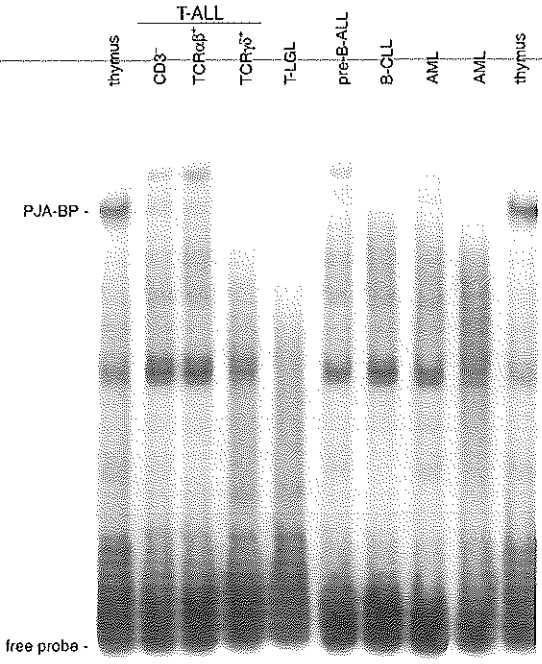


Figure 2. PJA-BP expression in human hematopoietic malignancies. EMSA of probe TCRAPJ-C1 with crude nuclear extracts from thymocytes and patients with a T-ALL ($TCR^-/CD3^-$, TCR-$\alpha\beta^+$, or TCR-$\gamma\delta^+$), TCR-$\alpha\beta^+$ T-LGL, precursor-B-ALL, B-CLL, and two patients with an AML.

We were able to detect weak PJA-BP expression in five out of six $TCR^-/CD3^-$ T-ALL tested, and in the two TCR-$\alpha\beta^+$ T-ALL tested. However, the two TCR-$\gamma\delta^+$ T-ALL tested were negative for PJA-BP expression. In contrast to our previous data (5), no full correlation was found between PJA-BP expression and TCR-δ deletion in these immature T cell malignancies; i.e. PJA-BP expression was observed in one patient with both TCR-δ genes in germline configuration and in one patient with two rearranged TCR-δ genes. Nevertheless, the absence of PJA-BP expression in TCR-$\gamma\delta^+$ T-ALL, still suggests a correlation of PJA-BP expression with T cells from the TCR-αß lineage.

We also performed EMSA experiments with nuclear extracts from cell samples of three patients with TCR-$\alpha\beta^+$ T-LGL and two patients with TCR-$\alpha\beta^+$ Sézary syndrome. No PJA-BP expression was found in these mature TCR-$\alpha\beta^+$ T cell malignancies. Because these malignancies are derived from peripheral T cells, whereas the T-ALL are assumed to be derived from cortical thymocytes (3, 12), we hypothesize that PJA-BP expression is restricted to thymocytes. The absence of PJA-BP expression in PBMNC from healthy volunteers and in the E-rozet$^+$ fraction of PBMNC (data not shown) supports this hypothesis.

We were not able to detect PJA-BP expression in precursor-B-ALL (n=5) or B-CLL (n=5) cell samples, but one patient with a precursor-B-ALL contained a very weak and vague retarded band of similar size as PJA-BP (data not shown). In contrast to our previous data using nuclear extracts from myeloid cell lines (5), no retarded band of similar size as the PJA-BP could be detected in five AML cell samples tested, representing different myeloid differentiation stages. Interestingly, a retarded band with only a slightly lower electrophoretic mobility compared to PJA-BP was observed

in these AML cell samples (Figure 2). Although we cannot fully exclude that this retarded band consists of a protein complex of PJA-BP with other proteins, we hypothesize that PJA-BP expression is T cell specific.

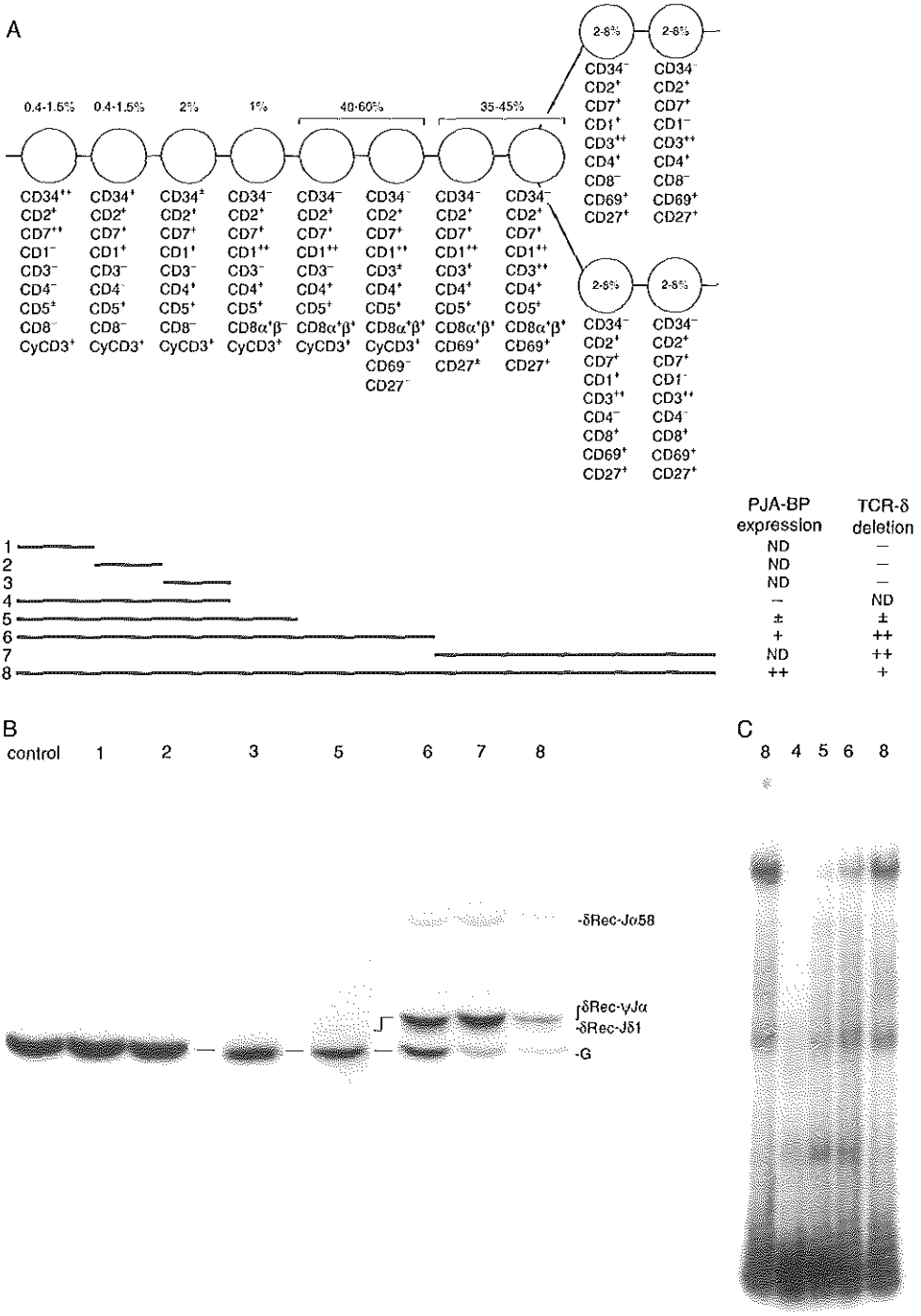
PJA-BP expression and TCR-δ deletion in human thymocyte subpopulations

Our EMSA experiments using human continuous T cell lines and T cell malignancies suggest that PJA-BP expression might be correlated with commitment to the TCR-αß lineage. Little is known about the lineage commitment of human thymocytes, although deletions of the TCR-δ gene (and in particular δRec-ψJα rearrangements) are thought to play an important role (3). Based on the very low frequency of δRec-ψJα rearrangements in human T cell lines and T cell malignancies, we recently hypothesized that these rearranged gene segments exist for only an extremely short period during thymocyte differentiation (Verschuren et al., submitted for publication). This might explain the weak PJA-BP expression in our T-ALL samples and the lack of correlation between PJA-BP expression and TCR-δ deletion in these cell samples. We therefore performed additional studies using freshly isolated human thymocytes to further investigate the correlation between PJA-BP expression and TCR-δ deletion.

We designed a hypothetical scheme for human T cell differentiation in the thymus, based on immunologic marker analysis (3, 13-20) (Figure 3A). According to this scheme seven thymocyte subpopulations were isolated (Figure 3A). DNA and nuclear extracts were isolated for Southern blot analysis with the δRec probe TCRDRE and EMSA experiments, respectively.

No preferential δRec-ψJα rearrangements could be detected by Southern blot analysis in the three most immature T cell subpopulations characterized by the phenotypes: $CD34^+/CD1^-/CD3^-/CD4^-/CD8^-$ (fraction 1), $CD34^+/CD1^+/CD3^-/CD4^-/CD8^-$ (fraction 2), and $CD34^\pm/CD1^+/CD3^-/CD4^+/CD8^-$ (fraction 3). A weak δRec-ψJα rearranged band, representing approximately 5% of the thymocytes, was detected in the thymocyte subpopulation obtained by depletion of the $CD8ß^+$ thymocytes (fraction 5). Based on these data we conclude that δRec-ψJα rearrangements start at the $CD34^-/CD1^+/CD3^-/CD4^+/CD8α^+ß^-$ thymocyte cell stage, which is consistent with the initiation of TCR-α rearrangements in mice (21, 22). The next thymocyte subpopulation (fraction 6) was obtained by depletion for the activation markers CD69 and CD27 (20). This subpopulation, in which the majority of the cells (~80%) are double positive ($CD4^+/CD8^+$), showed a strong δRec-ψJα rearranged band, suggesting that the major part of the δRec-ψJα rearrangements occur in these double positive thymocytes. Interestingly, our data therefore suggest that δRec-ψJα rearrangement occurs during or after thymocyte expansion (23). The

Figure 3. TCR-δ deletion and PJA-BP expression in human thymocyte subpopulations. A) Hypothetical scheme for human T cell differentiation based on immunological marker analysis (3, 13-20). The different isolated thymocyte subpopulations (fractions 1 to 7) are indicated with bars, directly below the scheme; the total thymocyte population is depicted as fraction 8. B) Southern blot analysis of filters containing *Eco*RI digested DNA hybridized with the δRec probe TCRDRE. C) EMSA of DNA probe TCRAPJ-C1 with crude nuclear extracts from thymocyte subpopulations. Numbering of the lanes in B and C is according to the subpopulations numbering outlined in A. ND, not determined.



*Eco*RI; TCRDRE probe

TCRAPJ-C1 probe

δRec-ψJα rearranged band was also present in de $CD69^+/CD27^+$ subpopulation (fraction 7), which contains more mature single positive thymocytes (20). We cannot determine whether this rearranged band represents new δRec-ψJα rearrangements or remaining rearrangements from earlier stages still present in genomic DNA or in circular excision products of ongoing Vα-Jα rearrangements.

Our EMSA experiments using nuclear extracts from the thymocyte subpopulations revealed that PJA-BP is not expressed in the thymocyte subpopulation depleted for $CD8\alpha^+$ cells (fraction 4), but PJA-BP expression could be detected in the thymocyte subpopulation depleted only for $CD8ß^+$ cells (fraction 5; Figure 3C). The thymocyte subpopulation obtained by depletion of $CD69^+$ and $CD27^+$ cells (fraction 6) showed the strongest expression of the PJA-BP. Because the magnetic beads severely hampered the isolation of nuclear extracts, we were not able to isolate nuclear extracts from the $CD69^+CD27^+$ thymocyte fraction (fraction 7).

Our study supports the idea that PJA-BP expression starts simultaneously with the occurrence of preferential δRec-ψJα rearrangements during T cell differentiation. The amount of PJA-BP protein in the nuclear extracts of the thymocyte subpopulations tested seemed to correlate with the amount of δRec-ψJα rearrangements in these subpopulations. These findings provide additional evidence for our hypothesis that PJA-BP is one of the factors involved the preferential recombination of δRec to ψJα.

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CHAPTER 7

T CELL RECEPTOR-δ DELETION IN HUMAN THYMOCYTES

CHAPTER 7

T CELL RECEPTOR-δ DELETION IN HUMAN THYMOCYTES

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INTRODUCTION

T lymphocytes recognize foreign antigens through the action of the heterodimeric T cell receptor (TCR). Although four TCR glycoproteins exist, only two types of heterodimers are usually formed, which consist of TCR-α and TCR-ß chains in most peripheral T lymphocytes, or TCR-γ and TCR-δ chains in 5-15% of the peripheral T lymphocytes. In both heterodimers one of the chains is encoded by a TCR locus with variable (V), diversity (D), and joining (J) gene segments (TCR-ß and TCR-δ), whereas the other chain is encoded by a TCR locus with V and J gene segments (TCR-α and TCR-γ) (1).

It is interesting to see that the Vα and Vδ gene segments are very homologous (2) and moreover that the gene segments of the TCR-α and TCR-δ genes are interspersed on chromosome 14q11 (3). Most of the Vδ gene segments have indeed been found rearranged to either Jδ or Jα gene segments in human thymocytes and/or peripheral T lymphocytes (4-9). Nevertheless, it appears that only three Vδ gene segments from the TCR-α/δ locus are preferentially utilized by TCR-γδ$^+$ T lymphocytes (10). Because the TCR-δ locus is located between the Vα and Jα gene segments (3) (Figure 1), Vα-Jα rearrangements will delete the TCR-δ chain, excluding co-expression of TCR-α and TCR-δ glycoproteins from one allele.

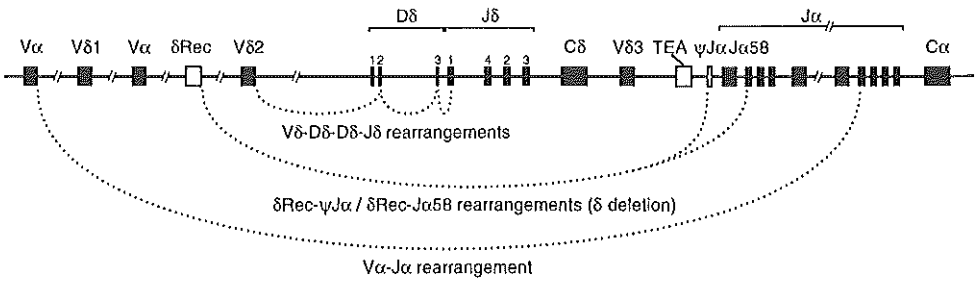

Figure 1. Schematic representation of the human TCR-α/δ locus. The position of the TCR-δ gene within the TCR-α gene complex is indicated as well as the TCR-δ-deleting elements (δRec and ψJα) and the TEA genetic element. The dotted lines indicate the possible consecutive gene rearrangements: Vδ-Dδ-Dδ-Jδ, δRec-ψJα or δRec-Jα58, and Vα-Jα.

In 1988 De Villartay et al. (11) identified the so-called TCR-δ-deleting elements δRec and ψJα. These gene segments flank the major part of the TCR-δ locus (Figure 1). Based on the strong rearranged δRec-ψJα band detected by Southern blot analysis, we and others suggested that most human thymocytes use this rearrangement for specific deletion of the TCR-δ gene to prepare the TCR-α/δ locus for subsequent Vα-Jα rearrangements (11-13 and Chapter 2). Furthermore, we also suggested that there might be a specific TCR-δ gene deletion recombination complex (14). The genomic position of the δRec gene segment remains however peculiar, because only two (Vδ2 and Vδ3) of the six Vδ gene segments are located downstream of the δRec gene segment (Figure 1). Therefore, rearrangements of the other Vδ gene segments will delete the δRec gene segment, thereby excluding subsequent TCR-δ deletion by δRec-ψJα rearrangements.

Several questions remain to be answered, such as: What is deleted by the δRec-ψJα rearrangements?; Is there a specific TCR-δ gene deletion recombination complex?; How are preferential TCR-δ-deleting rearrangements regulated?; and last but not least: Why do we need a specific mechanism for TCR-δ deletion?

WHAT IS DELETED BY THE δRec-ψJα REARRANGEMENT?

Our Southern blot analysis using DNA from thymus cell samples showed that the majority of δRec rearrangements represent δRec-ψJα rearrangements (Chapter 2). To identify the cell stage(s) at which these preferential rearrangements occur, we isolated several thymocyte subpopulations. Preferential δRec-ψJα rearrangements were first observed in the $CD34^-/CD1^+/CD3^-/CD4^+/CD8\alpha^+\beta^-$ thymocyte subset, but most of these rearrangements were found in the double positive ($CD4^+/CD8^+$) thymocyte subset (Figure 2A). This implies that preferential δRec-ψJα rearrangements occur during or after human thymocyte expansion (15, 16), which probably explains the high frequency of δRec-ψJα circular excision products in human thymocytes (Chapter 4).

The TCR-δ configuration in these thymocyte subsets might give some information about what actually is deleted by δRec-ψJα rearrangements. Therefore we performed additional Southern blot analyses using the TCRDJ1 probe (17). We observed only low frequencies of incomplete Dδ2-Dδ3 and Dδ2-Jδ1 rearrangements in the most immature thymocyte cell sample tested, containing $CD34^+/CD1^-/CD3^-/CD4^-/CD8^-$ thymocytes (Figure 2B). In this thymocyte subset most cells have their TCR-δ genes in germline configuration. Complete Vδ1-Jδ1 and Vδ2-Jδ1 rearrangements were observed in the more mature $CD34^+/CD1^+/CD3^-/CD4^-/CD8^-$ thymocyte subset. In this thymocyte subpopulation only a weak germline Jδ1 band was observed. Our Southern blot analyses showed that in the thymocyte subpopulations with preferential δRec-ψJα rearrangements both complete Vδ-Jδ1 and incomplete Dδ2-Dδ3 and Dδ2-Jδ1 rearrangements were detectable, and virtually no germline TCR-δ genes were observed. Those data suggests that δRec-ψJα rearrangements mainly delete rearranged TCR-δ genes instead of germline TCR-δ genes. These observations are in line with studies in mice, demonstrating the occurrence of rearranged TCR-δ genes within

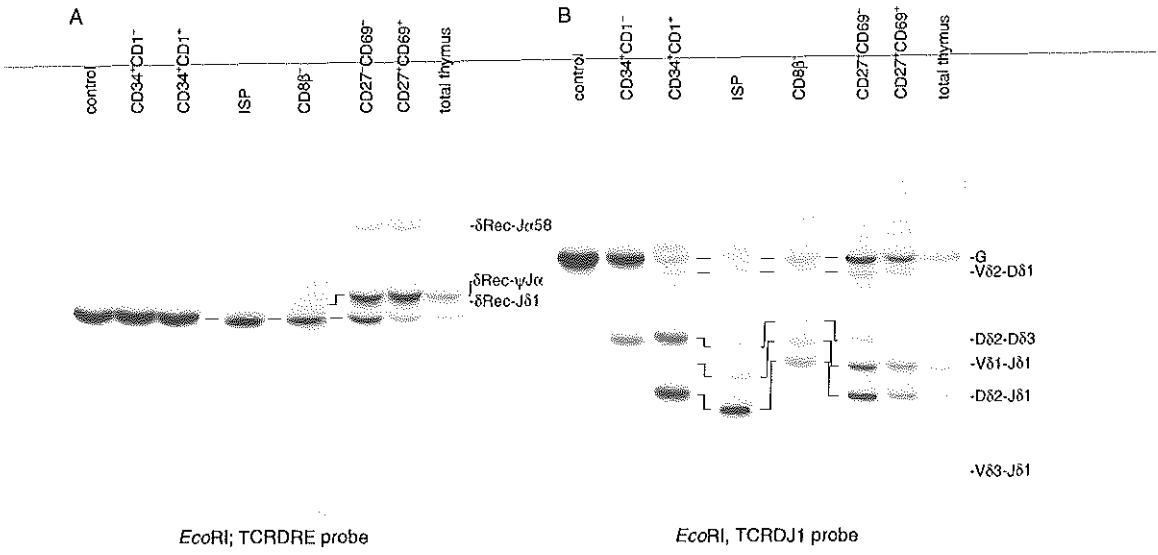

Figure 2. TCR-δ gene rearrangements in human thymocyte subpopulations. Human thymocyte subpopulations were isolated as described in Chapter 6. The $CD34^+/CD1^-$ and $CD34^+/CD1^+$ subpopulations consist of sorted $CD34^+/CD1^-/CD3^-/CD4^-/CD8^-$ and $CD34^+/CD1^+/CD3^-/CD4^-/CD8^-$ thymocytes, respectively. The immature single positive (ISP; $CD34^{\pm}/CD1^-/CD3^-/CD4^+/CD8^-$) fraction was obtained by depletion of total thymocytes with CD8 antibodies and sorting for $CD3^-/CD4^+/CD8^-$ cells. The $CD8\beta^-$ fraction was obtained by depletion of total thymocytes with CD8β antibodies. Therefore the most mature thymocytes in this fraction are $CD34^-/CD1^+/CD3^-/CD4^+/CD8\alpha^+\beta^-$. The $CD27^-/CD69^-$ and $CD27^+/CD69^+$ fractions were obtained by depletion and sorting, respectively, using CD27 and CD69 antibodies. DNA of these subpopulations and DNA from a total thymus cell sample were digested with *Eco*RI and hybridized with the TCRDRE probe (δRec) (**A**) and the TCRDJ1 probe (Jδ1) (**B**). Cell line HeLa was used as a germline control.

circular excision products of Vα-Jα rearrangements (4).

Additional analyses showed only minor differences in the signals of the rearranged bands between the initial cell stages with δRec-ψJα rearrangements and the more mature $CD69^+/CD27^+$ cell stage. This presence of TCR-δ rearrangements in the $CD69^+/CD27^+$ thymocyte subpopulation suggests that $TCR^-/CD3^-$ thymocytes committed to the TCR-αβ lineage and $TCR\text{-}\alpha\beta^+/CD3^+$ thymocytes indeed contain some remaining rearranged TCR-δ genes. This supports the stochastic model for thymocyte differentiation, in which thymocytes first attempt to rearrange TCR-γ and TCR-δ genes, and only attempt further TCR-α and TCR-β gene rearrangements if the former fail to successfully produce a TCR-γδ receptor (Chapter 1). Our data are also in line with previous studies on murine thymocytes (18-21).

A more detailed analysis of our thymocyte subsets is hampered by the presence of thymocytes from several differentiation stages in most of the subsets (Figure 2 and Chapter 6). Therefore we tried to identify clonal T cell populations with continuous activity of the δRec-ψJα rearrangement process. We identified two $CD3^-CD4^+CD8^+$ T cell acute lymphoblastic leukemia (ALL) patients with ongoing δRec-ψJα rearrangements (Chapter 4). In these patients both TCR-δ alleles contained complete Vδ-Jδ rearrangements. It has to be notified that only complete Vδ2-Jδ or

Vδ3-Jδ rearrangements can be deleted by a δRec-ψJα rearrangement (Figure 1). Indeed, the alleles containing the Vδ2-Jδ1 rearrangements were actively deleted in these patients. We also identified a T cell line (DND41) with ongoing δRec-ψJα rearrangements (Chapter 4). In this cell line, the allele with an unidentified rearrangement (or deletion) of a "gene segment" 5.2 kilobase pairs downstream of the Vδ2 gene segment to the Jδ1 gene segment is actively deleted by the δRec-ψJα rearrangement. Based on the combined thymocyte and T-ALL studies, we hypothesize that the δRec-ψJα rearrangement mainly deletes complete and sometimes incomplete TCR-δ rearrangements according to a stochastic model of T cell differentiation.

IS THERE A SPECIFIC TCR-δ GENE DELETION RECOMBINATION COMPLEX?

Our studies on the so-called *tal*-1 deletions in T-ALL revealed that they only occur in T-ALL with one or preferably two deleted TCR-δ genes, especially TCR-$\alpha\beta^+$ T-ALL, but that *tal*-1 deletions are absent in T-ALL without TCR-δ deletion, especially TCR-$\gamma\delta^+$ T-ALL (14). To explain the absence of *tal*-1 deletions in T-ALL without TCR-δ gene deletion, we postulated the existence of a special recombinase complex for TCR-δ deletion and/or TCR-α gene rearrangement, which is exclusively present in immature thymocytes committed to the TCR-αβ lineage (14).

The T cell line DND41 further supports the presence of a specific TCR-δ deletion mechanism. This cell line contains a productive TCR-ß and TCR-δ rearrangement and two out-of-frame TCR-γ genes. DND41 cells express the "illegitimate" TCR-ßδ heterodimer on its cell membrane (22, 23). We showed that this cell line exhibits continuous δRec-ψJα rearrangements (Chapter 4). The DND41 cells contain an in-frame Vδ1-Jδ1 rearrangement on one allele, which cannot be deleted by δRec-ψJα rearrangements. Therefore the deletions involve the other, non-productive allele (see above). Interestingly, the two T-ALL patients with continuous TCR-δ deletion activity both contain two out-of-frame TCR-δ alleles. These results suggest that a non-productive rearrangement on both alleles of either TCR-δ (T-ALL patients) and/or TCR-γ (DND41 cell line) induce TCR-δ gene deletion.

In order to further investigate the TCR-δ gene deletion mechanism, we performed extensive Southern blot analysis (Chapter 2) and polymerase chain reaction (PCR) analysis (Chapters 2, 3, and 4) on DNA from human thymocytes and DND41 cells. With our Southern blot analysis we were able to identify two types of preferential rearrangements of the δRec gene segment in human thymocytes; i.e. to Dδ and Jδ and to Jα. δRec rearrangements to Dδ and Jδ gene segments appeared to be Vδ-Jδ like, which parallel comparable preferential TCR-δ rearrangements (Vδ-Jδ) during human ontogeny (Chapter 2). These δRec rearrangements do not delete the TCR-δ gene and probably just represent side effects of the preferential TCR-δ rearrangement processes that occur during the same developmental stages. The preferential δRec to Jα rearrangements mainly represented δRec-ψJα and the newly identified δRec-Jα58 rearrangements, which are Vα-Jα like rearrangements (i.e. without involvement of Dδ gene segments), that indeed delete the TCR-δ genes.

In addition to the preferential δRec and Jα rearrangements, we also identified

TCR-δ deleting Vδ-Jα rearrangements by use of PCR analysis. The PCR technique is much more sensitive than Southern blot analysis, which probably explains that many Vδ-Jα primer sets showed a positive signal (Chapter 3). We and others observed an interesting phenomenon: the Vδ1 gene segment can rearrange to almost all Jα gene segments, whereas the Vδ2, δRec and Vδ3 gene segments only rearrange to the most 5′ Jα gene segments (5, 7, 8, 24; Chapters 2 and 3). The patterns observed in these TCR-δ-deleting rearrangements support the hypothesis that the distance between (or proximity of) the gene segments influences the choice of the gene segments in V(D)J recombination processes, as suggested previously from Vα-Jα rearrangement patterns in immature murine thymocytes (25-27).

To further investigate the possible relationship between "distance" and V(D)J recombination, we also performed PCR studies on rearrangements of the Vδ4, Vδ5, and Vδ6 gene segments to the Jα gene segments. As shown in Table I, there is an inverse correlation between "distance" and the usage of Jα gene segments. The Vδ gene segments proximal to the Jα region mainly rearrange to the most 5′ Jα gene segments, whereas the Vδ gene segments more distal of the Jα region rearrange to both upstream and downstream Jα gene segments. It might therefore be that rearrangements of distal Vδ gene segments to downstream Jα gene segments in fact replace preceding rearrangements of Vδ gene segments to upstream Jα gene segments (Figure 1).

TABLE I. PCR analysis of coding joints of Vδ rearrangements to the Jα gene segments[a]

	5′ gene segment[b]						
3′ gene segment[c]	Vδ4	Vδ6	Vδ1	Vδ5	δRec	Vδ2	Vδ3
ψJα	+	±	++	+	++	+	+
Jα60	±	−	++	+	+	+	−
Jα59	±	+	±	++	+	+	−
Jα58	++	+	+	++	++	+	+
Jα57	+	+	++	++	+	+	−
Jα53	++	++	++	+	+	+	−
Jα49	+	+	++	++	±	+	−
Jα43	+	+	+	++	±	±	−
Jα39	+	+	++	+	−	−	−
Jα36	++	+	++	++	−	−	−
Jα33	+	±	++	+	+	−	−
Jα27	+	±	++	−	±	−	−
Jα21	+	+	+	−	−	−	−
Jα14	−	−	−	−	−	−	−
Jα8	+	±	+	−	−	−	−
Jα4	+	−	±	−	−	−	−

a. Density of the PCR product was determined semi-quantitatively as previously described (47), and the following symbols were given to the measured values: ++; values > 1000; +, values between 300 and 1000; ±, values between 100 and 300; −, values < 100.

b. The order of the Vδ and δRec gene segments in this table correspond to the order in the genome. The Vδ3 gene segment is located proximal to the Jα region and Vδ4 is the most distal gene segment (Figure 3).

c. The order of the Jα gene segments in this table correspond to the order in the genome. The ψJα gene segment is the most 5′ Jα gene segment and Jα4 is the most 3′ gene segment used.

The presence of preferential δRec-ψJα and δRec-Jα58 TCR-δ-deleting rearrangements, in combination with the absence of preferential Vδ2-ψJα and Vδ2-Jα58 rearrangements suggests that besides "distance" additional factors play an important role in TCR-δ gene deletion process. Based on our combined Southern blot and PCR analyses, we will now elaborate a hypothetical scheme of rearrangement processes during T cell differentiation, which includes the presence of a specific TCR-δ gene deletion recombination complex. This hypothetical scheme consists of three stages (Figure 3):

Stage 1: TCR-δ rearrangements. In this initial stage the gene segments of the TCR-δ locus start to rearrange. There is evidence for sequential TCR-δ rearrangement pathways in the thymus during human ontogeny (28-31). As discussed above, our Southern blot analyses, using thymocyte subsets from postnatal thymi, showed that incomplete Dδ2-Dδ3 and Dδ2-Jδ1 rearrangements and complete Vδ2-Jδ1 rearrangements precede preferential δRec-ψJα rearrangements.

Stage 2: Deletion of the TCR-δ gene. Initiation of this intermediate stage is probably a consequence of the presence of two non-functional TCR-δ and/or TCR-γ chains as discussed above. Nevertheless, we cannot fully exclude that germline TCR-δ genes and/or incomplete TCR-δ rearrangements also can give rise to signals that initiate the TCR-δ deletion mechanism. The most prominent TCR-δ-deleting rearrangements are δRec-ψJα and δRec-Jα58. Activation of these rearrangements is probably initiated by several factors. Based on transgenic mice studies, it has recently been proposed that there is a regulatory sequence near the human δRec gene segment which is recognized by putative regulatory proteins and induces δRec rearrangements preferably in the TCR-αβ T cell lineage (32). Furthermore, transcription of the T-early-α (TEA) element is assumed to be responsible for opening the TCR-Jα locus to the V(D)J recombinase complex (33, 34). As discussed below, additional factors are probably necessary for regulating the observed preferential TCR-δ-deleting rearrangements.

Besides the δRec gene segment, other Vδ and may be some Vα gene segments can rearrange to the TCR-Jα locus at this stage (Chapters 2 and 3, Table I). These alternative TCR-δ-deleting rearrangements are not detectable by Southern blot analysis, indicating that they occur at low frequency. Based on the rearrangement patterns of Vδ3, Vδ2, and δRec in human thymocytes (Chapters 2 and 3), we now hypothesize that TEA expression only activates the most upstream Jα gene segments, in order to preserve maximum potential TCR-α diversity for subsequent Vα-Jα rearrangements.

We also studied the rearrangement patterns of Vδ-Jα and δRec-Jα in the T cell line DND41, which contains an active TCR-δ-deleting mechanism without detectability of, functional, ongoing Vα-Jα rearrangements. In this cell line, rearrangements of both δRec and Vδ3 gene segments to the Jα gene segments are comparable to those observed in thymocytes. However, we observed a difference in Jα gene segment usage for rearrangements of the Vδ1 gene segment. In DND41 cells, which expresses the TEA transcript, only rearrangements to the most upstream Jα gene segments were observed, whereas in normal thymocytes the Vδ1 gene segment can rearrange to nearly all Jα gene segments. This supports our hypothesis that the TCR-δ deletion mechanism, present in DND41 cells, is restricted to 5′ Jα gene

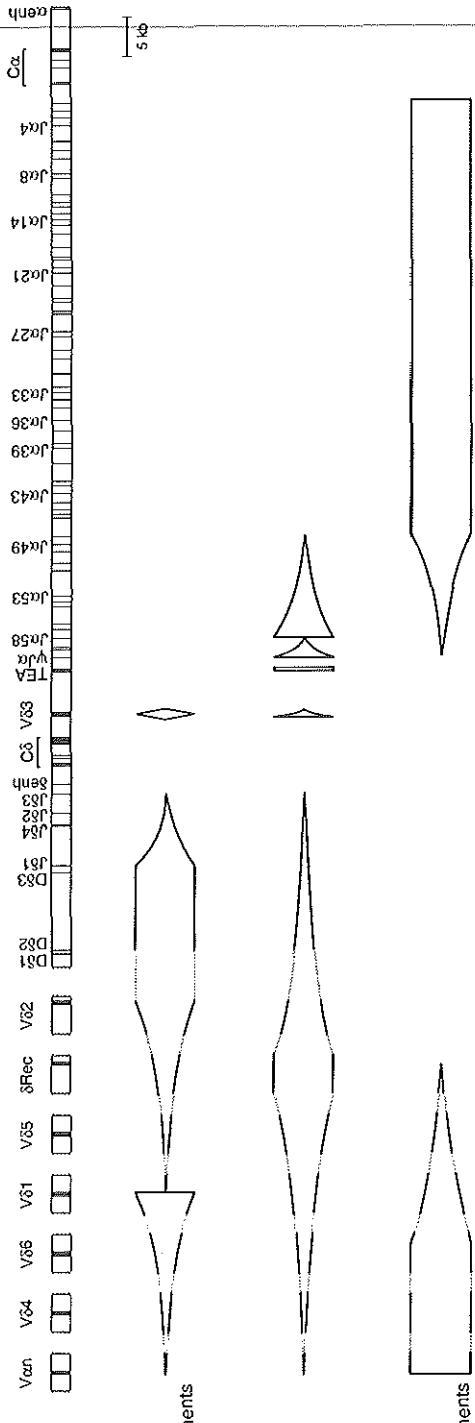

Figure 3. Hypothetical scheme of TCR-δ gene deletion in human T cell differentiation. The top of the figure shows a schematic representation of the TCR-α/δ locus. The three stages for: 1, TCR-δ rearrangement; 2, TCR-δ deletion; and 3, TCR-α rearrangement are indicated on the left. The horizontal bars under the TCR-α/δ locus represent the gene regions which can be involved in rearrangement processes during that particular stage. The size of the bar (height) represents the preference for the rearrangement process. See text for detailed explanation.

segments by the action of the TEA element. It also provides additional evidence that Vδ1 rearrangements to downstream Jα gene segments in human thymocytes probably are the result of subsequent Vα/δ-Jα rearrangements (see Stage 3).

Based on the very low frequency of δRec and ψJα rearrangements in T-ALL cells and T cell lines (Chapter 2), we hypothesize that rearranged TCR-δ-deleting elements exist for only an extremely short period during thymocyte differentiation; i.e. they are rapidly replaced by subsequent Vα-Jα rearrangements.

Stage 3: Ongoing TCR-α rearrangements. The rearrangements induced by the TCR-δ deletion mechanism will automatically delete the TEA element. Deletion of TEA probably activates the downstream part of the TCR-Jα locus, by making it accessible for V(D)J recombination. The loss of restriction of Jα gene segment usage is indeed observed. Vδ and Vα gene segments upstream of the δRec gene segment can now easily rearrange to both upstream and downstream Jα gene segments (Table I) (25-27). Thereby, the Vδ gene segments have actually become Vα gene segments. Indeed, functional rearrangements of these upstream Vδ gene segments to Jα gene segments are found, and the produced Vδ-Cα proteins can be expressed on the surface of the membrane together with TCR-ß proteins (2, 4, 5). To obtain additional evidence for ongoing TCR-α rearrangements, it might be interesting to further investigate the occurrence of Vα-Jα rearrangements and TCR-α protein chains in minor subclones from the DND41 cell line, if such subclones occur at all in this cell line.

Our hypothesis of a three-stage rearrangement process during T cell differentiation implies that rearrangement processes in the TCR-α/δ locus represent in fact a regulated cascade of replacement rearrangements, until a functional TCR heterodimer molecule is produced.

HOW ARE PREFERENTIAL TCR-δ-DELETING REARRANGEMENTS REGULATED?

Little is known about the regulation of (preferential) rearrangements in both T and B cells, but different factors have been proposed to play a role as reviewed previously (1). The most prominent TCR-δ-deleting rearrangement in human thymocytes is the δRec-ψJα rearrangement. As discussed above, we have obtained experimental evidence that this rearrangement is regulated by a specific TCR-δ gene deletion recombination complex. Two factors have been described that might play a role in regulating the δRec-ψJα rearrangement: a sequence near the δRec gene segment (32), and the expression of TEA (33, 34). If expression of TEA is important for regulating the δRec-ψJα rearrangement by opening the 5´ part of the TCR-Jα locus, why are not all Jα gene segments immediately downstream of the ψJα gene segment preferentially used?

We assume that specific sequences, representing binding sites for DNA-binding proteins, might be involved in regulating the preferential δRec-ψJα rearrangement. In order to find such regulatory sequences, we searched for homologous sequences in and around the two gene segments. We observed the same sequence of seven nucleotides in the spacer of the recombination signal sequences (RSS) of the δRec and

ψJα gene segments (Figure 4). RSS consist of conserved palindromic heptamer and nonamer sequences, separated by a non-conserved spacer region of 12 or 23 base pairs (bp). The presence of RSS is essential for both TCR and immunoglobulin V(D)J recombination (1, 35), but the exact function of the spacer has not been determined yet, although it is known that the length of the spacer plays an important role (36). The homologous sequence in the spacer region of the δRec and ψJα gene segments is conserved between man and mice (12), except for one bp which is the same in the murine δRec and ψJα gene segments (Figure 4). Besides the δRec and ψJα gene segments, other human and murine Vδ gene segments were observed to contain the homologous sequence (Vδ3 and Vδ6, Figure 4). However, no preferential Vδ3-ψJα or Vδ6-ψJα were detected using Southern blot analysis of human thymocytes (Chapter 2). Interestingly, the most common Vδ gene segments in T cell development (Vδ1 and Vδ2) do not contain this sequence. We tried to identify DNA-binding proteins that bind to these spacer sequences, but we were not able to detect such T cell specific DNA-binding proteins (Verschuren, unpublished results). However, this does not mean that these sequences do not play a regulatory role in δRec-ψJα rearrangement. More information about the function of these spacer sequences in preferential δRec-ψJα rearrangements can be obtained by investigation of rearrangement processes in plasmid constructs with mutated RSS which are introduced into the T cell line DND41.

We then performed additional studies to identify DNA-binding proteins recognizing sequences upstream or downstream of the ψJα gene segment. We detected a DNA-binding protein of approximately 180 kilodaltons, the so-called PJA-BP, in nuclear extracts from human thymocytes (Chapter 5) and murine thymocytes (Chapter 6), which recognizes a 46 bp sequence containing a TAAT/ATTA core mo-

gene segment	RSS[a]	SPACER[b]	RSS[a]
human			
δRec	7	ATGCATAGGCACCTCGACCCCGT	9
ψJα	7	G-G-------TT	9
Vδ3	9	TATGAC-T-C-G-------GC-T	7
Vδ6	7	C-C-CC--------GC-G--T--	9
murine			
δRec	7	G----C-*A*------GC----T--	9
ψJα	7	G-G*A*------TT-	9
Vδ5[c]	9	ACAGGC-C-C-G-------GC--	7
Vα2/Vδ8[c]	7	C-CTCC--------GT-G---A-	9

Figure 4. Sequence homology between the spacers of Vα/δ RSS. a. Heptamer (7) and nonamer (9) RSS are indicated to show the genomic orientation of the sequences. b. Spacer region of RSS. The mutations of the murine δRec and ψJα gene segments in the consensus GGCACCT sequence are given in italics. −, identical nucleotides after alignment. c. The murine Vδ5 gene segment is homologous to the human Vδ3 gene segment and the murine Vα2/Vδ8 gene segment is homologous to the human Vδ6 gene segment (48).

tif within the ψJα gene segment. The expression pattern of this protein in human continuous cell lines and malignancies suggests that this protein is probably thymocyte-specific and might be restricted to T cells of the TCR-αß lineage (Chapters 5 and 6). Furthermore, additional evidence for a role of this protein in the preferential δRec-ψJα rearrangements was obtained by the observation that PJA-BP expression and preferential δRec-ψJα rearrangements occur simultaneously in the same immature human thymocyte subpopulations (Chapter 6). Although we have to be careful to extrapolate these experiments to the *in vivo* function of this protein, we postulate that this protein might play an important role in the preferential δRec-ψJα rearrangement. Purification and cloning of PJA-BP should be performed in order to further investigate the role of PJA-BP in δRec-ψJα rearrangements.

During thymocyte development the δRec-Jα58 rearrangement appeared to be the second most important TCR-δ-deleting rearrangement (Chapter 2). The frequency of this rearrangement is three times less than the δRec-ψJα rearrangement based on our semi-quantitative Southern blot analysis (Chapter 2). The finding of this preferential rearrangement is in contradiction with the above mentioned function of the PJA-BP, because the Jα58 gene segment does not contain the TAAT/ATTA core domain necessary for PJA-BP binding (Chapter 2). If this gene segment is only used preferentially because of its close distance to the TEA gene segment (or the ψJα gene segment), why are the interjacent gene segments (Jα60 and Jα59) not preferentially used? We therefore looked for additional homologous sequences between the ψJα and Jα58 gene segments, which might represent binding sites for DNA-binding proteins. We observed a sequence immediately upstream of the RSS nonamer of the Jα58 gene segment which was homologous in 13 out of 14 bp with a sequence 27 bp upstream of the RSS nonamer of the ψJα gene segment (Figure 5). Comparable sequences were not observed in other Jα gene segments or in the δRec gene segment. Therefore, it would be very interesting to see whether there are DNA-binding proteins in human thymocytes that bind to this sequence!

We think that several factors are involved in regulating the preferential TCR-δ-deleting rearrangements, including regulatory sequences in or near the TCR-δ-deleting elements, expression of the PJA-BP, and transcription of TEA. Furthermore, the TCR-δ and TCR-α enhancers (37, 38) and the previously identified locus control region in the murine TCR-α/δ locus (39) undoubtedly can also play an important role. The T cell line DND41 will probably give us a good opportunity to further isolate and study the regulating factors of the human TCR-δ deletion mechanism.

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ψJα gene segment      AAGGTGATGCCACATCCCTTTCAACCATGCTGACACCTCTGGTTTTTGT
Jα58 gene segment     ---C----------.....--------C
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Figure 5. Homology between the ψJα and Jα58 gene segments. The nonamers of the RSS are depicted in bold. –, identical nucleotides after alignment; ., gap for alignment.

WHY DO WE NEED A SPECIFIC MECHANISM FOR TCR-δ DELETION?

One of the remaining issues that has to be elucidated is the need for a specific mechanism for TCR-δ deletion. This topic is especially intriguing because of the

peculiar position of the δRec gene segment in the genome, between the frequently used Vδ1 and Vδ2 gene segments. One of the explanations is that the TCR-δ deletion mechanism deletes some regulatory sequences that, if present, inhibit Vα-Jα rearrangements. Another less likely possibility is that TCR-δ deletion just brings the Vα gene segments proximal to the Jα gene segments for recombination.

We showed that most of the TCR-δ-deleting rearrangements are Vα-Jα like rearrangements, because of the lack of Dδ gene segments (Chapter 2). The Vδ2 gene segment seems to be the exception to this rule. PCR analysis with DNA from human thymocytes showed that Vδ2-ψJα and Vδ2-Jα58 rearrangements preferably contain Dδ gene segments, indicating that these rearrangements resemble Vδ-Jδ rearrangements (Chapter 2). Even more intriguing is the observation that DND41 cells lack ongoing Vδ2-Jα rearrangements (Chapter 4). The Vδ2 gene segment is still present on one allele in this cell line, but the Dδ gene segments are either rearranged or deleted on this allele. Based on these data we assume that the Vδ2 gene segment is the only Vδ gene segment that in principle has to rearrange via Dδ gene segments. Therefore we hypothesize that Vδ2 is the only true Vδ gene segment.

This is further supported by the observations of Giachino et al. (7), who showed that Vδ2-Jα-Cα transcripts are present in human thymocytes, but that they are completely absent in the periphery. They suggested that Vδ2-Cα chains might not be selected to form heterodimers with the TCR-ß chain. Previous studies have already shown the occurrence of the other Vδ-Cα transcripts in peripheral T lymphocytes and thymocytes (5, 6, 8, 24), which makes the Vδ2 gene segment a special gene segment.

Cross-lineage Vδ2-Dδ3 rearrangements are frequently observed in precursor-B-ALL (17, 40-43) and normal B cells (44), whereas cross-lineage rearrangements of the other Vδ gene segments are rare or absent (43, 45). Furthermore, it was shown that Vδ2-Dδ3-Jα rearrangements occurred in precursor-B-ALL, whereas Vδ2-Dδ-Jδ rearrangements were not observed (17, 42-44, 46). The absence of δRec-ψJα and Vα-Jα rearrangements in precursor-B-ALL (17) might be explained by the absence of factors involved in the TCR-δ-deleting mechanism like PJA-BP (Chapter 6).

The before mentioned data support the idea that the Vδ2 gene segment is a special gene segment, which might be regulated in a different way compared to the other Vδ gene segments. Because the Vδ2 gene segment is located downstream of the δRec gene segment, it might be that the δRec-ψJα rearrangement is especially suitable for deleting the Vδ2 gene segment or its nearby located TCR-δ gene regulating sequences. In that case, the observed preferential δRec-Dδ and δRec-Jδ rearrangements during human ontogeny (Chapter 2) can no longer be regarded as "side effects" of the preferential TCR-δ rearrangements processes that occur during the same developmental stages, as proposed above. In this point of view these rearrangements represent "normal TCR-δ-deleting rearrangements" which can be followed by subsequent Vα-Jα rearrangements.

Rearrangement of the Vδ1 gene segment, which is preferentially used in postnatal thymocytes (28, 31), automatically deletes the Vδ2 gene segment and the δRec gene segment. If deletion of the Vδ2 gene segment and its regulating sequences is sufficient to overcome inhibition of Vα-Jα rearrangements, then ongoing Vα-Jα rearrangements should occur after Vδ1 rearrangement. In this case the specific TCR-δ-deletion rearrangement is not needed anymore, which makes the function of TEA elusive. The

TEA might not be expressed or rearrangements of gene segments upstream of Vδ1 are still influenced by TEA, and therefore still rearrange to the most 5′ Jα gene segments. The latter might explain the presence of small amounts of PCR products of Vδ4 and Vδ6 to the most upstream Jα gene segments in human thymocytes (Table I).

The TCR-δ deletion mechanisms of human and mouse seem to differ (12, 32). Therefore studies using transgenic or knock-out mice will probably resolve only a part of the function of TCR-δ deletion. The DND41 T cell line probably will give us the opportunity to further investigate the TCR-δ deletion mechanism. It will be a challenge to investigate the deletion of certain sequences near the Vδ2 gene segment or deletion of the TEA gene segment, which might be accomplished by applying homologous recombination to DND41 cells.

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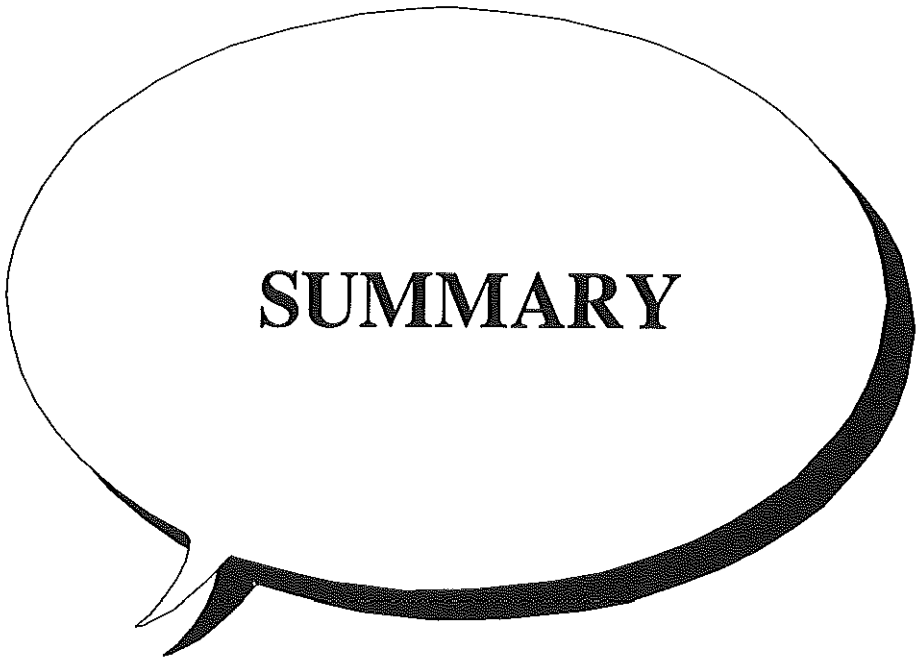

SUMMARY

SUMMARY

B and T lymphocytes are responsible for the specific immunity that protects the human body against infectious agents. These lymphocytes recognize foreign material (antigens) via specific surface receptor molecules, the so-called antigen-specific receptors. The specific recognition of antigens by T lymphocytes is mediated by the membrane bound T cell receptor (TCR), a heterodimer molecule composed of either TCR-α and TCR-ß or TCR-γ and TCR-δ protein chains. The four protein chains are encoded by four different TCR genes. These TCR genes contain sets of variable (V), diversity (D), and joining (J) gene segments in case of TCR-β and TCR-δ genes and sets of V and J gene segments in case of TCR-α and TCR-γ genes. The gene segments are joined (the so-called coding joint) via gene rearrangement processes. The interjacent DNA sequences are deleted as circular excision products, which contain the so-called signal joints. The large series of gene segments and the gene rearrangement process itself account for an enormous diversity of TCR-$\alpha\beta$ and TCR-$\gamma\delta$ molecules, with the potential of recognizing a very large number of foreign antigens.

Both TCR-$\alpha\beta^+$ and TCR-$\gamma\delta^+$ T lymphocytes are thought to be derived from a common precursor cell which differentiates in the thymus to become a mature T lymphocyte. The regulation mechanisms that force thymocytes into the TCR-αß or TCR-$\gamma\delta$ differentiation lineage are poorly understood, but rearrangement processes in the TCR-α/δ locus are likely to play an important role because of the special location of the TCR-δ gene segments between the Vα and Jα gene segments. The major part of the TCR-δ gene complex is flanked by the so-called TCR-δ-deleting elements, δRec and ψJα. Rearrangement of δRec to ψJα is assumed to delete the TCR-δ gene prior to Vα-Jα rearrangement. The non-productive δRec-ψJα recombination occurs at high frequency in both fetal and postnatal immature thymocytes, but is also detectable at low levels in peripheral blood mononuclear cells.

The aim of this thesis was to gain more insight in the mechanisms of TCR-δ deletion through rearrangement of the TCR-δ-deleting elements. We therefore performed studies to characterize which gene segments are involved in preferential rearrangements that delete the TCR-δ gene (**Chapters 2, 3, and 4**). Furthermore, we attempted to identify DNA-binding proteins that might be involved in the process of TCR-δ gene deletion (**Chapters 5 and 6**).

Based on our Southern blot analyses we identified two prominent TCR-δ-deleting δRec rearrangements: δRec-ψJα and δRec-Jα58 (**Chapter 2**). The δRec-ψJα rearrangement represents approximately 70% of all δRec rearrangements. The δRec-Jα58 rearrangement appeared to be the second most important δRec rearrangement in human thymocytes ($\sim$25% of the detected δRec rearrangements), which makes the Jα58 gene segment an important new TCR-δ-deleting element. The high frequency of these rearrangements during human ontogeny suggests that most human thymocytes use these rearrangements to further differentiate into the TCR-αß lineage. Based on the very low frequency of δRec, ψJα, and Jα58 rearrange-

ments in 4% of the T cell acute lymphoblastic leukemia patients (n=151) and 6% of the T cell lines (n=26), we hypothesize that rearranged TCR-δ-deleting elements exist for only an extremely short period during thymocyte differentiation, probably due to rapid subsequent Vα-Jα rearrangements.

The δRec gene segment has no sequence homology to Vδ or Vα gene segments. In order to compare rearrangements of the δRec gene segment with those of Vδ gene segments, we searched for similarities in rearrangement patterns of these gene segments (**Chapters 2 and 3**). Polymerase chain reaction analyses of δRec-ψJα, Vδ-ψJα, δRec-Jα58, and Vδ-Jα58 coding and signal joints revealed that except for Vδ2 rearrangements all other TCR-δ-deleting rearrangements are homologous to Vα-Jα rearrangements, because Dδ gene segments are absent in their junctional regions. Furthermore, we showed that the δRec, Vδ2, and Vδ3 gene segments rearrange only to the upstream Jα gene segments, whereas the Vδ1 gene segment can rearrange to virtually all Jα gene segments tested. δRec and Vδ1 rearrange more easily to Jα gene segments than to Dδ and Jδ gene segments. On the other hand, the Vδ2 gene segment rearranges more easily to Dδ and Jδ gene segments than to Jα gene segments. Our study suggests that rearrangement of the δRec gene segment can play an intermediate role between consecutive rearrangements of Vδ2 (the only true Vδ gene segment) and Vδ1 gene segments.

The T cell line DND41 and several T cell acute lymphoblastic leukemias were found to exhibit continuous activity of the TCR-δ deletion proces (**Chapter 4**) and therefore can serve as an experimental model to further study the process of TCR-δ deletion. Based on this study we suggest that TCR-δ deletion is an intermediate phase between consecutive TCR-δ rearrangements and TCR-α rearrangements.

Little is known about the regulation mechanisms of TCR-δ deletion. Although we showed that the δRec and ψJα gene segments can rearrange to different gene segments, they preferentially rearrange to each other. We therefore attempted to find cis-acting factors that recognize these gene segments (**Chapter 5**). We were able to detect a DNA binding protein of approximately 180 kDa (the so-called PJA-BP) in nuclear extracts from human thymocytes, that recognizes a sequence of 46 base pairs containing a TAAT core motif within the ψJα gene segment. Using a large series of human hematopoietic cell lines (n=19) (**Chapter 5**) and human hematopoietic malignancies (n=30) (**Chapter 6**), we showed that expression of the PJA-BP is probably thymocyte specific and might be restricted to thymocytes of the TCR-αß lineage.

Our electrophoretic mobility shift assays with a series of seven well-defined human thymocyte subpopulations (**Chapter 6**) showed that PJA-BP expression starts from the immature $CD34^-/CD1^+/CD3^-/CD4^+/CD8\alpha^+\beta^-$ thymocyte cell stage onwards. Southern blot analyses using these thymocyte subpopulations showed that preferential δRec-ψJα rearrangements in human thymocytes start simultaneously with the expression of the PJA-BP. We conclude from these studies that the PJA-BP is probably one of the factors involved in the preferential recombination of δRec to ψJα.

In conclusion, we can say that the work in this thesis provides new insight into the process of TCR-δ deletion in human thymocytes. The rearrangement patterns of the δRec and Vδ gene segments gave us the opportunity to propose a

new hypothetical model for TCR-δ deletion during T cell differentiation (**Chapter 7**). In this model, TCR-δ deletion is activated by a specific TCR-δ gene deleting recombinase complex which preferentially uses the TCR-δ-deleting elements δRec and ψJα or Jα58. In addition, other gene segments (Vδ gene segments and most Jα gene segments) can be used at low frequency for TCR-δ deletion. Rearrangement of the latter gene segments is probably influenced by several factors, including transcription of the T early alpha element and distance between the rearranging gene segments, in order to restrict TCR-δ deletion rearrangements to the most upstream Jα gene segments. This allows a large set of downstream Jα gene segments to be used for subsequent Vα-Jα rearrangements, in order to preserve maximal potential TCR-α diversity. The identification of a possible regulatory protein for preferential TCR-δ deletion rearrangements and the DND41 cell line with continuous TCR-δ-deletion recombinase activity give us new opportunities to further investigate the mechanisms for TCR-δ deletion.

SAMENVATTING

B- en T-lymfocyten zorgen voor de specifieke immuniteit die het lichaam beschermt tegen ziekteverwekkers. Lymfocyten herkennen lichaamsvreemde stoffen (antigenen) door middel van specifieke oppervlakte receptoren, de zogenoemde antigeen-specifieke receptoren. In geval van T-lymfocyten is de antigeen-specifieke receptor een membraan gebonden T-celreceptor (TCR), een molecule dat bestaat uit twee verschillende eiwit ketens, nl. TCR-α en TCR-β of TCR-γ en TCR-δ. De vier eiwitketens worden gecodeerd door vier verschillende TCR genen. Deze TCR genen bevatten een aantal "variable" (V), "diversity" (D) en "joining" (J) gensegmenten in geval van TCR-β of TCR-δ genen, en een aantal V en J gensegmenten in het geval van TCR-α en TCR-γ genen. De gensegmenten worden aan elkaar gekoppeld (de zogenaamde "coding joint") door middel van genherschikkingsprocessen. De tussenliggende DNA sequenties worden verwijderd (gedeleteerd) als cirkelvormige produkten, die de zogenaamde "signal joints" bevatten. Het grote aantal gensegmenten en het genherschikkingsproces zelf zorgen voor een enorme diversiteit aan TCR-$\alpha\beta$ en TCR-$\gamma\delta$ moleculen, die het vermogen hebben om een zeer groot aantal lichaamsvreemde antigenen te herkennen.

Er wordt gedacht dat zowel TCR-$\alpha\beta^+$ als TCR-$\gamma\delta^+$ T-lymfocyten afkomstig zijn van één voorlopercel die in de thymus differentieert tot een rijpe T-lymfocyt. Het is niet bekend welke regulatiemechanismen ervoor zorgen dat de thymocyten in de TCR-αß of TCR-$\gamma\delta$ differentiatielijn terecht komen, maar genherschikkings-processen in het TCR-α/δ locus spelen waarschijnlijk een belangrijke rol vanwege de speciale positie van de TCR-δ gensegmenten tussen de Vα en Jα gensegmenten. Het grootste gedeelte van het TCR-δ gencomplex wordt geflankeerd door de zogenoemde TCR-δ-deleterende elementen, δRec en ψJα. Er wordt aangenomen dat herschikking van δRec naar ψJα het TCR-δ gen deleteert voordat Vα-Jα genherschikkingen optreden. De niet-produktieve δRec-ψJα herschikking vindt in hoge frequentie plaats in zowel foetale als postnatale onrijpe thymocyten. Kleine hoeveelheden van deze herschikte genen worden echter ook waargenomen in de mononucleaire celfraktie van het perifere bloed.

Het doel van dit promotie-onderzoek was het verkrijgen van meer inzicht in het mechanisme van TCR-δ deletie door de TCR-δ-deleterende elementen. Daartoe werd onderzocht welke gensegmenten betrokken zijn bij preferentiële genherschikkingen die het TCR-δ gen deleteren (**Hoofdstuk 2, 3 en 4**). Verder hebben we getracht om DNA-bindende eiwitten te identificeren die mogelijk betrokken zijn bij het TCR-δ-deletieproces (**Hoofdstuk 5 en 6**).

Met behulp van onze Southern blot analyse hebben we twee belangrijke TCR-δ deletie genherschikkingen geïdentificeerd: δRec-ψJα and δRec-Jα58 (**Hoofdstuk 2**). De δRec-ψJα genherschikking vertegenwoordigt ongeveer 70% van alle δRec herschikkingen. De δRec-Jα58 genherschikking blijkt de op één na belangrijkste δRec genherschikking te zijn ($\pm$25% van de gedetecteerde δRec genherschikkingen), wat het Jα58 gensegment een nieuw belangrijk TCR-δ-

deleterend element maakt. De hoge frequentie van deze genherschikkingen tijdens de humane ontogenese suggereert dat de meeste humane thymocyten deze genherschikkingen gebruiken om verder te differentiëren in de TCR-$\alpha\beta$ differentiatielijn. Gebaseerd op de zeer lage frequenties van δRec, $\psi J\alpha$ en $J\alpha 58$ genherschikkingen in 4% van de T-cel acute lymphoblastaire leukemieën (n=151) en in 6% van de T-cellijnen (n=26), veronderstellen wij dat herschikte TCR-δ-deleterende elementen gedurende een zeer korte periode aanwezig zijn tijdens de differentiatie van thymocyten, omdat ze waarschijnlijk zeer snel worden vervangen door Vα-Jα genherschikkingen.

Het δRec gensegment heeft geen sequentie homologie met Vδ of Vα gensegmenten. Om genherschikkingen van het δRec gensegment te vergelijken met die van Vδ gensegmenten zochten wij naar overeenkomsten in genherschikkingspatronen tussen deze gensegmenten (**Hoofdstuk 2 en 3**). Polymerase ketting reactie analyse van δRec-$\psi J\alpha$, Vδ-$\psi J\alpha$, δRec-$J\alpha 58$, and Vδ-$J\alpha 58$ "coding" en "signal joints" liet zien dat behalve Vδ2 genherschikkingen alle andere TCR-δ-deleterende genherschikkingen homoloog zijn aan Vα-Jα genherschikkingen, omdat Dδ gensegmenten afwezig zijn in de koppelingsplaatsen tussen deze gensegmenten. Daarnaast hebben we laten zien dat δRec, Vδ2 en Vδ3 gensegmenten alleen naar de dichtstbijzijnde Jα gensegmenten herschikken, terwijl het Vδ1 gensegment kan herschikken naar bijna alle Jα gensegmenten die werden onderzocht. De δRec en Vδ1 gensegmenten herschikken makkelijker naar Jα gensegmenten dan naar Dδ en Jδ gensegmenten. Aan de andere kant herschikt het Vδ2 gensegment makkelijker naar Dδ en Jδ gensegmenten dan naar Jα gensegmenten. Ons onderzoek suggereert dat genherschikkingen van het δRec gensegment een tussenstap vormen tussen genherschikkingen van Vδ2 (het enige echte Vδ gensegment) en Vδ1 gensegmenten.

De T-cellijn DND41 en verschillende T-cel acute lymphoblastaire leukemieën bleken een continue activiteit van het TCR-δ-deletieproces te hebben (**Hoofdstuk 4**). Daarom kunnen ze dienen als een experimenteel model om het TCR-δ-deletieproces verder te bestuderen. Gebaseerd op deze studie hebben we gesuggereerd dat TCR-δ deletie een fase vertegenwoordigd tussen de achtereenvolgende TCR-δ genherschikkingen en TCR-α genherschikkingen.

Er is tot nu toe weinig bekend over het regulatiemechanisme van TCR-δ deletie. Alhoewel we hebben laten zien dat de δRec en $\psi J\alpha$ gensegmenten kunnen herschikken naar verschillende gensegmenten, herschikken ze toch voornamelijk naar elkaar. Daarom hebben we getracht om DNA-bindende eiwitten te vinden die deze gensegmenten herkennen (**Hoofdstuk 5**). We hebben in kernextracten van humane thymocyten een DNA-bindend eiwit gedetecteerd van ongeveer 180 kDa (het zogenaamde PJA-BP), dat een sequentie van 46 basenparen herkent in het $\psi J\alpha$ gensegment. Onderzoek van humane cellijnen (n=19) (**Hoofdstuk 5**) en humane maligniteiten (n=30) (**Hoofdstuk 6**) liet zien dat expressie van PJA-BP waarschijnlijk specifiek voorkomt in thymocyten, en dat het eiwit mogelijk alleen tot expressie komt in thymocyten van de TCR-$\alpha\beta$ differentiatielijn.

Ons electroforese-onderzoek met een serie goed gedefinieerde humane thymocyten subpopulaties (**Hoofdstuk 6**), liet zien dat de expressie van PJA-BP start vanaf het $CD34^-/CD1^+/CD3^-/CD4^+/CD8\alpha^+\beta^-$ thymocyten stadium.

Southern blot analyses met deze thymocyten subpopulaties hebben laten zien dat preferentiële δRec-ψJα genherschikkingen en expressie van PJA-BP tegelijkertijd starten in humane thymocyten. Daarom is het PJA-BP eiwit waarschijnlijk één van de factoren die betrokken is bij de preferentiële genherschikking van δRec naar ψJα.

Concluderend kunnen we stellen dat het onderzoek in dit proefschrift heeft geleid heeft tot nieuwe inzichten in het proces van TCR-δ gendeletie in humane thymocyten. De genherschikkingspatronen van de δRec en Vδ gensegmenten hebben ons de mogelijkheid gegeven een nieuw model voor TCR-δ gendeletie tijdens de T-cel differentiatie op te stellen (**Hoofdstuk 7**). In dit model wordt TCR-δ gendeletie geactiveerd door een specifiek TCR-δ gendeletie recombinatie-complex dat preferentiëel gebruik maakt van de TCR-δ-deleterende elementen δRec en ψJα of Jα58. Bovendien kunnen andere gensegmenten (Vδ gensegmenten en de meeste Jα gensegmenten) met een lage frequentie gebruikt worden voor TCR-δ gendeletie. Deze laatstgenoemde genherschikkingen worden waarschijnlijk beïnvloed door verschillende factoren, inclusief transcriptie van het T early alpha element, en de afstand tussen de herschikkende gensegmenten. Hierdoor worden de TCR-δ-deletie genherschikkingen beperkt tot de meest dichtstbijzijnde Jα gensegmenten, zodat een zo groot mogelijk aantal Jα gensegmenten kan worden gebruikt voor daaropvolgende Vα-Jα genherschikkingen, met als doel het bewaren van een maximale potentiële TCR-α diversiteit. De identificatie van een regulatie eiwit, dat mogelijk betrokken is bij preferentiële TCR-δ-deletie genherschikkingen, en de cellijn DND41 met continue TCR-δ-deletie recombinatie aktiviteit, geeft ons de mogelijkheid om het mechanisme van TCR-δ deletie verder te onderzoeken.

ABBREVIATIONS

ALL	:	acute lymphoblastic leukemia
AML	:	acute myeloid leukemia
bp	:	base pairs
BM	:	bone marrow
C	:	constant
CD	:	cluster of differentiation/cluster of designation
CIAP	:	calf intestinal alkaline phosphatase
CLL	:	chronic lymphocytic leukemia
D	:	diversity
DP	:	double positive ($CD4^+CD8^+$)
EMSA	:	electrophoretic mobility shift assay
FTOC	:	fetal thymic organ culture
G	:	germline
J	:	joining
kb	:	kilobase pairs
kDa	:	kilodaltons
NCB	:	neonatal cord blood
McAb	:	monoclonal antibodies
MHC	:	major histocompatibility complex
MNC	:	mononuclear cells
N region nucleotides	:	part of junctional region of randomly-inserted nucleotides
PAG	:	polyacrylamide gel
PAGE	:	polyacrylamide gel electrophorese
PB	:	peripheral blood
PCR	:	polymerase chain reaction
PJA-BP	:	ψJα-binding protein
P region nucleotide	:	junctional region nucleotides, which form a palindromic sequence together with the juxtaposed nucleotides of an untrimmed gene segment
RAG	:	recombination activating gene
RSS	:	recombination signal sequences
RT-PCR	:	reverse transcriptase-polymerase chain reaction
SP	:	single positive ($CD4^+$ or $CD8^+$)
TCR	:	T cell receptor
TdT	:	terminal deoxynucleotidyl transferase
TEA	:	T-early-α
T-LGL	:	T cell large granular lymphocyte leukemia
TN	:	triple negative ($CD3^-CD4^-CD8^-$)
V	:	variable

CURRICULUM VITAE

Martie C.M. Verschuren werd op 29 september 1966 in Breda geboren. Na het behalen van het H.A.V.O. diploma aan de Katholieke Scholengemeenschap Markenhage te Breda in 1984, volgde hij aan de Hogeschool West-Brabant de studierichting Chemie. Tijdens deze opleiding werd een tweetal Biochemie stages doorlopen, nl. bij Diosynth Int. B.V. te Oss (Dr. O.T. Schönherr) en bij het Medisch Biologisch Laboratorium TNO te Rijswijk (Dr. M. Posno en Dr. R.J. Leer). Het diploma werd *cum laude* behaald op 24 juni 1988. Aansluitend vervulde de auteur zijn militaire dienstplicht als hulpbeheerder reservedelen bij het 48^{e} pantser infanterie bataljon te 's Hertogenbosch. In september 1989 startte hij met de studie Scheikunde aan de Katholieke Universiteit Nijmegen. In het kader van deze studie werd het hoofdvak Biochemie bij de werkgroep Moleculaire Oncologie (Prof. dr. W.J.M. van de Ven en Dr. J.L.P. van Duijnhoven) uitgevoerd. Voor deze stage werd de Trigon-studenten-prijs 1990/1991 toegekend in het kader van de Hans Bloemendal Lectures. Het doctoraal examen werd op 26 augustus 1991 *cum laude* afgelegd. Van september 1991 tot april 1992 was de auteur werkzaam als wetenschappelijk medewerker bij de werkgroep Moleculaire Oncologie (Prof. dr. W.J.M. van de Ven en Dr. J.L.P. van Duijnhoven) aan de Katholieke Universiteit Nijmegen. Van mei 1992 tot mei 1996 was hij verbonden als assistent-in-opleiding aan de vakgroep Immunologie van de Erasmus Universiteit Rotterdam, alwaar hij onder begeleiding van Prof. dr. J.J.M. van Dongen het in dit proefschrift beschreven werk uitvoerde. In november 1993 werd het "Oxford examination in English as a foreign language (higher level)" behaald. Sinds juli 1996 is hij werkzaam als post-doctoraal medewerker bij de vakgroep Immunologie van de Erasmus Universiteit Rotterdam.

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