Molecular analysis of chromosomal translocations (12;22) and (12;21) in human leukemia

Moleculair onderzoek van chromosoom translocaties (12;22) en (12;21) in humane leukemie

Proefschrift

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van rector magnificus
Prof. Dr P.W.C. Akkermans M.A.
en volgens Besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
woensdag 18 juni 1997 om 9.45 uur

door

Arjan Buijs

Geboren te Schiedam

Promotiecommissie

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The studies described in this thesis were performed in (i) the Department of Genetics, St. Jude Children's Research Hospital, Memphis, U.S.A., supported in part by the NIH Cancer Center Support Core Grant P30 CA21765 and the American Lebanese Syrian Associated Charities (ALSAC) of St. Jude Children's Research Hospital, and (ii) the Department of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands.

Dit proefschrift is bewerkt binnen (i) de afdeling Genetics van St. Jude Children's Research Hospital, Memphis, Verenigde Staten, financiëel gesteund door NIH Cancer Center Support Core Grant P30 CA21765 en de American Lebanese Syrian Associated Charities (ALSAC) van St. Jude Children's Research Hospital, en de afdeling Celbiologie en Genetica van de Faculteit der Geneeskunde en Gezondheidswetenschappen van de Erasmus Universiteit Rotterdam

ISBN: 90-9010569-7
Printed by Haveka BV, Alblasserdam, The Netherlands.

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Introduction

Cancers, including those from the hematopoietic system such as leukemias and lymphomas, are characterized by the fact that affected cells differ from their non-cancerous neighbors in their genetic constitution. Environmental factors and/or genetic predisposition cause an accumulation of mutations, that will eventually result in neoplastic transformation. These mutations can be manifested by small changes in the DNA, such as pointmutations or microdeletions, or by more dramatic alterations that change the karyotype of the affected cells, such as chromosomal translocations, deletions, inversions and amplifications (Cleary, 1991; Drexler *et al.*, 1995; Rabbitts, 1994).

These deviations affect genes whose products interfere with cellular mechanisms that dictate the delicate balance between proliferation, differentiation or cell death (apoptosis). Proliferation can be defined as a continuous multiplication of cells, whereas differentiation is the process in which cells generally stop to proliferate and mature into nondividing cells with a specialized function. In chronic leukemia, for instance, hematopoietic cells have an enhanced proliferative potential, but are nonetheless capable of full maturation. In most acute leukemias, proliferating progenitor cells are arrested at an early stage of differentiation and are unable to convert to non-proliferating specialized cells (reviewed by Sawyers et al.(Sawyers *et al.*, 1991). Apoptosis, in the classical Greek Hippocratic corpus from the roots *apo-*, apart, and *ptosis*, falling, referred to the loss of leaves in the autumn. The allusion is to acceptable loss, with the anticipation of renewal (Squier *et al.*, 1995). To give an example of this, many hematopoietic cells eventually die due to activation of an intrinsic cellular destruction mechanism. However, due to continuous renewal an optimal hematopoietic cell population is maintained. In some types of leukemias and lymphomas apoptosis is prevented, thus interfering with disposal of unwanted cells (White, 1996; Yang & Korsmeyer, 1996).

Chromosomal translocations contribute to transformation by generation of oncogenes by two mechanisms: 1) activation of genes due to promoter or enhancer addition, 2) generation of fusion proteins (Drexler *et al.*, 1995). Although chromosomal translocations affect genes that encode proteins with a variety of functions, a major group of targeted genes belong to the class of transcription factors. *TEL*, also named *ETV6*, the subject of this thesis, belongs to a subgroup in this class of genes. It is part of the family of ETS transcription factors, encoding proteins that contain a specific homologous DNA binding domain, called the ETS domain.

This thesis describes experimental work dealing with the molecular and biochemical characterization of TEL, as well as with its involvement in two translocations, t(12;22)(p13;q11) in myeloid leukemia and t(12;21)(p13;q22) in childhood pre-B ALL. The experimental work is preceded by an introduction regarding the role of ETS transcription factors in development, hematopoiesis and oncogenesis, to place the role of TEL in leukemogenesis in a broader context.

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Aims of thesis

Chromosomal aberrations of the short arm of chromosome 12, involving both deletions and translocations, can be detected in 2% of AML and 10% of childhood ALL. Translocation (12;22)(p13;q11), associated with human myeloid leukemia, is a recurrent chromosomal translocation with a low incidence. The aims of this project were to characterize this translocation and to address the following questions: 1. Is t(12;22)(p13;q11) a variant Philadelphia translocation, involving the *BCR* gene on chromosome 22q11? 2. What is the role of t(12;22)(p13;q11) in leukemia? 3. Is the gene on chromosome 12p13 a common target for translocations involving this region? 4. Is the gene on chromosome 12p13 a candidate tumor suppressor gene, since this region is often deleted in childhood ALL? In this thesis I will present experimental work, that will address these questions.

Chapter 1. Role of ETS factors

1.1. General characteristics of ETS transcription factors

The founding member of the ETS family of transcription factors, *v-ETS* (mammalian homolog *ETS-1)*, was discovered as part of a chimeric GAG/v-MYB/v-ETS protein that is expressed by the E26 avian erythroblastosis virus (E26 transformation-specific)(Leprince *et al.*, 1983; Nunn *et al.*, 1983). Subsequent isolation of homologous genes defined a family, of which all members encode proteins that contain a highly homologous sequence of 85 aminoacids, the ETS domain (reviewed by Seth et al. and Wasylyk et al. (Seth *et al.*, 1992; Wasylyk *et al.*, 1993). This DNA binding domain interacts with the major groove of a specific DNA sequence that is characterized by the common core motif GGAA/T (Nye *et al.*, 1992). Nuclear magnetic resonance (NMR) studies revealed that the ETS domain of ETS-1 adopts a winged helix-turn-helix (HTH) DNA binding structure (Donaldson *et al.*, 1996). Subsequent determination of the crystal structure of the ETS domain of PU.1 in complex with DNA showed that the helix 3, the second helix of the HTH motif, contacts the major groove of the DNA, but, in addition, two loops on either side of this helix interact with the minor groove (see figure 1). Binding of PU.1 to its cognate site induces an 8 degree bend in the DNA (Kodandapani *et al.*, 1996). This protein-induced DNA distortion is thought to create a structural environment on which a ternary nucleoprotein complex can assemble which transactivates genes that regulate cellular processes determining cell fate. Recent studies with PU.1 confirmed the loop-helix-loop scaffold as a general model for DNA recognition by ETS proteins, defining ETS factors as a new class of HTH DNA-binding proteins (Pio *et al.*, 1996).

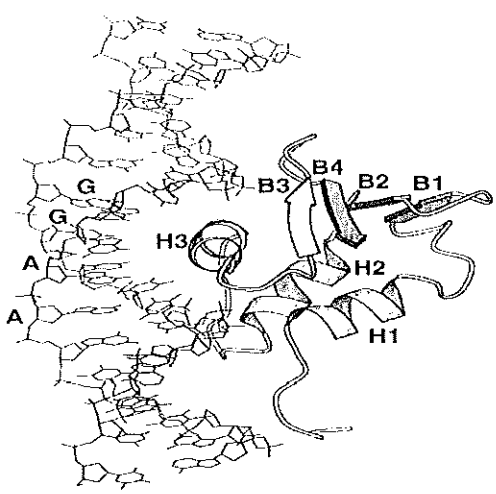

Figure 1. *Depiction of interaction of the ETS domain of ETS-1 with the DNA.* Interaction helix α3 contacts the major groove. (derived from Werner,1996 Cell October 1996, erratum). H1-H3: α-helices, B1-B4 β-plated sheets

ETS family members can be divided in different subgroups on the basis of their degree of homology within the ETS domain (see figure 2a,b), the position of the ETS domain within the protein, and by the presence of a helix-loop-helix (HLH) domain (see figure 2c,d)(Wasylyk *et al.*, 1993). The HLH domain of TEL has been demonstrated to function as an oligomerization domain (Jousset *et al.*, 1997)(chapters 2.2 and 3.2). DNA binding activity of ETS-1 can be repressed by an intramolecular mechanism (Jonsen *et al.*, 1996; Lim *et al.*, 1992; Petersen *et al.*, 1995), that can be relieved either by interaction with partner proteins, by posttranslational modifications, such as phosphorylation or by allosteric DNA interactions (Jonsen *et al.*, 1996; Petersen *et al.*, 1995; Rabault & Ghysdael, 1994).

As will be discussed below, the activity of ETS transcription factors is regulated by posttranslational modification, subcellular localization and interaction with partner proteins. For instance, transactivation activities of ETS-1 and ETS-2 are enhanced by RAS-mediated phosphorylation (Yang *et al.*, 1996), whereas PEA3 driven transcription activity was found to be influenced by two distinct MAPK cascades, the ERK and SAPK or JNK pathways (O'Hagan *et al.*, 1996). Transcription activity and localization of the *Drosophila melanogaster* ETS proteins YAN and POINTED is also regulated by RAS mediated phosphorylation (see chapter 1.2).

Transcription induction of c-*FOS*, an immediate early response gene of epidermal growth factor (EGF) stimulation, is mediated in part by a ternary nucleoprotein complex, consisting of serum response factor (SRF) (Norman *et al.*, 1988), ETS factor ELK-1 ($p62^{TCF}$) (Hipskind *et al.*, 1991) and the serum response element (SRE) within the promoter (reviewed by Treisman (Treisman, 1994). SAP1 and SAP2/NET/ERP are ELK-1 subfamily members and also form a ternary complex with SRF and SRE (Dalton & Treisman, 1992; Janknecht & Nordheim, 1992; Lopez *et al.*, 1994) (see figure 3). ELK-1 and SRF interact directly via a 30 aminoacids domain in ELK-1 (Janknecht & Nordheim, 1992; Shore & Sharrocks, 1994). Phosphorylation of ELK-1 by MAPK family members positively regulates ternary complex formation, DNA binding and transcription induction of c-*FOS* (Gille *et al.*, 1995; Janknecht *et al.*, 1993; Marais *et al.*, 1993; Whitmarsh *et al.*, 1995). Whereas SRF/ELK-1 is the target of a RAS/MAPK signaling pathway, SRF/SAP1 is the target of Ca^{2+}/calmodulin dependent kinase (CaMK) (Hipskind *et al.*, 1994; Miranti *et al.*, 1995). Each of the ELK-1 family members displays a certain degree of tissue specific expression. This suggests that distinct cytoplasmic signaling pathways can mediate *c-FOS* expression in diverse cell types via different ETS factors.

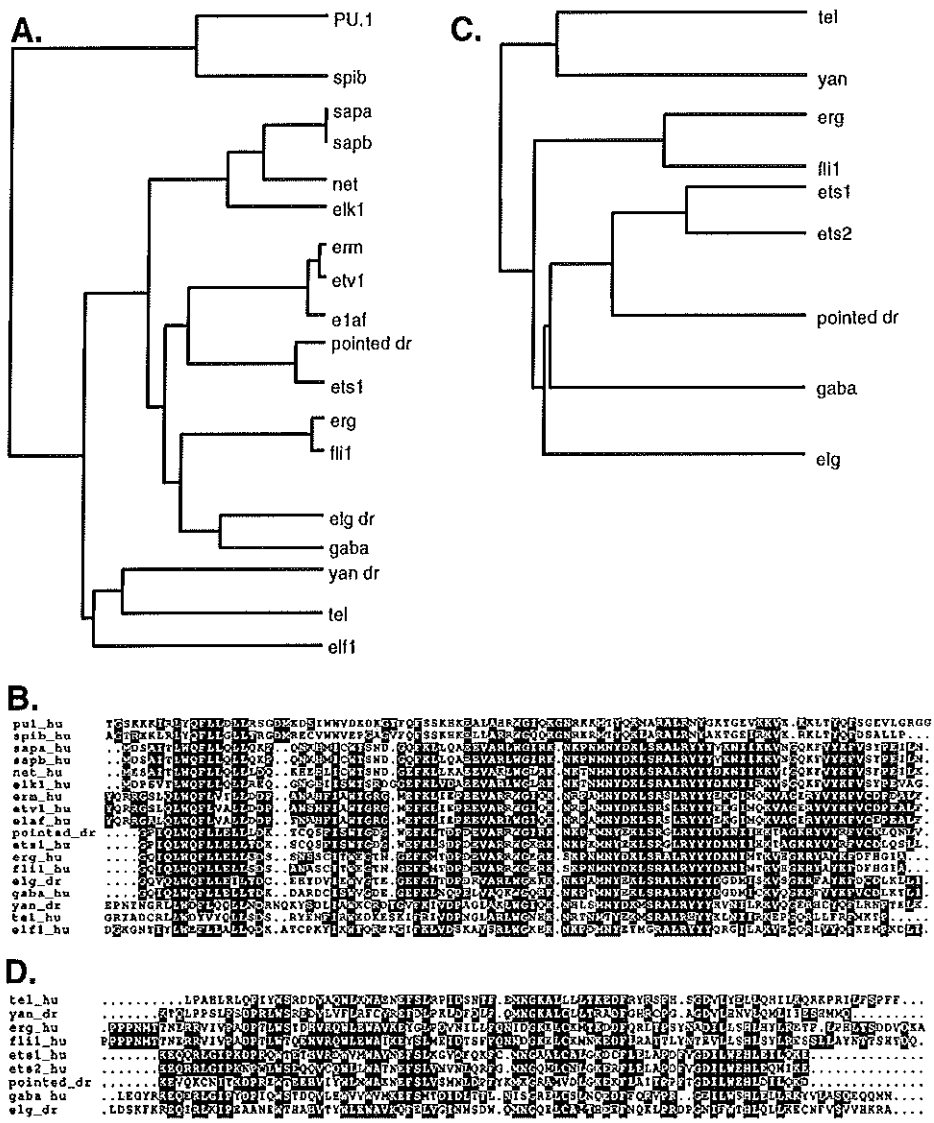

Figure 2. *Homology between ETS transcription factors.* A.Dendogram representing the level of homology within the ETS domain of ETS family members. B.Sequence alignment of the ETS domains. C.Dendogram representing the level of homology within the HLH domain of ETS family members D. Sequence alignment of Helix-Loop-Helix oligomerization domain of ETS family members. Dark boxes indicate identity. Gray boxes indicate similarity. Dendrograms and alignments were generated using Pileup (GCG) and Boxshade (Kay Hofmann)

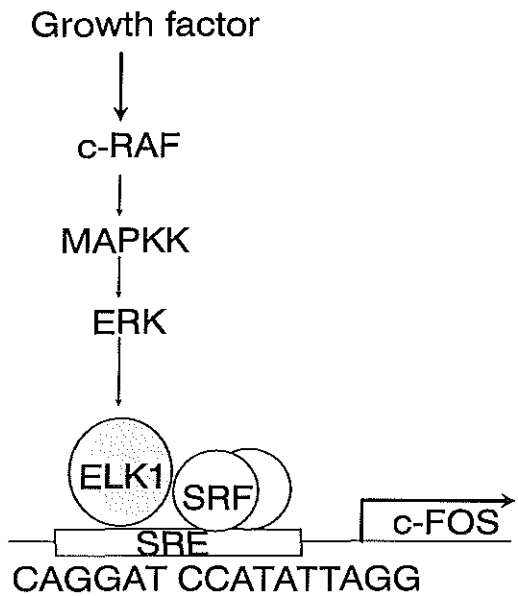

Figure 3. *SRE-mediated transcription of c-FOS by ELK1 and SRF.* Mitogen activated protein kinase (ERK) mediates complex formation of monomeric ETS factor ELK1 and homodimeric serum response factor (SRF) to the serum response element (SRE) in the c-*FOS* promotor, that contains ETS and SRF binding sites.

Alternatively, cell specific or lineage specific activity of ETS factors can also be accomplished by interaction with partner proteins. For instance, MafB, an AP-1 like protein, interacts with ETS-1 and inhibits erythroid differentiation, suggesting that it may establish and maintain a myelomonocytic phenotype of early myeloid cells by repression of ETS-1-induced erythroid-specific gene expression (Sieweke *et al.*, 1996). On the other hand, PAX-5, a key regulator of early B cell lymphopoiesis, recruits specific ETS factors, forming a ternary complex on B cell specific promoters (Fitzsimmons *et al.*, 1996). ETS factors can also influence the activity of other transcription factors. PIP, a lymphoid restricted interferon-regulatory-factor (IRF), acts as an activator or repressor of α/β-interferon-inducible transcription, dependent on the presence or absence of PU.1 (Brass *et al.*, 1996).

In addition to cytoplasmic kinase-dependent signaling pathways, steroid hormone receptors were found to mediate ETS factor activity. The androgen receptor (AR) is a member of the steroid hormone receptor family. Upon hormone binding the receptor migrates to the nucleus and directly regulates transcription of target genes. AR interacts with the ETS factor ERM (Schneikert *et al.*, 1996) and this interaction is thought to mediate the repression of matrix metalloproteinase-1 expression. Metalloproteinases are enzymes that degrade extracellular matrix and play an important role in tissue morphogenesis, cell differentiation and wound healing.

1.2. ETS factors in development

One of the best understood roles of members of the ETS family of transcription factors is in the development of the *Drosophila* eye (Wassarman *et al.*, 1995). In the developing eye the ETS factors YAN and POINTED have been identified as nuclear targets of RAS mediated phosphorylation in the signaling pathway initiated by receptor tyrosine kinases (RTK) (see figure 4). Furthermore, these two ETS factors are clear examples of how phosphorylation of ETS factors by mitogen activated protein kinases (MAPK) regulates their activity and influences cell fate.

The *Drosophila* eye is composed of approximately 800 identical units called ommatidia, each of which contains eight photoreceptor cells (R1-R8), four non-neuronal cone cells, and eight accessory cells. Neuronal differentiation of the R7 precursor cell is initiated by interaction of the R7 transmembrane RTK Sevenless (SEV), with the R8 specific Bride-of sevenless (Boss) ligand. Genetic evidence that an ETS factor was involved in differentiation of R7 photoreceptor cells came from the identification of *Drosophila* mutants. YAN/POK mutant flies have supernumerary photoreceptors, resembling the phenotype of flies, carrying an activated RAS1 gene. Independent cloning of the mutated YAN/POK gene by two groups revealed that it encodes an ETS family transcription factor, that is expressed in nuclei of undifferentiated photoreceptor cells. Upon differentiation, YAN/POK activity is downregulated (Lai & Rubin, 1992; Tei *et al.*, 1992). These data indicate that YAN/POK acts antagonistically to the signaling pathway mediated by SEV and RAS1.

Another target of the RAS/MAPK pathway is the ETS factor POINTED, that is necessary for normal development of the *Drosophila* embryonic nervous system, including eye development (Klämbt, 1993). *POINTED* encodes two ETS-DNA binding proteins P2 and P1 that are encoded by mRNAs transcribed from two different promoters (Klämbt, 1993). P2 contains a consensus recognition site (P-X-S/T-P) for phosphorylation of serine/threonine residues by MAPK. O'Neill et al. and Brunner et al. (Brunner *et al.*, 1994; O'Neill *et al.*, 1994) presented genetic and biochemical data that indicated that ETS transcription factors YAN/POK and POINTED are targets of the RAS1 mediated phosphorylation pathway. Whereas YAN seems to be a negative regulator of R7 photoreceptor cell development, POINTED appears to be a positive regulator. In agreement with this observation it was shown that stability and nuclear localization of YAN are negatively regulated upon activation of the SEV/RAS phosphorylation pathway (Rebay & Rubin, 1995). Further evidence for the regulation of YAN activity by phosphorylation comes from the observation that ectopic expression of a YAN mutant without consensus MAPK sites blocks neuronal development of photoreceptor cells. Mutant YAN may be responsible for a dramatic increase in cell death in the developing eye. Furthermore, it inhibited development of the embryonic CNS (Rebay & Rubin, 1995). Normal YAN was found to be expressed in non-differentiated mesoderm derivatives, but could not be detected in differentiated tissues such as gut and muscle. These observations suggest that YAN may play a more general role during development.

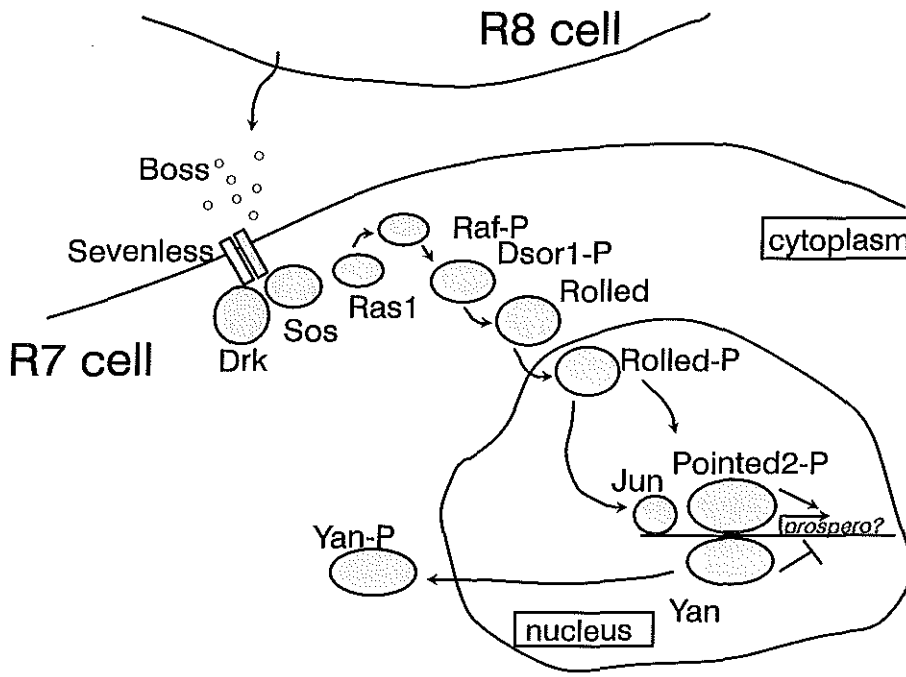

Figure 4. *Receptor tyrosine kinase/RAS1 signal cascade in the developing Drosophila eye.* Ligand dependent dimerization of the Sevenless tyrosine kinase receptor leads to subsequent phosphorylation of Raf (MAPKKK), Dsor1 (MAPKK) and Rolled (MAPK). Upon phosphorylation Rolled enters the nucleus and regulates activity of targets by phosphorylation (adapted from (Wassarman *et al.*, 1995)).

In addition to YAN and POINTED, a third transcription factor, c-JUN, was found to be involved in *Drosophila* eye development (Bohmann *et al.*, 1994). Treier et al.(Treier *et al.*, 1995) presented genetic and biochemical evidence that JUN and POINTED cooperate in R7 cell determination. The proteins are targeted to the same promoter and their activity is enhanced upon phosphorylation. Interestingly, JUN and POINTED cooperate in transcription activation of a reporter construct containing a single polyoma AP-1/ETS element, whereas YAN represses the cooperative activity of POINTED and JUN. A candidate target gene of YAN/POINTED mediated signal transduction pathway is *PROSPERO*. High levels of the homeodomain protein PROSPERO in R7 cells, required for proper connectivity of R7 photoreceptor axons to their synaptic targets, depend on SEV activation of the RAS1/MAPK pathway (Kauffmann *et al.*, 1996). Taken together, at least three transcription factors, YAN, POINTED and JUN are involved in *Drosophila* R7 receptor cell differentiation, and their antagonistic activities are tuned by phosphorylation induced by activation of the SEV/RTK pathway.

Similarly, another ETS factor, D-ELG, is required for egg chamber patterning and development during *Drosophila* oogenesis (Schulz *et al.*, 1993). In D-*ELG* mutant females, the spatial localization of OSKAR and GURKEN mRNA, involved in posterior and dorsoventral patterning of the egg, is disrupted. Furthermore, specialized follicle cells fail to migrate over the developing oocyte.

ETS-2 is expressed in a variety of cell types. During murine development it is highly expressed in newly forming cartilage, skull precursor cells and vertebral primordia (Kola *et al.*, 1993; Maroulakou *et al.*, 1994). ETS-2 is located on chromosome 21 and is overexpressed in Down's syndrome (trisomy 21) (Chumakov *et al.*, 1992). Sumarsono et al. (Sumarsono *et al.*, 1996) reported that ETS-2 transgenic mice develop neurocranial, visceral and skeletal abnormalities. These abnormalities have similarities with skeletal anomalies found in trisomy-16 mice and humans with Down's syndrome, in which the gene dosage of ETS-2 is increased by trisomy 21. These results indicate that ETS-2 has a role in skeletal development and implicate the overexpression of ETS-2 in the genesis of some skeletal abnormalities that occur in Down's syndrome.

1.3. ETS factors in hematopoiesis

Regulation of cell-specific transcription factors plays a crucial role in steering differentiation of pluripotent stem cells into certain hematopoietic lineages. The chacterization of chromosomal aberrations associated with malignancies of the hematopoietic system has revealed that transcription factors are often affected, indicating that altered transcription regulation plays a major role in leukemogenesis (reviewed by Cleary, Rabbitts and Barr (Barr, 1996; Cleary, 1991; Rabbitts, 1994)

A new method to study the role of transcription factors in hematopoietic development consists of mutation analysis of transcription factors by gene knock-out technology (reviewed by Shivdasani and Orkin (Shivdasani & Orkin, 1996)). The generation of nullizygous mice helped to elucidate the function of some ETS factors in hematopoiesis (figure 5).

ETS-1 is preferentially expressed in adult lymphoid tissue, with high levels in the thymus, but also in erythroid and myeloid cells. ETS-1 is expressed as two isoforms, p54 and p68, that are translated from alternatively spliced mRNAs. Both proteins bind DNA via the ETS domain, but have different transcription transactivation activities. Whereas both p54 and p68 are expressed in immature erythroid cells, p54 is expressed exclusively in B and T lymphocytes (Queva *et al.*, 1993). As mentioned above, ETS-2 is expressed in almost every tissue. However ETS-1 and ETS-2 are highly expressed in $CD4^+$ $CD8^-$ thymocytes. Interestingly, in resting human T cells ETS-1 is highly expressed, whereas ETS-2 expression is low. Upon antigenic or mitogenic stimulation, ETS-2 expression is dramatically upregulated, but ETS-1 expression goes down, suggesting an antagonistic role of both proteins in the thymus (Bhat *et al.*, 1990)(reviewed by Wasylyk et al. and Seth et al. (Seth *et al.*, 1992; Wasylyk *et al.*, 1993). As mentioned in chapter 1.1, ETS-1 is also implicated in erythropoiesis. MafB, a

myelomonocytic factor, prevents ETS-1 mediated activation of the transferrin receptor, which is essential for erythroid differentiation (Sieweke *et al.*, 1996). This inhibition is thought to prevent reactivation of the erythroid differentiation program by ETS-1 in myeloid cells. Nullizygous *c-ETS-1* mice displayed severe defects in both T- and B-cell lineages. T-cells numbers were dramatically reduced and failed to proliferated in response to mitogenic signals. T-cells were highly susceptible to apoptosis *in vitro*. B-cells were hampered in their terminal differentiation (Bories *et al.*, 1995; Muthusamy *et al.*, 1995).

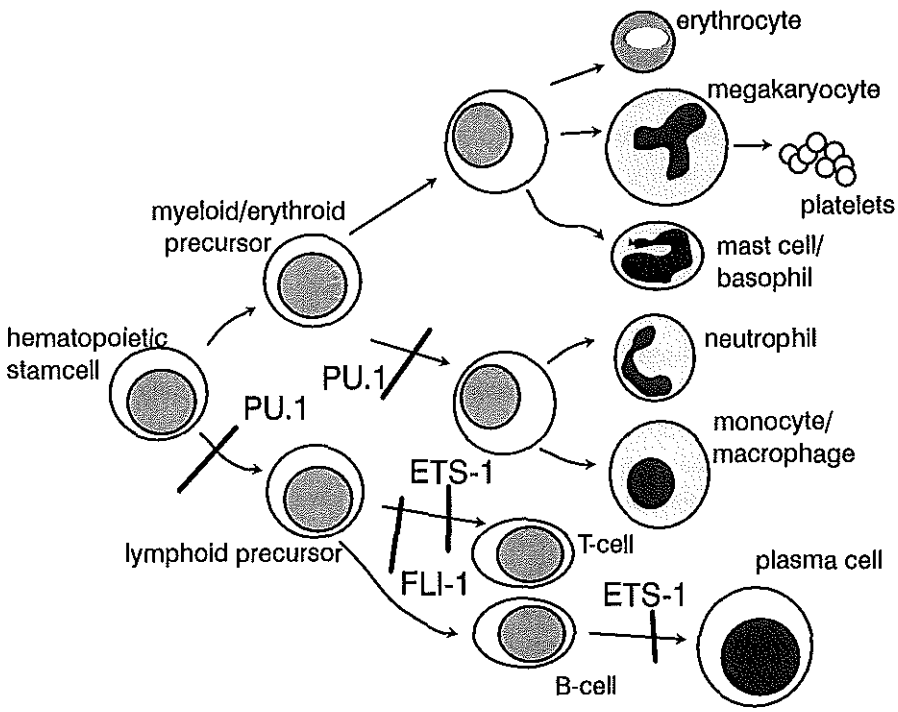

Figure 5. *Schematic representation of the position of essential function of ETS factors in hematopoiesis.* Position is based on earliest block in diffentiation (adapted from Shivdasani (Shivdasani & Orkin, 1996)).

PU.1, another ETS factor that is studied for its role in hematopoiesis, is expressed in a wide variety of hematopoietic cells, except for peripheral T cells. PU.1 activates a series of promoters of myeloid and lymphoid specific genes, including its own (summarized by Moreau-Gachelin et al.(Moreau-Gachelin *et al.*, 1996)). One of them is the receptor for macrophage colony stimulating factor (M-CSF-1), the c-*FMS* oncogene product (Reddy *et al.*, 1994). Interestingly, c-MYB represses this activation, suggesting that c-MYB mediates correct temporal expression of c-FMS during myeloid differentiation. Recently, PU.1 was found to be involved in CSF-1 dependent macrophage proliferation, probably by upregulation of the

expression of its receptor (Celada *et al.*, 1996). Nullizygous PU.1 mice died at late gestational age. Mutant embryos produced normal numbers of megakaryocytes and erythroid progenitors, but showed an impairment of erythroblast maturation. Furthermore, the mice displayed a multilineage defect in the generation of lymphoid progenitors, monocytes and granulocytes, suggesting that PU.1 acts in a multipotent progenitor in developmental programs of lymphoid and myeloid lineages during fetal liver hematopoiesis (Scott *et al.*, 1994). PU.1 was also found to be required in yolk sac myelopoiesis (Olson *et al.*, 1995). Interestingly, expression of genes associated with terminal myeloid differentiation (CD11b, CD64 and M-CSFR) was absent in PU.1 nullizygous embryos and differentiated ES cells. In an independent study, nullizygous PU.1 mice were born alive, but died of severe septicemia (McKercher *et al.*, 1996). In conclusion, it seems that PU.1 appears not to be essential for myeloid or lymphoid lineage commitment, but is required for terminal differentiation of most myeloid and lymphoid lineages.

Mice, supposedly deficient in FLI1, were viable, but exhibited thymic hypocellularity (Mélet *et al.*, 1996). This phenotype was not due to a deficiency in a specific subpopulation of thymocytes or to increased apoptosis, but rather suggested a defect in a prethymic T-cell progenitor. However, detailed analysis revealed that a truncated FLI1 protein was synthesized that arose from an internal translation initiation site and alternative splicing. It is therefore questionable whether the phenotype was caused by the absence of normal FLI1 or by expression of a novel truncated FLI1.

In conclusion, some members of the ETS transcription factors family appear to be key regulators in determining the lineage commitment as well as progression to full maturation of hematopoietic cells. The generation of nullizygous mice has proven to be a powerful tool to elucidate the function of some ETS factors in hematopoiesis. However, one drawback of generating a standard knockout is that a phenotype only shows the earliest stage at which a gene product is required, thereby loosing information about a possible function later in development. An approach that can be used to circumvent this problem, and to study the inactivation of a gene at a specific stage in development or in a specific cell type, is to make a conditional knockout based on the CRE-*lox*P system (Gu *et al.*, 1994; Kilby *et al.*, 1993). Another drawback may be that redundancy of the protein can mask its function.

1.4. ETS factors in oncogenesis

As mentioned in chapter 1.1, *v-ETS*, was discovered as part of a chimeric gene with *GAG* and *MYB*, that is expressed by the E26 avian erythroblastosis virus (Leprince *et al.*, 1983; Nunn *et al.*, 1983). ETS-1, the mammalian homolog of v-ETS, is amplified in MDS (Ohyashiki *et al.*, 1990) and transforms murine fibroblasts when overexpressed (Seth *et al.*, 1989). ETS-2 mRNA expression increases in AML(Santoro *et al.*, 1992). These observations set the stage for the involvement of ETS factors in malignant transformation. Uncontrolled expression of ETS factors may lead to aberrant proliferation, since ETS factors were found to influence CSF-1 receptor mediated mitogenic signaling by controling MYC expression (Langer *et al.*, 1992;

Roussel *et al.*, 1991; 1994). The *GAG-MYB-ETS* fusion oncogene, when expressed in a murine retroviral construct, was found to induce serum free proliferation of murine fibroblasts *in vitro* and erythroleukemia *in vivo* (Ruscetti *et al.*, 1992; Yuan *et al.*, 1989). Very recently, Athanasiou et al. (Athanasiou *et al.*, 1996) reported that expression of $p135^{GAG\text{-}MYB\text{-}ETS}$ in the IL3-dependent FDC-P2 myeloid cell line resulted in erythropoietin (Epo)-dependent inhibition of apoptosis upon withdrawal of IL3. This is the first report on an ETS factor associated with apoptosis.

Besides retroviral mobilization in birds, two different mechanisms directly activate cellular ETS factors oncogenically: proviral insertion in rodents and chromosomal translocations in humans. New born mice, infected with S FFV-P and -A and F MuLV develop erythroid leukemia with high frequency. This is caused by proviral integration in the vicinity of two ETS factors, *FLI1* and *SPI-1*/PU.1 in 75% and 95% of the tumors, respectively (Ben-David *et al.*, 1991; Moreau-Gachelin *et al.*, 1988). The proviral LTR acts as an enhancer and induces overexpression of FLI1 and/or PU.1. The involvement of *SPI-1*/PU.1 in leukemogenesis was confirmed by generation of mice transgenic for *SPI-1*/PU.1, of which 50% of the animals solely developed erythroleukemia (Moreau-Gachelin *et al.*, 1996).

The human homolog of *FLI1*, located on chromosome 11q24, fuses to *EWS*, a gene coding for a RNA binding protein as a result of t(11;22)(q12;q24) that can be detected by cytogenetic analysis in 85 % of Ewing's sarcoma (Delattre *et al.*, 1992). In EWS-FLI1 the RNA binding domain of EWS is replaced by the DNA binding ETS domain of FLI1. In contrast to normal FLI1, EWS-FLI1 transforms murine fibroblast. N-terminal sequences in EWS as well as the ETS domain of FLI1 are essential for transformation (May *et al.*, 1993a; 1993b). FLI1 and EWS-FLI1 bind DNA with the same sequence specificity. EWS-FLI1 is a more powerful transcription activator than FLI1 (Bailly *et al.*, 1994; May *et al.*, 1993b). Distinct domains in EWS contribute to the transforming and transactivating activity of the fusion protein (Lessnick *et al.*, 1995). EWS-FLI1 may influence proliferation or differentiation by more efficient activation of FLI1 target genes or by inappropriate regulation of genes normally not responsive to FLI1. FLI1 is expressed in hematopoietic tissues, including thymus and spleen (Klemsz *et al.*, 1993). This mainly hematopoietic expression of FLI1 suggests that ectopic FLI1 expression in Ewing's sarcoma is one of the important components of malignant transformation of cells of neuroectodermal origin. Denny and coworkers characterized stromelysin and cytokeratin as potential target genes of EWS-FLI1 using cDNA representational difference analysis of EWS-FLI-1 transformed fibroblasts (Braun *et al.*, 1995). Stromelysin-1, a member of the metalloproteinase family, digests a variety of extracellular matrix proteins. While metalloproteinases function normally in tissue remodelling during repair and embryogenesis, the ectopic expression of these proteins has also been linked to tumor invasion and metastasis. Therefore aberrant expression of stromelysin-1 by EWS-FLI-1 could very well contribute to the EWS-FLI-1 induced oncogenesis.

Interestingly, transformation of NIH3T3 by EWS-FLI1 is not so much determined by FLI1, but by an EWS-ETS transcription factor chimera, since a variant t(21:22)(q24;q12) in Ewing's sarcoma fuses the EWS gene to ERG in less than 10% of the cases (Sorensen *et al.*, 1994). ERG had been characterized previously and was demonstrated to contain two transcription activation domains, one N-terminal (i.e. an HLH protein-protein interaction domain) and a C-terminal domain (Rao *et al.*, 1987; Siddique *et al.*, 1993). In addition to its involvement in Ewing's sarcoma, *ERG* is also rearranged in AML (Shimizu *et al.*, 1993), where it was found to be fused with another RNA binding protein TLS/FUS as a consequence of t(16;21)(p11;q22) (Panagopoulos *et al.*, 1994; Prasad *et al.*, 1994). *In vitro* studies demonstrated that overexpression of ERG results in a mitogenic stimulation and transformation of NIH3T3 cells (Hart *et al.*, 1995). *ERG* gene expression is normally restricted to the thymus in adult humans, but it can be detected in KGa1, a early myeloid cell line, and in MOLT-4, a pre-T leukemic cell line, suggesting an association between malignancy and aberrant ERG expression (Watson *et al.*, 1992).

A third variant in Ewing's sarcoma fuses EWS to ETV1 (murine homolog ER81) as a result of t(7;22)(p12;q12). ETV1 is differentially expressed in different tissues (Jeon *et al.*, 1995). ER81 was demonstrated to be transcriptionally activated by the RAS/MAPK pathway. ER81 contains an N-terminal acidic transactivation domain which contains potential ERK phosphorylation sites. Sequences downstream of this transactivation domain are thought to have a negative regulatory effect on transactivation by ER81, suggesting a tight regulation of the transactivation activity of the protein (Janknecht, 1996). In t(7;22) the N-terminal transactivation domain of ETV1, including the potential ERK phosphorylation sites, and the inhibitory domain have been replaced by EWS, suggesting that one of the consequences of the translocation may be a RAS/MAPK independent transactivation of ETV1 target genes. Stromelysin-1 expression was also found to be upregulated by EWS-ERG and EWS-ETV1, suggesting that in addition to their structural similarities these fusion proteins have overlapping functional roles in transformation.

Recently, a fourth translocation was found to fuse EWS to ETV4 (murine homolog E1AF), as a result of t(17;22)(q12;q12) in a case of undifferentiated sarcoma (Kaneko *et al.*, 1996). ETV4 was found to confer an invasive phenotype upon transfection into the non-invasive human breast cancer cell line MCF-7 and to activate the transcription of MMP-9, a matrix metalloproteinase in these cells. Both ETV4 and MMP-9 mRNA expression was demonstrated to be elevated in some human invasive cell lines (Kaya *et al.*, 1996).

The fifth translocation that fuses EWS to an ETS factor in Ewing tumors has been described by Delattre and coworkers (Peter *et al.*, 1997). As a result of a t(2;21;22)(q33:q22;q12) EWS is fused to FEV, a new member of the ETS family of transcription factors. FEV expression seems to be restricted to prostate and small intestine. In general, it can be concluded that ectopic or aberrant expression of various ETS factors critically contribute to malignant transformation and tumor progression.

1.5. Chromosome 12p13 aberrations involving the *TEL* gene

Chromosomal translocations and deletions, involving chromosome region 12p11-13 have been described in 0.5 to 2 % of AML (Adriaansen, 1992) and in 10 % of childhood ALL (Raimondi *et al.*, 1986). The cytogenetic detection of chromosome 12p13 translocations with several different chromosome partners suggests that this locus may harbor one or more genes that could be targets of the distinct translocations. Because of the frequent deletions in childhood ALL this region may also contain one or more tumor suppressor genes.

Characterization of the breakpoints of four different translocations t(5;12)(q33;p13), t(12;22)(p13;q11), t(9;12)(q34;p13) and t(12;21)(p13;q22) by four teams of researchers resulted in the independent cloning of the *TEL (translocation ETS leukemia)* (also named *ETV6: ETS translocation variant 6*) gene on chromosome region 12p13. This provided powerful tools to study *TEL*s involvement in other translocations in leukemias and lymphomas using molecular cytogenetic techniques (FISH), and Southern, Northern and RT-PCR analysis. In addition, *TEL*s candidacy for a tumor suppressor function could be further substantiated by LOH studies, molecular cytogenetic analysis and biochemical investigations. Furthermore, the clinical relevance of *TEL* rearrangements could be studied by using them as prognostic indicators for the leukemia. In this chapter I will present clinical and molecular data of chromosomal aberrations involving the *TEL* gene and I will discuss putative mechanisms by which TEL and TEL chimeric proteins contribute to leukemogenesis. The first part of this chapter describes chromosomal translocations listed in numerical order of the partner chromosome. The second part deals with deletions of the *TEL* locus. The *TEL* gene spans about 240 kb and encodes 8 exons. Exon 1, 2 and 3 are separated by two large introns of approximately 100 kb each (Baens *et al.*, 1996). Exon 3 and 4 encode a putative helix-loop-helix (HLH) protein-protein interaction domain, whereas exon 6-8 encode the ETS DNA binding domain (figure 6).

Translocations

t(1;12)

A single case of t(1;12) has been mentioned by Golub et al. (Golub *et al.*, 1996a) in which the breakpoint in *TEL* occurs between exons 5 and 6 (see figure 6). This case is mentioned here for the sake of completeness. No clinical data of the malignancy associated with the translocation were presented and the fusion partner of *TEL* has not been identified.

t(3;12)(q26;p13)

Raynaud and coworkers (Raynaud *et al.*, 1996c) described a recurrent t(3;12)(q26;p13) in four patients with acute transformation of MDS and one with blast crisis of cALL. FISH analysis indicated that in 3 cases the 12p13 breakpoint occurred within the first and second introns of *TEL* and the 3q26 breakpoints were scattered 5' and 3' of *EVI1* gene. This heterogeneity of 3q26 breakpoints was also observed in t(3;21)(q26;q22) in which *AML1*, located at chromosome region 21q22, is rearranged in fusion transcripts with two unrelated genes at 3q26, *EAP* and *MDS1*, as well as with *EVI1* (reviewed by Nucifora and Rowley, 1995)(Nucifora & Rowley,

1995). RT-PCR analysis of RNA of two leukemic samples associated with t(3;12), revealed that the translocations result in chimeric TEL-EVI1 and TEL-MDS1-EVI1 transcripts (Drs P. Peeters and P. Marynen; personal communication). The fusion transcripts only contain *TEL* exon 1 or 1 and 2, arguing that the *TEL* promoter induces ectopic transcription of the *EVI1* gene which is normally not expressed in hematopoietic cells (Morishita *et al.*, 1990).

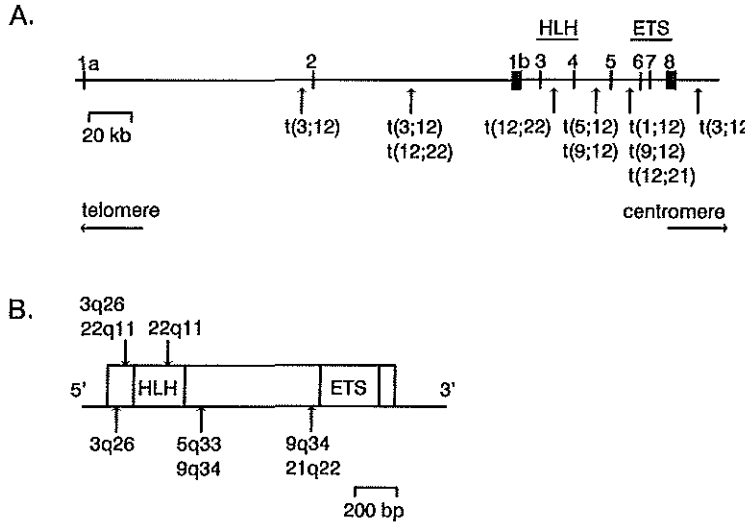

Figure 6. *Localization of translocation breakpoints in TEL.* A. Localization of breakpoints of translocations on the physical map of *TEL* . B. Localization of fusion points in TEL cDNA.

t(5;12)(q33;p13)

The first published translocation involving *TEL* was t(5;12)(q33;p13) , associated with a subgroup of chronic myelomonocytic leukemia (CMML) (Golub *et al.*, 1994). CMML is a form of MDS that is characterized by abnormal clonal myeloid proliferation and progression to AML. The translocation breakpoint on chromosome 12 occurs between exon 4 and 5 of *TEL* (Golub *et al.*, 1994; Wlodarska *et al.*, 1995b). The translocation results in an in-frame fusion of *TEL* and the gene encoding the PDGFβ receptor on chromosome 5q33. The fusion protein consists of the N-terminal part of TEL, including its HLH oligomerization domain, and the transmembrane and tyrosine kinase domains of the receptor (figure 7). Although the ligand binding domain of the receptor is absent, activation of the kinase domain occurs as a result of oligomerization of the fusion protein via the TEL HLH domain. This ligand independent self-association and constitutive activation of the kinase domain is thought to activate signal transduction pathways leading to neoplastic transformation. Furthermore, TEL-PDGFβR confers IL3 independent growth to the IL-3 dependent hematopoietic cell line Ba/F3 (Carroll *et al.*, 1996).

t(6;12)(q23;p13)

TEL was reported as the target of t(6;12)(q23;p13) in a cell line that was derived from leukemic cells of a patient with ALL (Suto *et al.*, 1995). Two different types of fusion cDNAs were characterized. The first type contained exon 1 and 2 of TEL fused to what is thought to be the 3' untranslated region of the *STL* (six-twelve-leukemia) gene. A second cDNA contained sequences of STL fused to 3' TEL starting with exon 6.

t(7;12)(p15;p13)

TEL involvement was demonstrated by FISH analysis in two cases of t(7;12)(p15;p13) (Wlodarska *et al.*, 1995a). In a case of AML M0 the 12p13 breakpoint was localized between *TEL* exons 2 and 8, whereas in a case of ALL L2 the breakpoint is located near exon 1. Since the gene on chromosome 7p15 is not known, the functional implication of these two translocations remains to be determined.

t(9;12)(q34;p13)

The initial experiment that resulted in the discovery of *TEL* involvement in t(9;12)(q34;p13) was not the molecular analysis of this cryptic translocation *per se*, but a series of immune complex-kinase reactions to study protein tyrosine kinase activity of BCR-ABL fusion proteins. In a case of cALL without the Philadelphia chromosome, an ABL protein with elevated protein kinase activity was detected (Chan *et al.*, 1987). RACE-PCR cloning identified a TEL-ABL fusion transcript as a result of a t(9;12)(Papadopoulos *et al.*, 1995). The breakpoint in *TEL* occurred between exons 3 and 4, similar to these found in cases of t(5;12). No reciprocal ABL-TEL product could be amplified. The resulting TEL-ABL fusion protein contained N-terminal TEL sequences, including the HLH domain, fused to the kinase domain of ABL, a non-receptor tyrosine kinase (figure 7). This configuration is very similar to t(9;22) BCR-ABL, in which BCR provides a coiled-coil homodimerization domain (McWhirter *et al.*, 1993). In a case of AUL t(9;12;14), a TEL-ABL fusion has been characterized in which the *TEL* breakpoint was located between exons 5 and 6 (Golub *et al.*, 1996b). Screening of 186 adult ALL and 30 childhood ALL patients for a TEL-ABL fusion suggested that it is not a common event in ALL (Janssen *et al.*, 1995). This is probably due to the opposed transcription direction of the genes on the involved chromosomes, i.e. towards the centromere for *TEL* on 12p13 and towards the telomere for *c-ABL* on 9q34. In order to generate a TEL-ABL fusion a more complex translocation is necessary, that involves inversion of one of the genes.

The increased protein tyrosine kinase activity of ABL in the case described by Papadopoulos et al. (Papadopoulos *et al.*, 1995) already suggested that the HLH domain mediated oligomerization of TEL-ABL was responsible for this effect. *In vitro* experiments indeed demonstrated that TEL-ABL is constitutively phosphorylated, depending on the presence of TELs HLH domain (Golub *et al.*, 1996b). Like BCR-ABL (McWhirter & Wang, 1993), TEL-ABL exhibits all the properties of an activated kinase; it localizes to actin stress

fibers, it transforms fibroblasts and primary bone marrow cells and confers IL3 independency to the hematopoietic cell line Ba/F3 (Golub *et al.*, 1996b). These data are consistent with the interpretation that ABLs kinase domain activates downstream signal transduction cascades that are necessary for neoplastic transformation. Although TEL-ABL is thought to be a rare translocation, study of TEL-ABL could be important to reveal the mechanism by which ABL contributes to leukemogenesis. Indeed, it was recently demonstrated that TEL-ABL and BCR-ABL activate similar transduction pathways in hematopoietic cell lines (Okuda *et al.*, 1996).

In one of the TEL-ABL translocations mentioned above, the second *TEL* allele was deleted, resulting in loss of wildtype TEL function. Golub and coworkers suggested that loss of TEL function alone may be a leukemogenic event and that *TEL* may act as a tumor suppressor gene. Arguing against this hypothesis is the fact that TEL-ABL transforms in a dominant fashion. Golub et al. favor the hypothesis that interaction of TEL-ABL with TEL may inhibit the activity of TEL-ABL. Deletion of the second *TEL* allele would circumvent that phenomenon. An alternative theory I favor is that both mechanisms may act synergistically. Deletion of the second *TEL* allele would lead to maximal activation of ABLs phosphotyrosine kinase activity that is further complemented by loss of the alleged tumor suppressor activity of TEL.

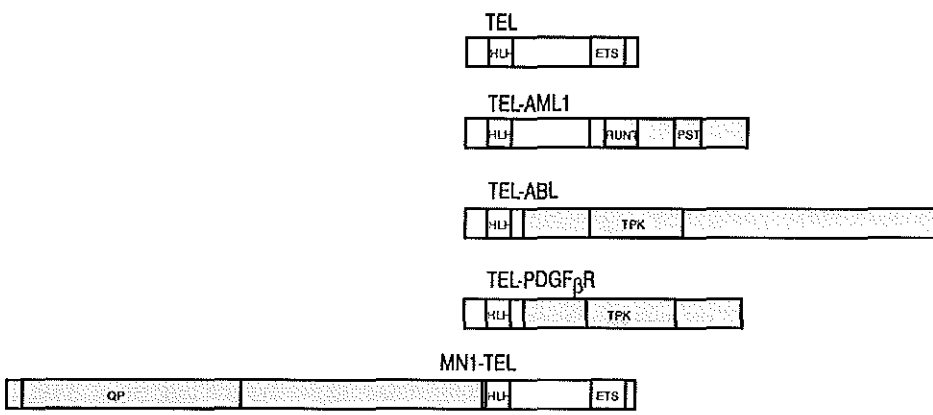

Figure 7. *Schematic representation of chimeric TEL proteins, associated with human leukemias.* The TEL sequences are indicated in white boxes, those of the fusion partners in shaded boxes. HLH = Helix-Loop-Helix domain, ETS = ETS DNA binding domain, RUNT = RUNT homology DNA binding domain, PTK = phosphotyrosine kinase domain, QP = glutamine/proline rich region

t(10;12)(q24;p13)

TEL involvement has been documented in a case of progressive MDS, with eosinophilia and monocytosis, associated with a t(10;12)(q24;p13)(Wlodarska *et al.*, 1995b). FISH analysis using cosmids containing 5'TEL and 3'TEL exons revealed that the 12p13 breakpoint was located within the *TEL* gene. Target genes on chromosome 10q24 have not been identified. The authors propose that in addition to t(5;12), variant translocations like t(10;12), could contribute to this subtype of MDS.

t(12;13)(p13;q14)

Tosi et al. (Tosi *et al.*, 1994) reported two cases of adult AML of FAB subtype M0, with a cytogenetically detectable t(12;13)(p13;q12). Previously, cases with this translocation were reported in leukemia of lymphoid origin. At the time of publication only the status of the RB-1 tumor suppressor gene at 13q14 had been analyzed by FISH. However, no definitive conclusion about the involvement of this gene could be established. By Northern analysis of patient RNA using a 3' TEL cDNA probe an aberrant transcript could be detected in both cases. Furthermore, the YAC 964c10 containing the entire *TEL* gene was found to be split in FISH analysis (Dr Biondi; personal communication). These data suggest *TEL* involvement in the translocation, but to date efforts to clone a chromosome 13q14 fusion partner have not been successful.

t(12;14)(p11;q32)

In a case of B non-Hodgkin lymphoma (NHL), FISH revealed a translocation t(12;14)(p11;q32), with a microdeletion of *TEL* sequences. The second chromosome 12 was involved in three different translocations, one of them a t(5;12;?)(p11;p11p13;?) with the *TEL* breakpoint in the 5' end of the gene, upstream of the HLH domain. This was the first case of *TEL* rearrangement in NHL (Dr. Wlodarska; personal communication).

t(12;21)(p13;q22)

Translocation (12;21)(p13;q22) can be detected in 0.05% of childhood ALL by standard cytogenetic analysis (Raimondi, 1993) and was first described by Soekarman et al. in 1992 (Soekarman *et al.*, 1992). It was independently characterized by two groups (Golub *et al.*, 1995; Romana *et al.*, 1995a), who found a fusion of *TEL* to the *AML1* gene on chromosome 21q22. The breakpoints in *TEL* are mainly clustered in an intron between exons 5 and 6. The TEL-AML1 chimeric transcript encodes a fusion protein containing the HLH oligomerization domain of TEL and the RUNT DNA binding domain and transactivation domain of AML1 (figure 7). Molecular analysis of ALL by FISH, Southern analysis and RT-PCR analysis revealed that the translocation can be detected in up to 25% of childhood ALL, in both Caucasian (see chapter 3)(McLean *et al.*, 1996; Romana *et al.*, 1995b; Shurtleff *et al.*, 1995) and Chinese populations (Liang *et al.*, 1996). It defines a subgroup of pediatric ALL of an age between 1 and 10 years with a B lineage immunophenotype, a nonhyperdiploid DNA content and an excellent prognosis (chapter 3.1). TEL-AML1 seems to be restricted to childhood ALL

as it is rarely detected in adult ALL (Raynaud *et al.*, 1996b; Shih *et al.*, 1996). A detailed description of the incidence of the translocation, its possible clinical significance and the molecular mechanism by which the fusion protein is thought to contribute to leukemogenesis can be found in chapter 3.1 of this monograph. In short, TEL-AML1 was found to repress AML1 dependent transcription of TCRβ enhancer reporter plasmids *in vitro* (Hiebert *et al.*, 1996). Furthermore, it oligomerizes with TEL and in transfected fibroblasts it colocalizes with endogenous TEL in the cytoplasm. These characteristics are dependent on the presence of the HLH domain in the fusion protein (see chapter 3.2).

The translocation is frequently accompanied by deletion of the second *TEL* allele (Golub *et al.*, 1995; Raynaud *et al.*, 1996a; Romana *et al.*, 1995a; Wlodarska *et al.*, 1996). This deletion is a secondary event in the leukemia (Kim *et al.*, 1996; Raynaud *et al.*, 1996a; Wlodarska *et al.*, 1996), suggesting that it may provide a proliferative advantage to a TEL-AML1 positive leukemic subclone.

t(12;22)(p13;q11)

In hematopoietic malignancies of myeloid lineages, the rare translocation(12;22)(p13;q11) results in the fusion of the *MN1* gene on chromosome 22q11 to *TEL* (Buijs *et al.*, 1995)(see chapter 2.1). *MN1* was characterized by the group of Zwarthoff (Lekanne Deprez *et al.*, 1995) by cloning of the breakpoint at chromosome 22q11 of a reciprocal t(4;22)(p16;q11) in a patient with multiple meningiomas. The gene contains two exons, separated by an intron of 60 kb. The first exon encodes the main body of a nuclear protein that shows no homology to protein sequences in databases, except for two homopolymeric glutamine stretches. These are encoded for by repeated CAG sequences of which the inadvertent amplification in several genes leads to gene inactivation and causes certain inherited diseases, such as Huntington's chorea(Nance, 1996). However this mechanism does not apply to *MN1*, since no instability in the CAG repeats of *MN1* could be observed in tens of cases of meningioma (Drs Riegman and Zwarthoff, personal communication). Another characteristic of MN1 is that its N-terminal one third is rich in proline residues. Together, these characteristics suggest that MN1 is involved in transcription regulation (Gerber *et al.*, 1994). Since t(12;22)(p13;q11) is the subject of chapter 2 of this thesis, a detailed description of cloning of the translocation, characterization of the fusion transcripts and biochemical analyses of the fusion proteins can be found there. Here I will restrict myself to a summery of the data.

Breakpoints in the *TEL* gene have been localized between exons 2 and 3 and exons 3 and 4, i.e. 5' of or within the HLH domain encoding exons. Both reciprocal chimeric transcripts are expressed: TEL-MN1 and MN1-TEL. The MN1-TEL fusion protein contain almost the entire MN1 protein fused to the ETS domain of TEL (figure 7). We hypothesize that these fusion proteins interfere with expression of TEL target genes. Indeed, MN1-TEL type I was found to transform a murine fibroblast cell line, similar to the Ewing's sarcoma associated fusion protein EWS-FLI1. The transforming capacity of the fusion protein is dependent on N-terminal MN1 sequences. Furthermore, the transforming potential of MN1-TEL was reduced

by mutating the ETS domain. Taken together these observations are in agreement with the above hypothesis. MN1 and MN1-TEL were found to upregulate transcription of the Moloney sarcoma virus LTR. However, N-terminal MN1 sequences, essential for transformation do not coincide with sequences of MN1 necessary for upregulation of transcription. Based on the structure of the fusion protein and its activities we favor the hypothesis that, in contrast to the fusion proteins mentioned above in which the HLH domain of TEL is instrumental, in MN1-TEL the ETS DNA binding domain is instrumental in neoplastic transformation.

Deletions

Deletions of chromosome 12p13 in pediatric ALL could be detected in approximately 5 % of the cases (1991; Raimondi *et al.*, 1986). Using FISH analysis with probes derived from this region, it was possible to map their smallest region of overlap to a genomic region that is bordered distal to the centromere by *TEL* and proximal by *KIP1* (Hoglund *et al.*, 1996; Kobayashi *et al.*, 1994; 1995; Sato *et al.*, 1995). Secondly, LOH of this region was found to be very common in childhood ALL (Cavé *et al.*, 1995; Stegmaier *et al.*, 1995; Takeuchi *et al.*, 1996). However, mutation analysis of both *TEL* and *KIP1* revealed a germline configuration of both genes (Seriu *et al.*, 1996; Stegmaier *et al.*, 1996), suggesting that neither of these genes would be the critical tumor suppressor gene in pediatric ALL.

It has already been mentioned above that t(9;12) TEL-ABL and t(12;21) TEL-AML1 are frequently accompanied by a deletion of the non-rearranged *TEL* locus or in some cases by an intragenic *TEL* deletion. To date, deletions of *TEL* have only been observed in combination with a translocation of the other allele. It remains an interesting question whether hemizygosity of *TEL* is associated with genomic instability that could subsequently lead to deletion of the non-rearranged *TEL* locus and/or other genetic defects that promote progression of the leukemia.

Concluding remarks

In this chapter an overview has been presented of chromosomal aberrations in human hematopoietic neoplasms in which the *TEL* gene on chromosome 12p13 has been implemented. In addition to the translocations mentioned above, *TEL* is a likely target of other translocations, such as t(2;12)(q37;p13), t(4;12)(q12;p13), dic(9;12)(p11-13;p11-12), t(12;17)(p13;q11), t(12;18)(p13;p11), dic(12;19)(p13;q11), t(12;20)(p13;q12) (Mitelman, 1991). *TEL*s involvement in these translocations and their possible contribution to leukemogenesis is the focus of ongoing research.

The biochemically distinct HLH domain and the ETS domain of the protein contribute to different fusion proteins (see figure 6). *TEL* may also contribute to leukemogenesis by promoter addition, as seems to be the case in t(3;12) *TEL-EVI1*. Furthermore, cytogenetic and biochemical data support a model in which the dosage of nuclear TEL may influence the progression of the disease. The diversity by which TEL is thought to contribute to the various hematopoietic malignancies is an intriguing aspect. In this respect TEL is unique.

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Chapter 2. Chromosomal translocation (12;22)(p13;q11)

Chapter 2.1.

Translocation (12;22)(p13;q11) in myeloproliferative disorders results in fusion of the ETS-like *TEL* gene on 12p13 to the *MN1* gene on 22q11. (1995). *Oncogene* **10**:1511-1519.

Translocation (12;22) (p13;q11) in myeloproliferative disorders results in fusion of the ETS-like *TEL* gene on 12p13 to the *MN1* gene on 22q11

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In myeloid and lymphoid leukemias recurrent chromosomal aberrations can be detected in chromosome region 12p13. We characterized the genes involved in t(12;22) (p13;q11) in two patients with myeloid leukemia and one with myelodysplastic syndrome (MDS). *MN1*, a gene on chromosome 22q11 was shown to be fused to *TEL*, a member of the family of ETS transcription factors on chromosome 12p13. The translocation results in transcription of the reciprocal fusion mRNAs, MN1-TEL and TEL-MN1, of which MN1-TEL is likely to encode an aberrant transcription factor containing the ETS DNA-binding domain of TEL. In addition to fusion of TEL to the PDGFβ receptor in t(5;12) in chronic myelomonocytic leukemia (CMML), our data suggest that the involvement of this protein in myeloid leukemogenesis could be dual; its isolated protein-protein dimerization and DNA-binding domains may be crucial for the oncogenic activation of functionally different fusion proteins.

Keywords: translocation; *TEL*; *MN1*; myeloid leukemia; chromosome 12p

Introduction

The translocation (12;22)(p13;q11) has been associated with myeloid malignancies of different FAB subtypes. Originally it was found as an alternative translocation in chronic myeloid leukemia (CML) and it was proposed that the 22q− chromosome was an alternative Philadelphia chromosome (Engel *et al.*, 1977; van der Blij-Philipsen *et al.*, 1977; Verma and Dosik, 1979). However, later the same specific translocation was also found in rare cases of acute myeloid leukemia (AML) M1, M7, M4 and MDS (Hagemeijer *et al.*, 1981; Johansson *et al.*, 1990; Callen *et al.*, 1991; Mitelman, 1991; Kashimura and Minamihisamatsu, 1993). The chromosomal region 12p11-13 has been implicated in the genesis of 0.5–2% of different myeloid (Adriaansen, 1992) and 10% of childhood acute lymphoblastic leukemias (ALL) (Raimondi *et al.*, 1986). In the case of myeloid leukemias, this often involves balanced translocations with a collection of different partner chromosomes (Mitelman, 1991), which is also true for the specific sub-band 12p13, involved in t(12;22). In ALL the region is often deleted, but also balanced translocations have been described (Raimondi *et al.*, 1986; Mitelman, 1991; Krance *et al.*, 1992; van der Plas *et al.*, 1995). With respect to the cytogenetic data, different loci, present on 12p11, 12p12 and 12p13, may be involved in these malignancies (Mitelman, 1991).

Our interest in this specific but rare translocation in myeloid malignancies was raised by the 22q11 breakpoint and its association with aspecific CML, which indicated a possible involvement of the *BCR* gene. However, initial mapping experiments with BCR cDNA probes excluded this possibility and the use of rodent/human hybrid cell lines, containing the segregated chromosomes of a patient with t(12;22) AML, suggested that the translocation breakpoint mapped telomeric from the *BCR* gene on chromosome 22 (DvdP, unpublished results). We set out to molecularly identify t(12;22) with the idea that it represented a crucial tumorigenic step in the genesis of myeloid malignancies and that the same locus on 12p13 may be involved in other chromosome translocations.

Using hybrid cell lines from a patient with t(12;22) AML-M4, we could map and clone the genomic chromosomal breakpoint on 22q11. It was situated in the intron of *MN1*, a candidate gene for meningioma, encoding a protein with features characteristic of transcription factors (Lekanne Deprez *et al.*, 1995, this issue). This allowed us to clone chromosome 12 sequences and to isolate fusion cDNAs, using a RT-PCR cloning procedure. The translocation results in the fusion of the *MN1* gene to the recently identified *TEL* gene on 12p13, a member of the ETS gene family of transcription factors (Golub *et al.*, 1994). The amino-terminal part of the TEL protein, containing a putative protein-protein dimerization domain, was found to be fused to the tyrosine kinase domain of the PDGFβ receptor by the t(5;12)(q34;p13) in chronic myelomonocytic leukemia (CMML). In two out of three patients with different myeloid malignancies, carrying t(12;22), both the TEL-MN1 and MN1-TEL fusion mRNAs are expressed. In hybrid cell lines of the third patient, only expression of MN1-TEL mRNA could be found. This transcript will encode a fusion protein, containing an ETS DNA-binding domain, that may act as an altered transcription factor, similar to EWS-FLI1 in Ewing's sarcoma (Bailly *et al.*, 1994). Our data suggest that the involvement of TEL in human myeloid malignancies is dual. Distinct domains of the protein seem to be essential constituents of functionally different fusion proteins.

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Received 21 December 1994; revised 16 January 1995; accepted 16 January 1995

Results

Localization of chromosomal breakpoint on 22q11

To localize the 22q11 breakpoint of t(12;22), probes of known localization in the 22q11-q13 region were hybridized to DNA of somatic cell hybrids, containing the segregated translocation chromosomes of a t(12;22) AML patient. To this end, bone marrow cells of a patient (p1) with t(12;22) AML were fused to the hamster cell line A3 using standard procedures (Geurts van Kessel *et al.*, 1981). After selection, individual clones were picked and characterized cytogenetically, using Reverse banding techniques. The chromosomal content of the relevant hybrid cell lines is shown in Table 1. DNA of human thymus, hamster cell line A3

Table 1 Hybrid cell lines containing segregated t(12;22) chromosomes scored for chromosome 22q-specific markers

	Chromosomes				centromere		Markers 1.8 kb *Eco*RI76A4	2.2 MN1	17.13′ MN1				telomere	
Cell Line*	12	12p+	22	22q-	5′BCR	D22S1	Cosmid	cDNA	cDNA	D22S193	D22S56	LIF	D22S15	c-*sis*
A3JA 19A	–	–	+	–	+	+	+	+	+	+	+	+	+	+
A3JA 19C	+	–	–	–	–	–	–	–	–	–	–	–	–	–
A3JA 13B	–	+	–	–	–	–	+	+	–	–	+	+	+	+
A3JA 12A	+	–	–	+	+	+	–	–	+	+	–	–	–	–
A3JA 1C	+	+	–	–										
A3JA 2B	–	+	+	–										
A3JA 12C	–	+		+										
A3JA 20A	+	+	–	–										
A3JA 14B	+	–	+	+										

Chromosome; +chromosome seen in at least 5/10 metaphases. Marker: +positive hybridization signal; – no hybridization signal. *: the hybrid cell lines have additional human chromosomes not listed in this table

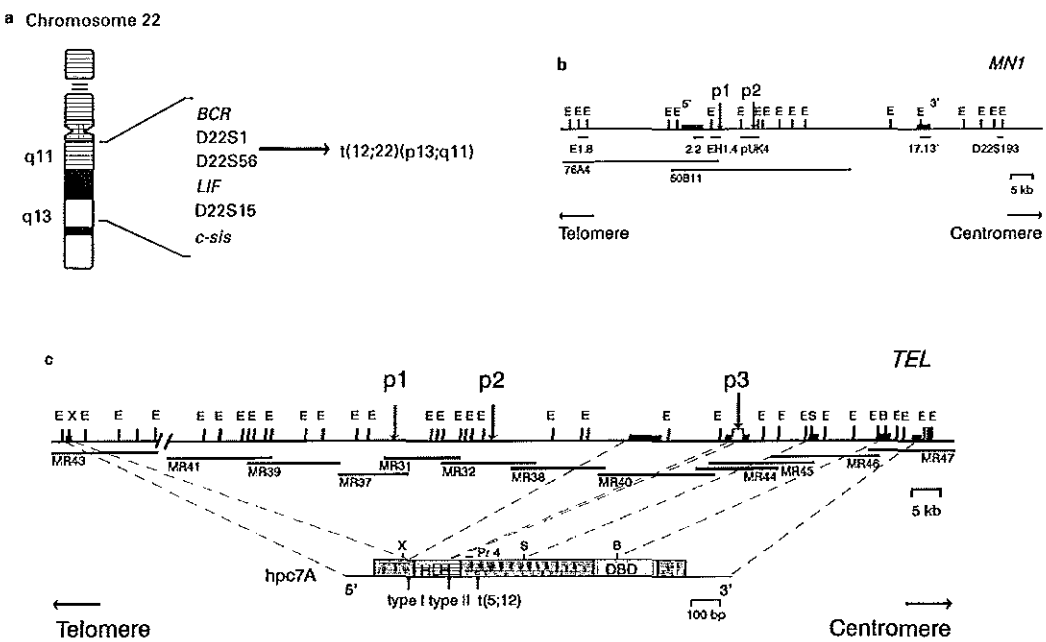

Figure 1 (a) Schematic representation of human chromosome 22. Indicated are several loci with respect to t(12;22)(p13;q11) (b) Restriction map of *MN1* on chromosome 22. Indicated below the map are probes E1.8, cDNA 2.2, EH1.4, pUK4, cDNA 17.1 3′ and D22S193 and cosmids clones 76A4 and 50B11. Localization of exons are indicated with black boxes. (c) Restriction map of *TEL* on chromosome 12. The t(12;22) breakpoints of patients 1, 2 and 3 are shown (p1, p2 and p3). Below the map, the phage contig is shown of which clones MR41 to MR40 were isolated by chromosome walking starting with chimeric phages containing the (12;22) breakpoint of p2. MR43 to MR47 were isolated by hybridization with TEL cDNA clone hpc7A to a genomic phage library. The cDNA hpc7A is depicted underneath the phage contig. The positions of the type I and II in t(12;22) and the t(5;12) fusions are indicated by arrows. Dashed lines indicate genomic fragments (black boxes) that cross-hybridize to hpc7A subfragments. These genomic fragments were delimited by various restriction enzyme sites, not shown in this map. HLH=Helix–Loop–Helix domain; DBD=ETS DNA-binding domain; E=*Eco*RI, S=*Sac*I, B=*Bam*HI, X=*Xho*I

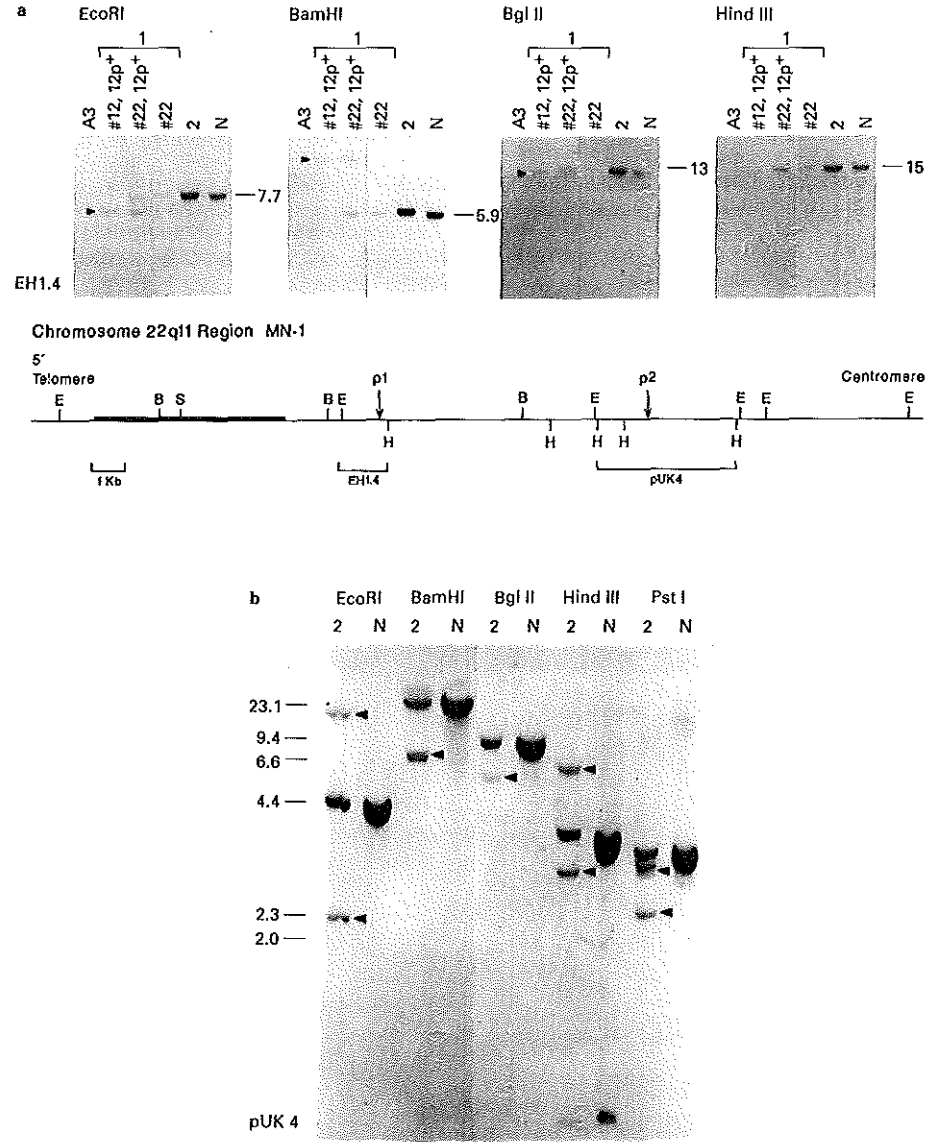

Figure 2 Localization of breakpoints on chromosome 22 in p1 and p2 (**a**) A Southern blot containing DNA of hamster cell line A3, hybrid cell lines with the segregated translocation chromosomes derived from bone marrow of p1, bone marrow of p2 (2) and human thymus DNA (N) was digested with *Eco*RI, *Bam*HI, *Bgl*I or *Hind*III. Southern blots were hybridized with a 1.4 kb *Eco*RI–*Hind*III fragment, located at the 5' side of the 60 kb intron in *MN1*. Sizes of normal fragments are indicated (in kb) and arrowheads indicate aberrant fragments (**b**) DNA of bone marrow from p2(2) and human thymus (N), digested with *Eco*RI, *Bam*HI, *Bgl*II, *Hind*III or *Pst*I was hybridized with 4.4 kb *Eco*RI fragment pUK4. Sizes of molecular weight markers are shown (in kb). Arrowheads indicate aberrant fragments. A map of the *MN1* region with localization of probes and the breakpoints of p1 and p2 is shown in the middle of the figure

and hamster/human hybrid cell lines A3JA 19A (#22), A3JA 19C (#12), A3JA 13B (#12p$^+$) and A3JA 12A (#12,22q$^-$) was digested with *Eco*RI and *Bam*HI. Southern blots were hybridized to 5'BCR, D22S1, D22S56, LIF, D22S15 and c-*sis* (for relative position of probes, see Figure 1a), respectively. The former two probes hybridized with DNA of hybrid cell lines A3JA 19A and A3JA 12A, whereas the latter four hybridized with DNA of hybrid cell lines A3JA 19A and A3JA 13B (Table 1), indicating that the breakpoint was located between D22S1 and D22S56. Subsequent hybridization with probes from this area, D22S193 and a single copy 1.8 kb *Eco*RI fragment, mapping between D22S1 and D22S56, revealed that D22S193 was not translocated in p1, while the 1.8 kb *Eco*RI fragment was. These two probes are derived from a

cosmid contig spanning the translocation breakpoint on chromosome 22q11 of a meningioma associated balanced translocation t(4;22)(p16;q11), in a patient with sporadic meningiomas (Lekanne Deprez *et al.*, 1991) (Lekanne Deprez *et al.*, 1995, this issue). The physical distance between the probes is 100 kb (Figure 1b), spanning a region containing a gene called *MN1*, consisting of two large exons separated by an intron of 60 kb, oriented with its 3′ end toward the centromere of chromosome 22 (Figure 1b). To analyse whether *MN1* was directly involved in t(12;22), we hybridized blots containing DNA of our hybrid cell lines with MN1 cDNA probes 2.2 (1.5 kb) and 17.1 3′ (1.4 kb), derived from the 5′ and 3′ exon, respectively (see Figure 1b). As shown in Table 1, cDNA 17.1 3′ hybridizes with DNA derived from cell line A3JA 12A (# 12,22q$^-$), but not with DNA derived from A3JA 1C (# 12,12p$^+$), while cDNA 2.2 hybridized with DNA derived from cell line A3JA 13B (# 12p$^+$). Thus the probes are separated by the translocation, indicating that *MN1* contains the 22q11 breakpoint of the t(12;22) in p1. To map the position of this breakpoint, genomic subfragments of cosmid 50B11 (see Figure 1b) were hybridized to Southern blots containing DNA of the hybrid of this patient digested with *Eco*RI, *Bam*HI, *Bgl*II and *Hind*III. One of these, probe EH 1.4 (Figures 1b and 2 a and b), detects a normal 7.7 kb *Eco*RI fragment in DNA of hybrid cell line A3JA 19A (#22), A3JA 2B (#22,12p$^+$) from p1, in bone marrow DNA of p2 and normal human thymus DNA (Figure 3b), but an aberrant fragment of 5.7 kb in cell lines A3JA 1C (# 12,12p$^+$) and A3JA 2B. To exclude an *Eco*RI polymorphism, aberrant fragments were also detected in *Bam*HI and *Bgl*II digested DNA of these cell lines (20 and 12 kb, respectively).

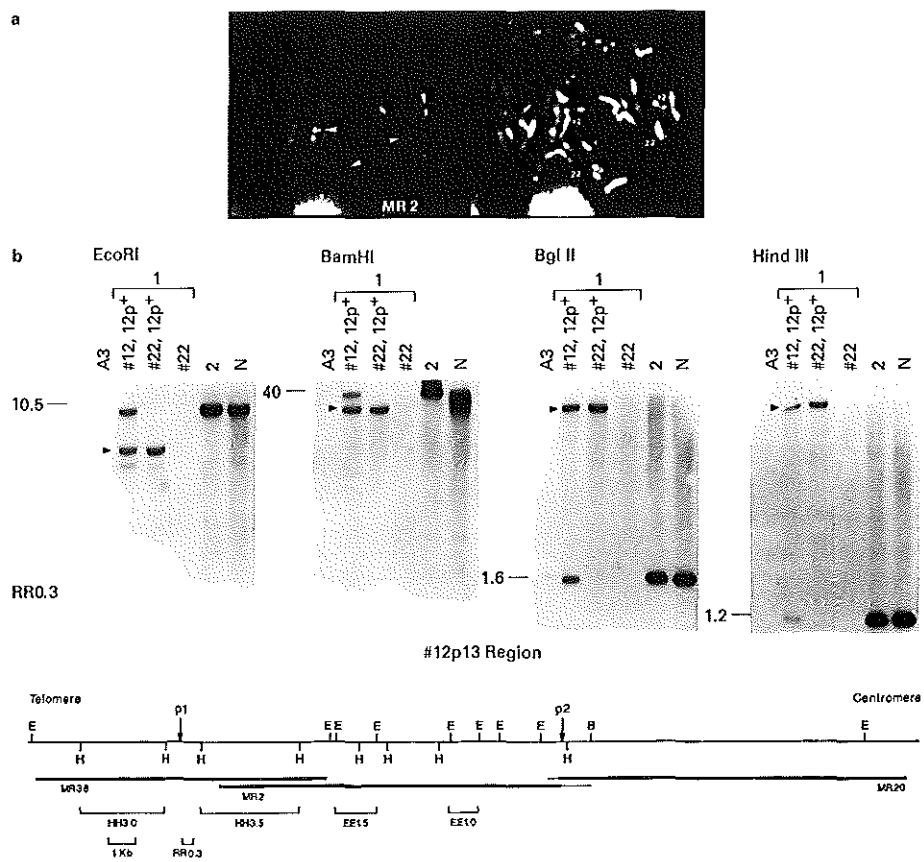

Figure 3 Fluorescent *in situ* hybridization and localization of the breakpoint on chromosome 12p13 in p1. (**a**) Chimeric *TEL-MN1* 12-22 phage clone MR2 from p2 was hybridized to metaphase chromosomes of normal human blood. Right panel shows position of chromosomes 12 and 22 with DAPI staining. Hybridization of MR2 to the short arm of chromosome 12 (strong signal) and the long arm of chromosome 22 (weak signal) is indicated with an arrow. Localization of chimeric phage clones MR 20 and MR2 on the physical map of the 12p13 region is shown at the bottom of (**b**). Sequences derived of chromosome 12 are indicated with bold lines whereas sequences derived from chromosome 22 are indicated with thin lines (**b**) DNA derived from hamster cell line A3, hybrid cell lines containing the relevant translocation chromosomes of p1 (1), bone marrow of p2 (2) and normal thymus (N) was digested with *Eco*RI, *Bam*HI, *Bgl*I or *Hind*III. The Southern blot was hybridized with a single-copy 0.3 kb *Rsa*I fragment (RR0.3). Sizes of normal fragments are indicated (in kb), aberrant fragments are indicated by an arrow. The physical map of the chromosome 12p13 region is shown at the bottom of the figure and the position of phage clones MR36 and MR2 and MR20 is indicated. Probes used to localize the 12p$^+$ breakpoint of p1 (1) are shown underneath

A similar approach was followed to find the breakpoint in the *MN1* gene in the bone marrow DNA of p2. The 4.4 kb *Eco*RI intronic fragment (pUK4; see Figure 1b and 2b) detects aberrant fragments of 20 kb and 2.2 kb in addition to the normal *Eco*RI fragment of 4.4 kb (Figure 2b). Aberrant fragments were also detected in *Bam*HI, *Bgl*II, *Hind*III and *Pst*I digested DNA of p2. These results indicate that the breakpoints on chromosome 22 in p1 and p2 are located in close proximity to one another at the 5′ side of the 60 kb intron of MN1.

Localization of the breakpoints on chromosome 12p

To identify the breakpoints of these patients on chromosome 12p13, a genomic λEMBL3 library (10^5 pfu) was constructed of bone marrow DNA from p2 (MERO 12/22 library). From the recombinant phages hybridizing with probe pUK4, eight out of 13 showed restriction patterns that in part diverged from the wild-type *MN1* restriction map and contained *Eco*RI fragments of aberrant size. Of these phages, MR20 and MR2 appeared to contain the chromosome 12p13 and 22q11 specific breakpoints, respectively (Figure 3b). To verify whether MR2 indeed contained sequences derived from chromosome 12, the phage was used as probe in a fluorescent *in situ* hybridization experiment (FISH) on metaphase chromosomes from human peripheral blood. As shown in Figure 3a, MR2 clearly hybridized to chromosome 12 band p13, confirming its alleged chromosomal position. Since this phage clone also contains 0.7 kb of chromosome 22 sequences, a faint signal on chromosome 22 is visible. Similar data were obtained with phage MR20 (not shown). Mapping of phage clones MR2, MR20 and wild-type phages of a contig derived from this region (Figure 1c), revealed that the breakpoint of p2 is located in a 1.0 kb *Eco*RI–*Hind*III fragment (see Figure 1c). The breakpoint on chromosome 12 in p1 could be detected by Southern blot analysis of the hybrid cell lines using chromosome 12-specific probes (HH3.0, HH3.5, EE1.5 and EE1.0, Figure 3b). Of these, HH3.5, EE1.5 and EE1.0 remain on the $12p^+$ chromosome, while HH3.0 is translocated to the $22q^-$ chromosome (not shown). A single copy 0.3 kb *Rsa*I fragment (RR0.3, Figure 3b) from this region, detected aberrant fragments containing the chromosome 12p13 breakpoint in *Eco*RI, *Bam*HI, *Bgl*II and *Hind*III digested DNA of hybrid cell lines A3JA 1C and A3JA 2B (Figure 3b). Exact mapping of the breakpoint showed that it is situated 13.5 kb telomeric from the 12p13 breakpoint in p2.

RACE-PCR cloning

Due to restricted amounts of patient material, we analysed the presence of aberrant mRNAs by RACE-PCR (rapid amplification of cDNA ends) (Frohman *et al.*, 1988; Belyavsky *et al.*, 1989). To analyse expression of 5′ chromosome 12/3′ MN1 chimeric mRNA, 1 μg of poly(A)$^+$ RNA of p2 was primed with a 3′ MN1-specific antisense primer for first strand cDNA synthesis (Figure 4a, primer 1). This product was then amplified using a 5′ anchor primer and a nested 3′ MN1 primer (Figure 4a, primer 2). A PCR fragment of 440 bp (not shown) was subcloned and sequenced. The fragment contains 282 bp of new 5′ sequences spliced to the 3′ exon of MN1. In the reading frame of the MN1 moiety of the clone, the new sequence contains an open reading frame with a possible AUG start codon 163 bp 5′ of the MN1 sequence. Comparison of the predicted protein sequence with the recently published sequence of TEL (Golub *et al.*, 1994), showed that they were identical. TEL is a member of the ETS family of transcription factors and contains, in addition to the carboxyl-terminal ETS DNA-binding domain, a putative helix–loop–helix (HLH) protein dimerization domain, located at the amino-terminal side of the protein. In t(5;12) in CMML the HLH domain of TEL is fused to the kinase domain of PDGFRβ. However, in our case, the breakpoint in the *TEL* gene occurs at a position 5′ of the HLH coding sequence. Therefore the TEL-MN1 mRNA would encode a protein of 12 kDa containing the first 54 amino acids of TEL fused to the last 59 amino acids of MN1, not including the HLH domain of TEL.

Expression of the complementary MN1-TEL fusion mRNA, was tested by RT-PCR analysis using antisense TEL primers (Figure 4a, primers 4 and 5). Poly(A)$^+$ RNA of p2 (1 μg) was used to perform a RT-PCR reaction with MN1 sense primer 3 and TEL antisense primer 4 (see Figure 4a). Part of this reaction was used for a second round of PCR with the same MN1 primer 3 and a nested antisense TEL primer 5 (see Figure 4a). After gel electrophoresis, a 550 bp product was visible (not shown), matching the size expected for the MN1-TEL fusion cDNA which was subsequently confirmed by sequence analysis. Therefore, the respective mRNA is calculated to encode a fusion product of 177 kDa, containing the first 1259 amino acids of MN1 fused to the last 397 amino acids of TEL, including the helix–loop–helix (HLH) protein dimerization domain (Figure 4a; type I).

Consistent expression of both fusion mRNAs was analysed in the other two t(12;22) patients, p1 and p3. RT-PCR analysis was done on 5 μg of total RNA of hybrid cell lines A3JA 2B (#22,$12p^+$), A3JA 1C (#12,$12p^+$), A3JA 20A (#12,$12p^+$) and A3JA 12C (#$22q^-$, $12p^+$) for p1 and on bone marrow RNA for p3. Unfortunately, RNA from hybrid cell lines had to be used for p1, since leukemic cells were no longer available. In all hybrid cell lines containing the $12p^+$ chromosome, the same 550 bp MN1-TEL cDNA was amplified, while in cell lines A3JA 14B (#12,22,$22q^-$) and A3JA 12C (#$12p^+$,$22q^-$) no TEL-MN1 cDNA could be amplified. RT-PCR amplification of p3 RNA from bone marrow cells produced both a TEL-MN1 and a MN1-TEL-specific product, but the first appeared 165 bp longer and the second 165 bp shorter than the concomitant fusion cDNA products of p1 and p2. Cloning and sequence analysis of these amplified fragments showed that the breakpoint in the TEL coding sequence occurs 165 bp farther 3′, explaining the discrepancy in size of the amplified cDNA fragments. The breakpoint in the TEL coding sequence is located within the HLH region, indicating that neither of the predicted fusion proteins will contain the intact HLH domain of TEL. We designate the fusion in p1 and p2 as type I and p3 as type II (Figure 4a). Figure 4b shows the sequence around the breakpoint areas of both types of reciprocal fusion cDNAs, which in type I occurs at TEL codon 55 and

in type II at codon 110. As a result the type II TEL-MN1 and MN1-TEL mRNAs would encode for 19 kDa and 170 kDa fusion proteins, respectively.

cDNA cloning and partial genomic organization of the TEL *gene*

A human placenta cDNA library of a complexity of 10^6 pfu was hybridized with the 5′ TEL-specific 246 bp *Eco*RI–*Xho*I fragment of the TEL-MN1 fusion cDNA fragment of p1. Many hybridizing plaques were isolated of which we analysed one with an insert of 1.7 kb (hpc7A) that contained the entire open reading frame of TEL as well as 5′ and 3′ UTRs of 106 bp and 199 bp, respectively. This cDNA clone was hybridized to the genomic MERO 12/22 λEMBL3 library of p2 and out of 100 hybridizing plaques 20 were isolated and analysed. A partial physical map of *TEL* is shown in Figure 1c. Restriction analysis of phage MR44 revealed that it has overlap with MR40, which belongs to a phage contig of 100 kb that was isolated with the 12p13 breakpoint of p2 as a starting point. Subfragments of hpc7A were hybridized to the entire phage contig to identify fragment containing exons. The 541 bp *Xho*I–*Sac*I TEL cDNA fragment hybridizes with the 2.2 kb *Xho*I–*Eco*RI fragment of MR43, the 12.5 kb *Sal*I–*Eco*RI fragment of MR40, the 8 kb *Eco*RI fragment of MR44 and the 4.3 kb *Eco*RI fragment of MR46. Sequencing of the 2.2 kb *Xho*I–*Eco*RI fragment of MR43 showed that the exon/intron border coincides with the type I fusion point in the cDNA. The breakpoints in the TEL fusion cDNAs of our p1 and p3 and the published CMML patients (Golub *et al.*, 1994) (i.e. 5′, within, and 3′ of the TEL HLH sequence, respectively) mark the borders of different *TEL* exons. Since the type II and t(5;12) CMML breakpoint map at least one exon apart and primer 4 (located between those breakpoints) hybridizes with the 8 kb *Eco*RI fragment in phage MR44 (Figure 1c), we deduce that the breakpoint of p3 is located within an intron between these exons within this fragment. From the hybridization pattern of 5′ and

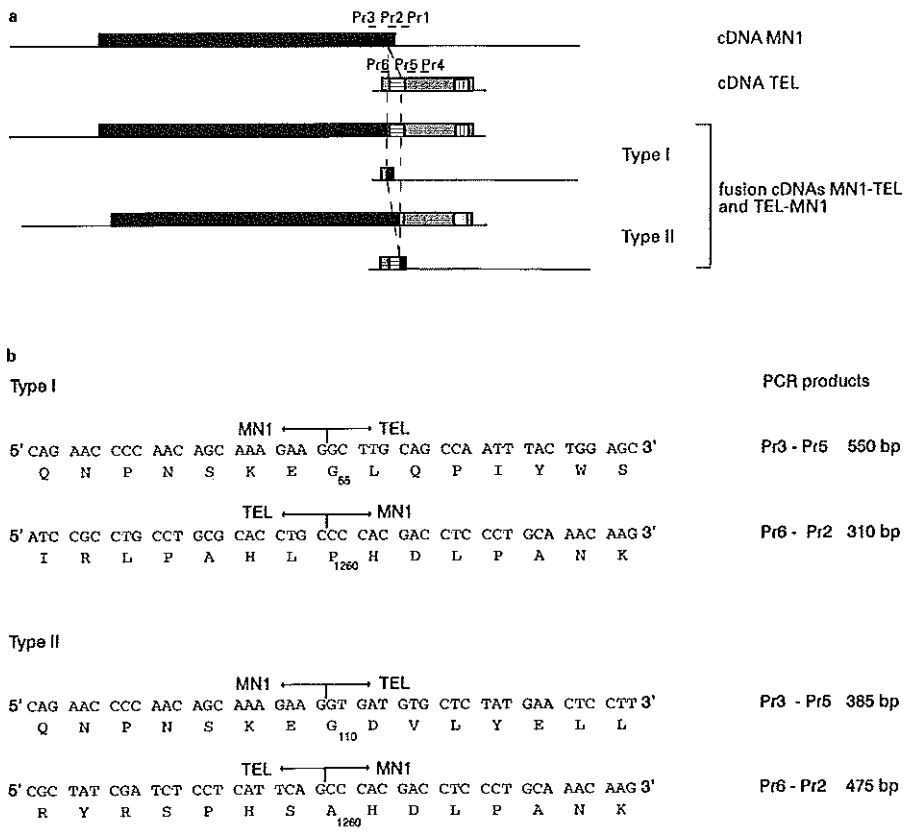

Figure 4 Schematic representation of cDNAs and breakpoint regions in t(12;22) (a) cDNAs of normal MN1 and TEL and the reciprocal type I and II fusion products. ORFs of MN1 and TEL are shown in black and gray, respectively. Horizontally striped boxes indicate the HLH domain of TEL, whereas vertically striped boxes indicate the ETS-domain. Primers used in RT-PCR reactions are indicated above the MN1 and TEL cDNAs. (b) Sequences of reciprocal fusion cDNAs around the type I and II breakpoints. Amino acid sequences deduced from the nucleotide sequence are shown in one-letter code below. Numbers indicate in which codon of TEL and MN1 the fusion occurs for both types of translocations. The number on the right side indicates the length of the PCR fusion cDNAs with the different primer combinations

3′ genomic *TEL* fragments to the panel of hybrid cell lines of p1 we conclude that the gene is located in a 5′ telomeric 3′ centromeric orientation on 12p13. This orientation is in agreement with the orientation of the PDGFRβ (Morris *et al.*, 1992) on chromosome 5q33 and *MN1* on chromosome 22q11 (Lekanne Deprez *et al.*, 1995, this issue), predicting that fusion of these genes with *TEL* are the product of conventional balanced translocations.

Discussion

In this paper we show the existence of new leukemia specific fusion genes *TEL-MN1* and *MN1-TEL* as the result of the t(12;22) in different myeloid malignancies. The fact that in three out of three patients the same two genes are involved in the translocation provides compelling evidence for a direct role of the fusion products in the leukemic process. In contrast to most chromosome translocations both chimeric gene derivatives are expressed in patient bone marrow cells. However, the observation is not unique as it was consistently found in acute promyelocytic leukemia (APL) (Alcalay *et al.*, 1992), where both PML-RARα and RARα-PML RNAs are expressed. In the hybrid cell lines of p1, containing chromosome 22q$^-$, no TEL-MN1 transcript could be amplified. Since the transcript was present in bone marrow cells of the two other patients, we suspect that its absence is due to silencing in the hybrid cell lines. Although we find both fusion RNAs using RT-PCR, we do not know their relative levels of expression in patient cells. Due to lack of patient material, we were not able to perform Northern blot hybridizations and we did not attempt to quantitate the RT-PCR approach.

The translocation involves the recently described ETS-related gene, *TEL*, and a gene with a possible role in meningioma, *MN1*. Golub *et al.* (1994) found *TEL* to be fused to the PDGFRβ gene in CMML, a specific subtype of myelodysplastic syndrome (MDS). TEL mRNA encodes a typical ETS-related transcription factor belonging to the subgroup that have the DNA-binding domain at the carboxyl-terminal end and a HLH protein-protein interaction domain at the amino-terminal side (Wasylyk *et al.*, 1993). In CMML, only the TEL-PDGFRβ fusion transcript is expressed, while the complementary fusion gene, which would contain the DNA-binding domain, is silent. Because the breakpoint occurs just 3′ of the HLH-domain, Golub *et al.* (1994) suggested that, in addition to ectopic expression of the PDGFβ receptor by the *TEL* promoter, the putative dimerization motif may be important for ligand independent tyrosine kinase activation of the TEL-PDGFRβ fusion protein. In our fusion products, the breakpoint occurs just 5′ or within the HLH region (depending on the type of fusion), suggesting that this dimerization domain is nonessential for the function of the fusion proteins in t(12;22) leukemic cells.

The possible function of the MN1 protein is much less clear. As shown by Lekanne Deprez *et al.* (1995, this issue) the gene was cloned from a t(4;22) in an isolated case of meningioma and the encoded protein does not show significant homology to any known protein sequences. The only clue obtained so far is its alleged nuclear localization. This preliminary observation in combination with the glutamine/proline-rich amino-terminal sequence of the protein, a feature found in the activation domains of transcription factors (Gerber *et al.*, 1994), may indicate a role in transcription activation. However, whether MN1 would function as a classic DNA-binding transcription factor or as transcription factor associating protein remains to be determined.

As yet there are no clues whether TEL-MN1, MN1-TEL or both are important for transformation of early myeloid precursors in t(12;22). Upon evaluation of their features we favor the latter product as a potential oncogene, since it has the structure of an altered transcription factor. It contains the carboxyl-terminal part of the TEL protein providing the ETS DNA-binding domain linked to almost the entire MN1 protein, including the glutamine/proline-rich stretches. This structure is highly reminiscent of the EWS-FLI1 and EWS-ERG1 fusion proteins in Ewing's sarcoma, where the glutamine/proline-rich amino-terminal part of the EWS protein is linked to the ETS domains of FLI1 or ERG (Delattre *et al.*, 1992; Sorensen *et al.*, 1994). It was shown that substitution of normal amino-terminal FLI1 by EWS sequences creates a transcription factor with much stronger transactivation potential (May *et al.*, 1993; Bailly *et al.*, 1994). Although it has not been analysed, it is reasonable to speculate that this is in part due to its glutamine/proline-rich sequences of EWS, making it plausible that MN1-TEL could have similar characteristics. In addition, fusion of MN1 could enhance the DNA binding affinity of the TEL moiety of the protein, by interference with a possible intra-molecular interaction in TEL that was shown to regulate DNA binding in several other ETS family members (Lim *et al.*, 1992; Wasylyk *et al.*, 1993). Both possibilities are currently under investigation.

Regulation of transcription activity of several members of the ETS family, like Elk1 and SAP in mouse (Janknecht *et al.*, 1993; Marais *et al.*, 1993; Hipskind *et al.*, 1994) and pointed and yan in *D. melanogaster* (Brunner *et al.*, 1994), is directed by phosphorylation by mitogen activated protein kinase (MAPK), that in turn is activated by several signal transduction pathways, one of which is the RAS pathway. It is interesting to note that TEL contains three candidate MAPK sites (Clark-Lewis *et al.*, 1991; Gonzalez *et al.*, 1991) at amino acid positions 20–23, 200–204 and 255–258 indicating that activity of the protein may be regulated by MAPK, via the RAS pathway. In this respect it is interesting that a number of t(12;22) patients, including p2, presents with a CML-like disease (Mitelman, 1991) while in CML tumorigenicity of the BCR-ABL protein in part depends upon continuous activation of the RAS signaling pathway (Pendergast *et al.*, 1993). In much the same way, the supposedly activated tyrosine kinase of the TEL-PDGFRβ protein in CMML would result in activation of the RAS pathway. This indicates that RAS activation (Sawyers and Denny, 1994) and thereby probably activation of members of the ETS family of transcription factors plays a central role in the genesis of myeloid leukemia. Also the description of an altered *ERG* gene in AML carrying a t(16;21) (Shimizu *et al.*, 1993) could conform to this general

picture. Although TEL mRNA is ubiquitously expressed, it remains to be analysed whether the protein is a natural target of the RAS pathway. Alternatively, if TEL is not a downstream target of RAS, the activated MN1-TEL protein may feed into the same downstream processes by competition for DNA binding sites that would normally be occupied by RAS-responsive ETS proteins, activating genes essential for myeloid transformation.

To date, we have isolated 155 kb of genomic *TEL* sequences. Since the map of the large intron is incomplete and genomic DNA encoding the 5′ and 3′ UTRs still needs to be isolated, the gene may extend much farther. The involvement of *TEL* in t(5;12) (Golub *et al.*, 1994) and t(12;22), in combination with detection of 12p12-13 aberrations in 0.5–2% of myeloid (Adriaansen, 1992) and 10% of pediatric lymphoid (Raimondi *et al.*, 1986) malignancies make this gene a prime candidate for other chromosomal translocations or deletions involving this region. This possibility is currently under investigation. In contrast to most cases with chromosome 12p12-13 abberations (Mitelman, 1991), t(12;22) is often the sole karyotypic abnormality, despite the variable phenotype of the leukemia. We favor the idea that additional, undetected mutations lead to diversification, while activation of TEL is only one of the necessary steps in malignant transformation. In this respect it is worthwhile to mention that p2 and p3 showed the translocation at relapse after treatment with cytostatic agents.

The most remarkable feature of TEL in these translocations seems that separate domains of the protein contribute to distinct fusion proteins that, although different in function, could lead to deregulation of the same downstream targets needed for transformation of myeloid progenitors.

Materials and methods

Patients and cell lines

Patient 1 (p1), a 19-year-old woman was diagnosed with AML-M4. Cytogenetic analysis on bone marrow showed a karyotype 47,XX, +8, t(12;22)(p13;q11). Patient 2 (p2), a 58-year-old man, was diagnosed with (Ph chromosome negative) CML. Cytogenetic analysis of bone marrow showed a normal karyotype. Treatment with hydroxyurea was started and clinical status improved. However, 2 years later the patient relapsed with accelerated disease. Cytogenetic analysis of blood and bone marrow revealed a karyotype 46,XY, t(12;22)(p13;q12). Clinical and cytogenetic status has recently been described in detail (Geurts van Kessel *et al.*, 1994). Patient 3 (p3), a 15-year-old boy, was diagnosed with myelodysplastic Syndrome (RAEB). At diagnosis the karyogram was normal. Antileukemic treatment was started. One year later cytogenetic analysis revealed a karyogram 46,XY, t(12;22)(p13;q11).

A hamster/human hybrid cell line panel was generated by fusion of hamster fibroblast cell line A3 with bone marrow cells of p1 using inactivated Sendaivirus (Geurts van Kessel *et al.*, 1981). Hybrid cell lines were cultured in Ham's F10 medium + 10% FCS supplemented with 1 × HAT. Cytogenetic analysis of patient material and hybrid cell lines was done according to standard methods (Hagemeijer *et al.*, 1979).

Southern blotting

DNA of relevant hybrid cell lines was digested with various enzymes and electrophoresed on a 0.7% agarose gel. DNA was blotted onto nylon membranes (Hybond N^+, Amersham, UK). Filters were hybridized according to standard procedures (Sambrook *et al.*, 1989) with probes labeled by random priming (Feinberg and Vogelstein, 1983). Filters were washed at a stringency of 0.3 × SSC at 65°C.

Construction of genomic λEMBL3 library

A genomic λEMBL3 library was constructed of 300 μg DNA of bone marrow from p2 according to Frischauf *et al.* (1983). Recombinant phages (10^6) were plated and screened with a 4.4 kb *Eco*RI genomic fragment (pUK4) of *MN1*. Phages that showed a restriction pattern diverging from wild-type *MN1* phages were considered as putative breakpoint containing recombinants and were analysed for chromosomal localization by FISH (Hagemeijer *et al.*, 1993). A genomic map of chromosome the 12p13 region was generated by chromosome walking. Before hybridization, probes were competed with 100 μg of human competitor DNA (Sigma, St. Louis, MO) for 1.5 h in 100 μl 5 × SSC at 65°C.

Fluorescent in situ *hybridization*

FISH was done according to Hagemeijer *et al.* (1993). Chromosomes were identified by 4′, 6′-diamino-2-phenyl indole (DAPI) banding, using a fluorescence microscope equipped with FITC and DAPI filters.

RNA isolation

Total RNA was isolated from bone marrow of patients or from A3JA hybrid cell lines using the acidic guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was isolated using the PolyA Tract mRNA Isolation System III (Promega, Madison, WI).

RACE cloning

Poly(A)$^+$ RNA (1 μg) of p2 was used to clone 5′ TEL–3′ MN1 fusion cDNA using the 5′-AmpliFINDER™ RACE Kit (Clontech Laboratories, Palo Alto, CA). First strand cDNA was synthesized with MN1 antisense primer 1 (5′-AGTTAGGGCAGCCACGAATG-3′). A nested antisense MN1 primer 2 (5′-CTTGAATTCCCAAATCTGTTG-GAG-3′) was used in the PCR reaction. PCR was performed using three cycles of denaturation (45 s, 94°C), annealing (45 s, 45°C) and extension (2 min, 72°C, followed by 32 cycles of denaturation (45 s, 94°C), annealing (45 s, 62°C) and extension (2 min, 72°C). PCR products were visualized by electrophoresis on a 2% agarose gel. PCR products were phenol/chloroform extracted, precipitated and digested with *Eco*RI. After digestion the products were subcloned in pBluescript KS$^-$ (Stratagene, La Jolla, CA) and sequenced using USB Sequenase™ Version 2.0 DNA sequencing Kit (USB, Cleveland, OH).

cDNA cloning

A human placenta cDNA library in λgt11 (10^6 pfu) (Clontech Laboratories, Palo Alto, CA) was screened using a 5′ TEL-specific 246 bp *Eco*RI–*Xho*I cDNA fragment of p2, resulting in the isolation of a 1.7 kb TEL cDNA clone hpc7A.

RT-PCR

MN1-TEL and TEL-MN1 junctions were amplified using two sets of primers. 5 μg total RNA or 1 μg poly(A)$^+$ RNA was used to synthesize first strand cDNA with MN1 antisense primer 1 with AMV Reverse Transcriptase (Promega, Madison, WI) at 42°C. PCR was performed using one cycle of denaturation (5 min, 94°C), annealing (5 min, 59°C) and extension (5 min, 72°C), followed by 24 cycles of denaturation (2 min, 94°C), annealing (2 min, 59°C) and extension (3 min, 72°C) with TEL sense primer 6 (5'-AGTGTAGCATTAAGCAGGAACG-3'). 2.5 μl of PCR reaction was used in a second round of PCR with TEL primer 6 and MN1 primer 2 (Figure 4b), using three cycles of denaturation (2 min, 94°C), annealing (2 min, 47°C) and extension (3 min, 72°C) followed by 30 cycles of denaturation (2 min, 94°C), annealing (2 min, 60°C) and extension (3 min, 72°C). In case of MN1-TEL junction cDNA fragments TEL antisense primer 4 (5'-GTATGACCTCCGGCTG-3') was used to synthesize first strand cDNA. PCR was performed using MN1 sense primer 3 (5'-TCCAGCTACAGAGGCA-3') for one cycle of denaturation (5 min, 94°C), annealing (5 min, 47°C), and extension (5 min, 72°C) followed by 24 cycles of denaturation (2 min, 94°C), annealing (2 min, 47°C) and extension (3 min, 72°C). 2.5 μl of this PCR reaction was used in a second amplification with nested antisense TEL primer 5, (5'-CCAGGGTGGAAGAATG-3'), for 30 cycles of denaturation (2 min, 94°C), annealing (2 min, 47°C) and extension (3 min, 72°C). 10 μl of the PCR reaction was electrophoresed on a 2% agarose gel and blotted onto nylon membranes (Hybond N$^+$, Amersham, UK). Membranes were hybridized using either MN1 cDNA 2.2 probe and a 246 bp *Eco*RI–*Xho*I TEL-specific 5' cDNA fragment. PCR products were subcloned and sequenced.

Acknowledgements

Part of the work of authors AB, SVB and GG was performed at the Department of Cell Biology and Genetics, Erasmus University Rotterdam, The Netherlands. The authors wish to thank Bep Smit, Ellen van Drunen, Susan Ragdale and Virginia Valentine for cytogenetic analysis of hybrid cell lines and fluorescent *in situ* hybridization experiments. Drs ten Haaft (Maastricht, The Netherlands), Oosten (Nijmegen, The Netherlands) and Verhoef (Leuven, Belgium) for patient material, Prof Dr Bootsma and Robbert Rottier for helpful suggestions and discussions and Peggy Burdick for secretarial assistance. These studies were supported in part by the NIH Cancer Center Support CORE Grant P30 CA21765 and the American Lebanese Syrian Associated Charities (ALSAC).

EMBL Nucleotide Sequence Database accession numbers: X85024 TEL-MN1 (type II): X85025 MN1-TEL (type I): X85026 TEL-MN1 (type I): X85027 MN1-TEL (type II).

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Chapter 2.2.

The MN1-TEL fusion protein, resulting from the t(12;22)(p13;q11) in myeloid leukemia, is a chimeric transcription factor that transforms NIH3T3 cells

The MN1-TEL fusion protein, resulting from the t(12;22)(p13;q11) in myeloid leukemia, is a chimeric transcription factor that transforms NIH3T3 cells

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Abstract

Recently we have characterized the *TEL* gene (or *ETV6*) as the target of the t(12;22)(p13;q11) in myeloid leukemia. TEL is a member of the ETS family of transcription factors and contains a Helix-Loop-Helix (HLH) protein interaction domain and an ETS DNA binding domain. By contrast to other chimeric proteins containing TEL, such as TEL-PDGFβR in t(5;12)(q33:p13), TEL-ABL in t(9;12)(q34:p13) and TEL-AML1 in t(12;21)(p13;q21) that all contain TELs HLH domain, MN1-TEL contains the ETS DNA binding domain of TEL. Its N-terminal MN1 moiety is rich in proline residues and contains two polyglutamine stretches. These characteristics suggest that MN1-TEL may function as an activated transcription factor (e.g., EWS-FLI1 in Ewing sarcoma). We analyzed the subcellular localization, transforming potential, and transcriptional transactivation properties of MN1-TEL to understand its role in leukemogenesis. Using immunofluorescence analysis, we showed that MN1-TEL localizes predominantly in nuclear speckles. In contrast, MN1 and TEL were expressed in a more diffuse nuclear pattern. Unlike TEL and MN1, MN1-TEL transformed NIH3T3 cells as assayed by growth in soft-agar assays. This activity required N-terminal MN1 sequences as well as a functional ETS domain in TEL. Both MN1 and MN1-TEL greatly increased transcription from the Moloney sarcoma virus LTR. The transactivating capacity of MN1-TEL depended on both the ETS domain and sequences in MN1; these MN1-associated sequences differed from those essential for transformation of NIH3T3 cells. Because the fusion protein was functionally dependent on the ETS domain for both activities we characterized the consensus DNA recognition sequence of TEL. We demonstrate that TEL shows homotypic interaction dependent on its helix-loop-helix (HLH) domain. Since in MN1-TEL this domain seems not to be functional, our results suggest that MN1-TEL contributes to leukemogenesis by a mechanism distinct from that of other chimeras containing TEL.

Introduction

Nonrandom chromosomal abnormalities are hallmarks of underlying gene alterations that have a direct causative role in tumorigenesis (Rabbitts, 1994). Translocations affecting the 12p13 region are associated with myeloid and lymphoid malignancies (Mitelman, 1991). TEL (or ETV6), a member of the ETS family of transcription factors, is the target of several translocations involving chromosome 12 band p13, including t(5;12)(q33;p13) and t(9;12)(q34;p13), in which the C-terminus of TEL (including its ETS DNA binding domain) is replaced by the kinase domains of PDGFβR or ABL, respectively (Golub *et al.*, 1994; Papadopoulos *et al.*, 1995). The t(12;21)(p13;q22) combines the RUNT DNA binding domain of AML1 with the N-terminal region of TEL (Golub *et al.*, 1995; Romana *et al.*, 1995). The oncogenic activation of these three fusion proteins may rest (at least in part) upon the helix-loop-helix (HLH) protein interaction domain in the N-terminal portion of TEL. For example, the TEL-ABL fusion proteins interact via the HLH domain *in vitro*, and activation of the tyrosine kinase activity of ABL requires this interaction (Golub *et al.*, 1996).

We recently characterized the t(12;22)(p13;q11), which is associated with myeloid leukemias and myelodysplastic syndromes (Buijs *et al.*, 1995). In t(12;22), N-terminal sequences of TEL are replaced by almost the entire MN1 protein. Depending on the breakpoint in TEL two distinct fusion proteins may be encoded, in which fusion of MN1 occurs either N-terminal of (type I) or within (type II) its HLH oligomerization domain. Although its function is unknown, MN1 seems to be a nuclear protein (Lekanne Deprez *et al.*, 1995), and its N-terminal region is rich in prolines and contains two polyglutamine stretches. These features are common to many transcription factors (Gerber *et al.*, 1994).

In contrast to the translocations mentioned above, the product of t(12;22) retains the ETS domain of TEL. In this way, MN1-TEL is similar to EWS-FLI1, an activated transcription factor that is associated with Ewing sarcoma. Like TEL, FLI1 is a member of the ETS family of transcription factors, and the ETS domain is retained in EWS-FLI1. Further, expression of EWS-FLI1 causes transformation of NIH3T3 cells *in vitro* (May *et al.*, 1993a; 1993b), and this activity requires the ETS domain of FLI1 as well as transactivation- and transformation-specific sequences in EWS (Bailly *et al.*, 1994; Lessnick *et al.*, 1995).

In light of structural similarities to EWS-FLI1, MN1-TEL may function as an activated transcription factor. Therefore, we analyzed the subcellular localization, transforming potential, and transactivation properties of MN1-TEL to understand its role in leukemogenesis. Our results suggest that MN1-TEL is implicated in leukemogenesis by disregulation of the TEL transcription cascade.

Materials and Methods

Cell lines

The NIH3T3 (mouse fibroblast), COS-1 (simian kidney carcinoma), HeLa (human cervical carcinoma), and 293T (human embryonic kidney) cell lines were grown in DMEM supplemented with 10% fetal bovine serum.

Plasmids

See Figure 1 for schematic diagrams of the various cDNA constructs used in our experiments. These inserts were cloned into the CMV promoter containing expression vector pSCTOP (Fornerod *et al.*, 1995) or into the retroviral vector pSRαMSVtkCD8 (Hirai *et al.*, 1995). This second vector enables selection of transduced cells based on the expression of murine CD8. The integrity of all mutant cDNAs was verified by sequence analysis. N-terminal tagged TEL was generated by cloning of a triple influenza hemagglutinin tag (Fornerod *et al.*, 1995) into the *Afl*III site within the first codon of the TEL cDNA clone hpc7a (Buijs *et al.*, 1995). The deletion mutant TELΔ53-116 was generated by the in-frame deletion of the 192 bp *Fsp*I-*Xmn*I fragment of TEL. We created a full-length MN1-TEL type I cDNA (MN1-TEL I) by a three way ligation of the 3736 bp *Sac*II-*Nsp*I MN1 cDNA fragment (Lekanne Deprez *et al.*, 1995), the 1244 bp *Cla*I-*Eco*RI TEL fragment and the *Nsp*I-*Cla*1 MN1-TEL fusion cDNA fragment obtained from patient 2 (Buijs *et al.*, 1995). To generate MN1-TEL type II (MN1-TEL II), we ligated the same 3736 bp *Sac*II-*Nsp*I MN1 cDNA fragment to a 1209 bp *Xmn*I-*Eco*RI TEL cDNA fragment and to the *Nsp*I-*Xmn*1 fusion cDNA product from patient 3 (Buijs *et al.*, 1995). The various MN1-TEL deletion mutants were obtained by deleting internal restriction fragments (shown in parentheses) from the MN1 moiety of MN1-TEL I. These mutants included MN1-TELΔ692-1123 (1296 bp *Pml*I-*Srf*I), MN1-TELΔ18-1123 (3335 bp *Hin*cII-*Srf*I), MN1-TELΔ12-228 (651 bp *Hin*cII), MN1-TELΔ18-454 (1311 bp *Msc*I), MN1-TELΔ12-951 (2803 bp *Msc*I-*Pml*I), and MN1-TELΔ229-1223 (2985 bp *Hin*cII-*Eco*47III). To generate a TEL mutant that was incapable of binding DNA (TEL-DBDM), we performed site directed mutagenesis on full-length TEL cDNA in bacteriophage M13. We changed codons 396 and 399 from arginine to leucine by using the oligonucleotide 5'-GAGAAAATGTCCTTAGCCCTGCTCCACTACTACAA-3' and obtained mutant phage according to manufacturers recommendations (Biorad, Hercules, CA). These arginine residues are conserved among all ETS factors and interact with the DNA α-helix (Kodandapani *et al.*, 1996). The VP16-TEL fusion construct was created by PCR amplification of herpes simplex virus 1 (HSV1) VP16 codons 413-489 (Dalrymple *et al.*, 1985) with the primers VP16COOH (5'-CCCAAGCTTGCCGCCACCATGGCCCCCCCGACCGAT-3') and VP16*Bbs*I (5'-CAGGCGGATCGAGTCTTCGTACTCGTCAATTCCA-3'), using pRG50 as template (kindly provided by Dr O'Hare). Primer VP16COOH introduces a *Hin*dIII cloning site, a Kozak consensus sequence for initiation of translation (Kozak, 1989), and substitutes an ATG for codon 412 of VP16 to provide a translation initiation site in the VP16-

TEL cDNA construct. Primer VP16*Bbs*I contains codons 485-489 of VP16, followed by a *Bbs*I restriction site. The cycling conditions were 94°C x 2 min, 55°C x 2 min, and 72°C x 3 min for 2 cycles followed by 24 cycles of 94°C x 2 min, 65°C x 2 min, and 72°C. The resulting 263 bp *Hin*dIII-*Bbs*I HSV1 VP16 cDNA fragment was cloned into the *Bbs*I site at codon 45 of TEL. We created the pMSVluc reporter plasmid by cloning the 1.2 kb *Xho*I-*Hin*dIII fragment, containing the 5' MSV LTR of pSRαMSVtkneo (Muller *et al.*, 1991), into pGL2-Basic (Promega, Madison,WI).

Retroviral transduction

Retroviruses were generated by using calcium phosphate transfection (Graham & Eb, 1973) of $3x10^6$ 293T cells (in a 10 cm dish) with 10 μg of the appropriate pSRαMSVtkCD8-based construct (Hirai *et al.*, 1995) and 10 μg of the ecotropic, replication-defective helper virus pSV-ΨE-MLV DNA (Muller *et al.*, 1991). After 20 hours, the precipitates were removed, and virus-containing supernatants were harvested for 42 hours at 4-8 hour intervals. The supernatants were filtered over 0.45 μm gauze filters. We then overlaid $2x10^5$ NIH3T3 fibroblasts for 3 hours (in a 10 cm dish) with 1.5 ml of high-titer supernatant that contained 6 μg/ml polybrene. CD8-expressing cells were collected by using fluorescence-activated cell sorting (FACS) 60 hours after infection.

Antibodies

A synthetic peptide containing the 10 C-terminal amino acids of TEL (Golub *et al.*, 1994) was conjugated to keyhole limpet hemocyanin and injected into New Zealand White rabbits (Rockland, Gilbertsville, PA) according to standard techniques (Harlow and Lane, 1988). Immunopurified α-TEL antibodies were obtained using affinity purification of the α-TEL serum 32 over a synthetic C-terminal TEL peptide-coupled Affi-Gel 10 column (Biorad, Hercules, CA). Monoclonal antibody (MoAb) 2F2 was raised against a bacterially expressed N-terminal MN1 fusion protein, and will be described in detail elsewhere (A.M.and E.Z.; manuscript in preparation). MoAb 12CA5, recognizing an influenza hemagglutinin tag has been described before (Wilson *et al.*, 1984).

Immunofluorescence analysis

Pools of $1x10^5$ virus-infected, $CD8^+$-positive cells were seeded on microscope slides. After 24 hours, the cells were fixed in 3% paraformaldehyde for 15 min and permeabilized with 0.2% Triton in PBS for 10 min. The fixed cells were then incubated for 2 hours at room temperature with immunopurified α-TEL (1:1250 in PBS/1% BSA), 2 μg/ml 12CA5 or MN1 MoAb 2F2 (1:1000 in PBS/1%BSA). Bound antibodies were visualized by using fluorescein isothiocyanate-conjugated goat anti-rabbit or Texas Red-conjugated goat anti-mouse secondary antibodies as described (Fornerod *et al.*, 1995). Images were obtained by using confocal microscopy (BioRad MRC1000 Laser Scanning confocal microscope).

Transformation analysis

For each transduced construct, we plated triplicate samples of $2x10^4$ CD8-positive fibroblasts into soft agar (3 cm dish); the culture medium was Iscove's medium supplemented with 15% fetal bovine serum (Lugo & Witts, 1989). Colonies were counted 21 days after plating.

Immunoprecipitation and Western blotting

By using calcium phosphate precipitation, $2x10^5$ HeLa or COS-1 cells (6 cm dish) were transfected with 10 µg of the appropriate pSCTOP-based expression vector. After 20 hours, the precipitate was removed and 36 hours later, the cells were metabolically labeled for 12 hours with 100 µCi ^{35}S-methionine/cysteine *in vivo* labelling mix (Dupont NEN, Wilmington, DE) or ^{3}H-leucine (Amersham Corp., Arlington Heights, IL) in 1.4 ml of methionine/cysteine or leucine-free DMEM, supplemented with 8% dialyzed fetal calf serum. The labeled cells were washed twice with ice-cold PBS, then lysed and immunoprecipitated with MoAb 12CA5 (BAbCo, Richmond, CA) as described, except that the lysisbuffer contained 1% IGEPAL CA630 (Sigma Chemical Corp. St. Louis, MO) in stead of 1% NP40 (Fornerod *et al.*, 1996). Immunoprecipitates were separated by SDS-PAGE and visualized by using autoradiography or electroblotted unto PVDF membrane (Millipore, Bedford, MA). Blots were blocked overnight in PBS containing 1% BSA, and then incubated for two hours with α-TEL serum 32 diluted 1 in 1000 in PBS/1%BSA. Bound antibody was visualized by using alkaline phosphatase-conjugated antirabbit IgG secondary antibodies using colorimetry.

Determination of the consensus DNA-binding sequence of TEL

A full-length cDNA representing the TEL open reading frame was PCR-amplified from TEL cDNA clone hpc7a by using the primers 5'-AGATCTGAGACTCCTGCTCAG- 3' and 5'-AGATCTGCATTCATCTTCTTGGTA-3'. A cDNA corresponding to the TEL ETS domain (codons 315-452) was generated through PCR amplification of the same template by using primer 5'-AGATCTATGAACCACATCATGGTC-3' and the antisense primer used for the full-length cDNA. All three primers contained *Bgl*II restriction sites for the in-frame cloning of the *Bgl*II-digested fragments into the *Bam*HI site of pGEX-2T (Pharmacia Biotech, Uppsala, Sweden). The GST-TEL and GST-ETS domain fusion proteins were expressed in *E.coli* and purified as previously described (Smith & Johnson, 1988). The binding site was selected as previously described (Inaba *et al.*, 1994).

DNA binding assays

Electrophoretic mobility shift assays (EMSA) of the purified GST-TEL and GST-ETS fusion proteins were done by using 3 fmol ^{32}P-endlabeled double-stranded probes (the consensus TEL DNA-binding site or the mutant oligonucleotide probes 5'-GCATGTCCGGAAGTAGTGCC-3' and 5'-GCATGTCCCCAAGTAGTGCC-3'). GST-fusion protein was incubated with 1 µl of anti-TEL serum 32 for 2 hours on ice. The appropriate probe and protein were incubated on ice for 20 minutes in 20 mM HEPES-KOH (pH 7.9), 1 mM EDTA, 1 mM EGTA, 4% Ficoll (total

volume, 20 µl). Complexed and free probe were separated on a 4% polyacrylamide gels in 0.25 x TBE and visualized by using autoradiography (Meijer *et al.*, 1992).

Transactivation analysis

For each expression construct, we transfected triplicate dishes of $1.5x10^5$ NIH3T3 cells (6 cm dish) with 3 µg of the appropriate pSCTOP-based vector, 1 µg pMSVluc, 150 ng of rat β-actin promoter driven secreted alkaline phosphate expression construct and 5.9 µg pBluescript (as carrier). The medium was changed after 20 hours, and luciferase assays (Promega, Madison WI) were performed on transfected cells 24 hours later. Luciferase activity was measured with a Optocomp illuminometer. To control for transfection efficiency, the alkaline phosphatase activity in the medium was measured as described (Owen *et al.*, 1990). Induction of transactivation equaled the corrected activity associated with insert-containing pSCTOP plasmid divided by the activity associated with empty pSCTOP.

Results

Subcellular distribution of TEL, MN1, and MN1-TEL

Because the primary sequences of TEL and MN1 have features that suggest their involvement in the regulation of transcription, we used indirect immunofluorescence analysis to study the subcellular localization of various TEL, MN1, and MN1-TEL products using immunopurified α-TEL antibodies or MN1 MoAb 2F2 (Figure 1). Endogenous TEL was found predominantly in the nucleus (excluding the nucleoli) of NIH3T3 and HeLa cells, but some protein was also detected in the cytoplasm (figure 2a, b). The specificity of the TEL antibodies was verified by competing with bacterially expressed GST-TEL (figure 2c). No endogenous MN1 could be detected in NIH3T3 cells (figure 2d). Exogenously expressed TEL was detected in the nucleus (excluding the nucleoli) or cytoplasm or both subcellular compartments in TEL-retrovirus transduced NIH3T3 cells (figure 2e). The ETS DNA binding domain mutant, TEL-DBDM, which contained leucines instead of arginines at residues 396 and 399 (within the ETS domain), was expressed exclusively in the cytoplasm (figure 2f). At present it is not known whether these mutations directly target the NLS of TEL or whether aberrant folding of the protein prevents nuclear transfer by masking its NLS. The deletion mutant TELΔ53-116 (which lacks almost the entire HLH oligomerization domain) was concurrently expressed in both the cytoplasm and the nucleus (figure 2g).

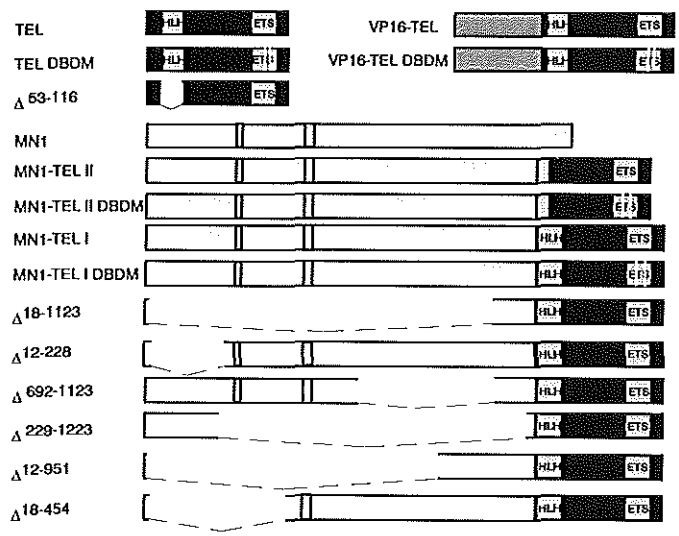

Figure 1 Schematic representation of TEL, MN1, MN1-TEL and VP16-TEL cDNA constructs. ETS is ETS DNA binding domain, HLH is Helix-Loop-Helix oligomerization domain, DBDM is DNA binding domain mutant; white lines represent mutated codons. Gray boxes in MN1 sequences represent glutamine stretches.

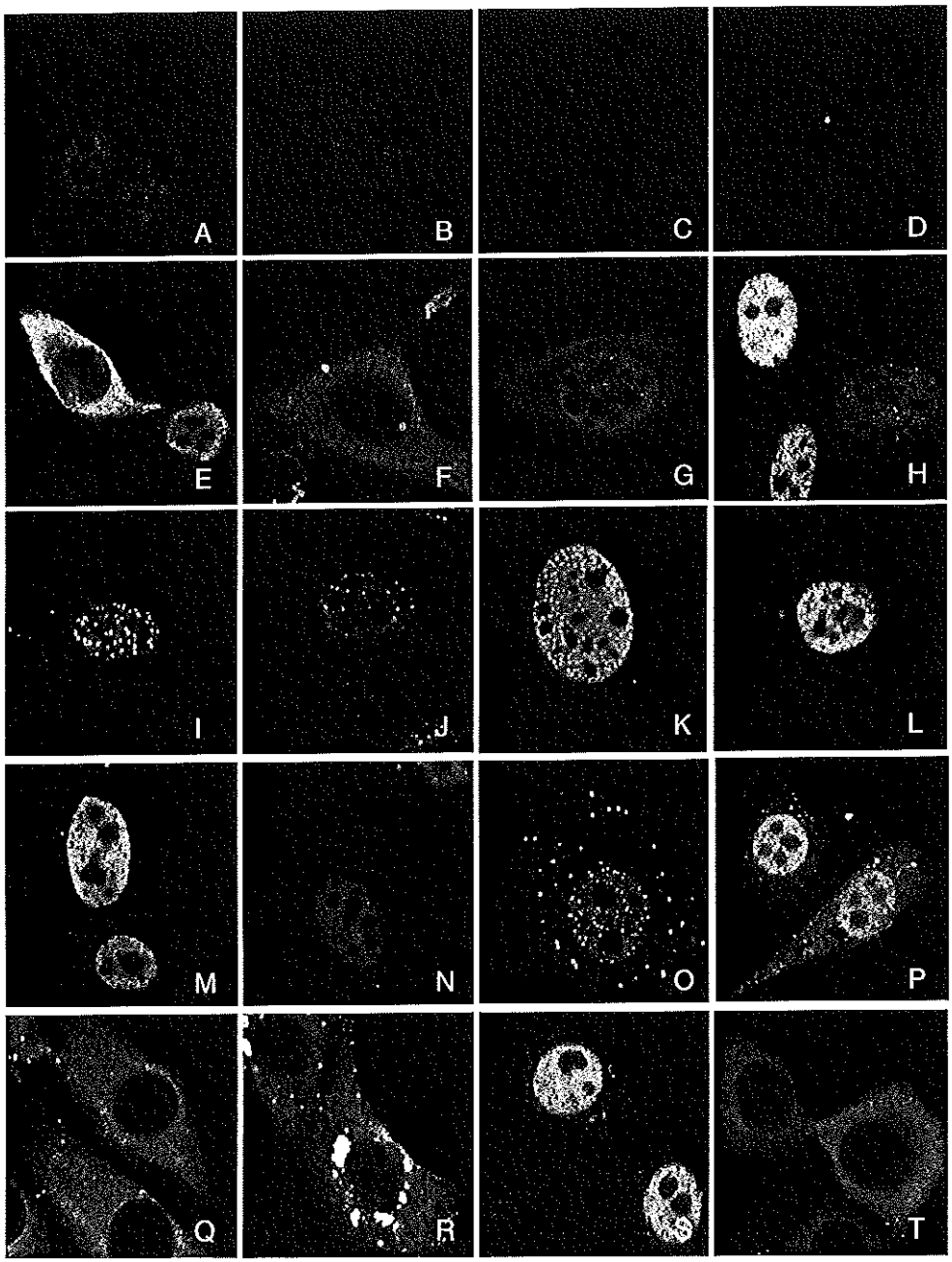
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Figure 2 Subcellular distribution of endogenous TEL and virally transduced TEL, MN1, MN1-TEL and VP16-TEL proteins. **A-C** Indirect immunofluorescence analysis of endogenous TEL using immunopurified α-TEL antibodies in NIH3T3 cells (**A**) and HeLa cells (**B,C**); analysis of competed α-TEL antibodies using bacterially expressed GST-TEL fusion protein on HeLa cells (**C**). Endogenous and exogenous expression of MN1 in NIH3T3 cells (**D, H**). Distribution of virally transduced TEL, TEL DBDM, and TELΔ53-116 (**E-G**), MN1-TEL I and MN1-TEL I DBDM (**I,J**), MN1-TEL II and MN1-TEL II DBDM (**K,L**), MN1-TEL I deletion mutants Δ18-1123, Δ12-228, Δ692-1123, Δ229-1223, Δ18-454 and Δ12-951(**M-R**), VP16-TEL and VP16-TEL DBDM (**S,T**) in NIH3T3 cells was analyzed using α-TEL antibodies (**E-G,I,J,L,M-T**) or MN1-specific MoAb 2F2 (**D,H,K**). Proteins were visualized using FITC-conjugated second antibody. Images were obtained by using confocal microscopy. Signals of **A-D** have been electronically amplified.

Exogenous MN1 was diffusely present throughout the nucleus (excluding the nucleoli, figure 2h). In contrast, α-TEL identified speckles of MN1-TEL I predominantly in the nucleus (figure 2i). The same pattern was found for MN1-TEL I DBDM (figure 2j). MN1-TEL II and MN1-TEL II DBDM were expressed in a similar speckled nuclear pattern using MN1 specific MoAb 2F2 or α-TEL antibodies, respectively (figure 2k,l). In double labeling experiments signals obtained with 2F2 and α-TEL were overlapping (data not shown). Preliminary analyses to identify whether these speckles colocalize with known nuclear domains demonstrated that they are different from PML Oncogenic Domains (PODs) (data not shown)(Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994).

We then analyzed the localization of the MN1-TEL deletion constructs to provide a control for the transformation and transactivation studies. The MN1-TELΔ18-1123 (figure 2m), MN1-TELΔ12-228 (figure 2n), MN1-TELΔ692-1123 (figure 2o), and MN1-TELΔ229-1223 (figure 2p) constructs were all solely or predominantly expressed in the nucleus. In contrast, MN1-TELΔ18-454 and MN1-TELΔ12-951 were expressed in the cytoplasm. Whereas MN1-TELΔ12-951 displayed a diffuse cytoplasmic staining (figure 2q), MN1-TELΔ18-454 was expressed in large, perinuclear plaques as well (figure 2r). Although VP16-TEL was expressed in the nucleus (figure 2s), VP16-TEL DBDM (like TEL DBDM) localized in the cytoplasm (2t). These results demonstrate that MN1-TEL is expressed in the nucleus in distinct speckles, which argue in favor of the hypothesis that MN1-TEL may act as an aberrant transcription regulator. Furthermore, analysis of subcellular localization of deletion mutants is a crucial control for the correct interpretation of our functional assays. Despite the presence of an alleged NLS in the ETS domain of the mutants, some of them do not transfer to the nucleus.

Expression of MN1-TEL leads to transformation of NIH3T3 fibroblasts

The MN1-TEL fusion protein resembles t(11;22) EWS-FLI1, which is associated with Ewing sarcoma and transforms NIH3T3 cells (Lessnick *et al.*, 1995; May *et al.*, 1993a). We therefore virally transduced MN1-TEL type I, TEL and MN1 into NIH3T3 fibroblasts to compare their transforming potential. Indirect immunofluorescence using TEL and MN1 specific antibodies showed that more than 95% of the sorted $CD8^+$ cells expressed the various cDNA constructs (data not shown). The morphology of the MN1-TEL-infected cultures differed from that of the mock-, TEL- and MN1-transduced cells (figure 3a). Whereas the mock-, TEL- and MN1-

infected cells grew in a contact-inhibited monolayer, the cells expressing MN1-TEL were not contact-inhibited and had a more rounded up, spiky morphology (figure 3a).

To assess the transforming capacity of the various proteins, we plated retrovirally transduced cells in soft agar. As shown in figure 3b and table 1, only cells transduced with MN1-TEL type I formed colonies above background. The same result was obtained in four independent experiments (table 1).

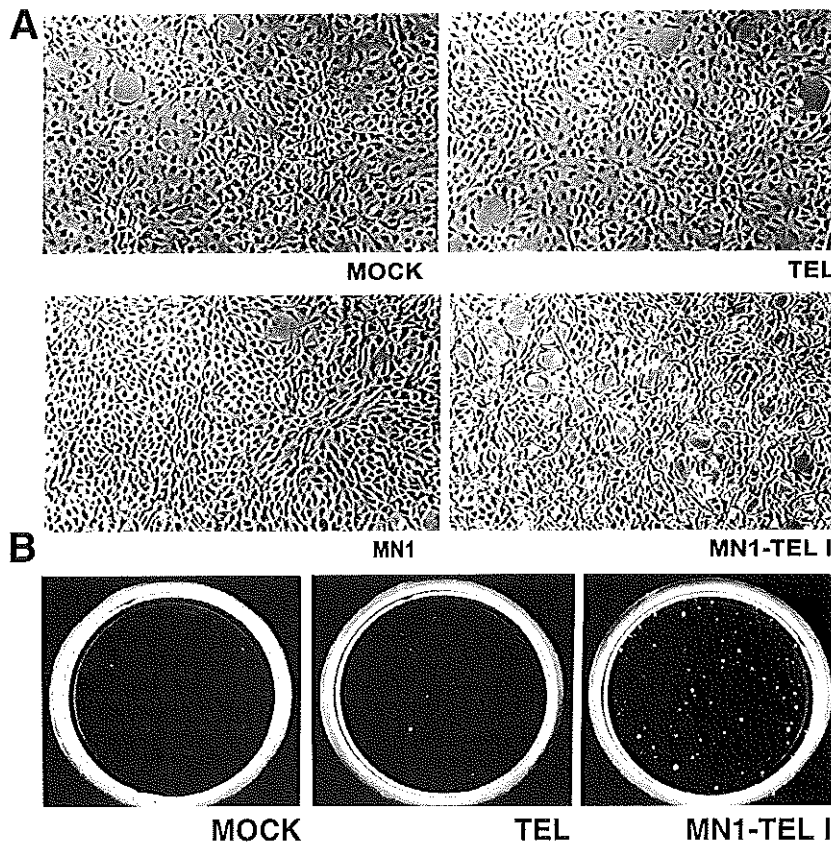


Figure 3 Morphologic analysis and soft agar assays demonstrating transformation of NIH3T3 cells by retrovirally transduced MN1-TEL I. **A** Polyclonal populations of sorted CD8-positive NIH3T3 cells infected with mock, TEL, MN1 or MN1-TEL I expressing retroviruses were seeded on plastic culture dishes. Only MN1-TEL I infected NIH3T3 cells were not contact-inhibited and displayed an aberrant morphology. **B** MN1-TEL I infected CD8-positive NIH3T3 cells form colonies in soft agar. Mock or TEL infected cells did not form colonies when plated in agar. Cells were seeded into agar at a density of 20,000 cells per plate and at a serum concentration of 15%.

N-terminal sequences of MN1 and the ETS domain of MN1-TEL are necessary for transformation of NIH3T3 cells

To determine sequences of MN1-TEL necessary for transformation of NIH3T3 cells, we generated a series of MN1-TEL I deletion constructs (figure 1). Cells infected with retroviruses expressing mutant MN1-TEL proteins were assayed for colony formation in soft agar. Deletion of almost all MN1 sequences (MN1-TELΔ18-1123) abolished the transforming activity of MN1-TEL (table 1), demonstrating that they are necessary for transformation. More subtle deletions showed that removal of aminoacids 12-228, 18-454, or 12-951 from the N-terminus of MN1-TEL was also sufficient to eliminate colony formation (table 1). Removal of aminoacids 692-1123 or 229-1223 greatly reduced the transforming capacity of MN1-TEL. However, a considerable increase in the number of colonies smaller than the macroscopically visible 150 μm standard were generated (table 1; right column), suggesting that these two mutants have some, but impaired, growth stimulating potential.

TABLE 1. Number colonies scored in soft agar assays.

Number of colonies	> 150μm			total		
Construct	mean	stddev	n	mean	stddev	n
MOCK	1	1	4	17	9	3
TEL	1	3	3	1	1	2
TELΔ53-116	4	4	2	8	0	1
MN1	0	0	2	5	2	2
MN1-TEL I	49	5	4	146	63	3
MN1-TEL I Δ18-1123	10	3	2	22	0	1
MN1-TEL I Δ12-228	0	0	3	0	0	2
MN1-TEL I Δ18-454	5	3	2	17	7	2
MN1-TEL I Δ12-951	1	0	3	2	2	2
MN1-TEL I Δ692-1123	7	0	1	39	0	1
MN1-TEL I Δ229-1223	7	0	1	44	0	1
MN1-TEL I DBDM	2	0	1	65	0	1
VP16-TEL	4	0	2	22	0	1

stddev = standard deviation, n = number of independent experiments

In addition, the subcellular localization of each mutant was determined by indirect immunofluorescence analysis. As mentioned above, full-length MN1-TEL I, MN1-TEL I DBDM, MN1-TELΔ18-1123, MN1-TELΔ12-228, MN1-TELΔ692-1123 and MN1-TELΔ229-1223 were found in the nucleus (figure 2). Because MN1-TELΔ18-454 and MN1-TELΔ12-951 were exclusively expressed in the cytoplasm, these two deletion mutants were non informative for mapping domains of MN1-TEL, involved in transformation and transactivation.

To test whether the ETS domain of MN1-TEL is necessary for transformation of NIH3T3 cells, we introduced the mutated ETS DNA binding domain into MN1-TEL. Although the subcellular expression of the mutant protein was identical to wildtype MN1-TEL I (figure 2 i,j), cells transduced with MN1-TEL I DBDM did not form colonies, bigger than the 150 µm standard (table 1; middle column). However, like MN1-TEL deletion mutants 692-1123 and 229-1223, gave rise to an increased number of smaller colonies (table 1: right column). This result indicates that the ETS domain is instrumental for MN1-TEL-mediated transformation of NIH3T3 cells, since mutating the DNA binding domain greatly reduced its transforming potential.

Addition of the VP16 transactivation domain to TEL or deletion of the HLH domain fails to induce transformation of NIH3T3 cells

To test whether MN1 confers transforming activity to TEL by addition of a strong transactivating domain, we fused the acidic transactivating domain of the HSV1 VP16 protein to the N-terminal side of the TEL sequences present in MN1-TEL type I. The resulting VP16-TEL fusion protein is similar to VP16-FLI1, which transforms NIH3T3 cells (Lessnick *et al.*, 1995). As shown in table 1, VP16-TEL failed to transform NIH3T3 cells, indicating that addition of a strong heterologous transactivating domain alone is insufficient to confer transforming ability to TEL. Further, cells transduced with a TEL mutant lacking the HLH putative oligomerization domain (TELΔ53-116) failed to form colonies (table 1), suggesting that TELs inability to form colonies is not due to oligomerization via the HLH domain.

TEL recognizes a canonical ETS recognition sequence CCGGAAGT

Because TEL belongs to the ETS family of transcription factors, we wished to determine its DNA-binding site. Constructs that fused the GST protein to the ETS domain of TEL (i.e., the 138 C-terminal amino acids), GST-ETS, or to the whole TEL ORF, GST-TEL, were expressed in bacteria. The GST-ETS fusion protein was used to identify a TEL consensus binding site by random oligomer amplification (Inaba *et al.*, 1994). All but 1 of the 26 amplified clones identified after six rounds of selection contained the canonical ETS recognition sequence GGAA (figure 4). After comparing the adjacent sequences, we determined that the consensus TEL recognition sequence is CCGGAAGT.

AGCATGTCC GGAA GTAGTGCCTTATCCTGTTTGTC
GACTAGATCGGAAGTTGGGTACAAGGTGC GGAA GTACT
GACTACGGCTATC GGAA GTGAATTGCCCCAGTACT
GACCCA GGAA GAACCTCCAACAAGTATGAAGTACT
GACGGATGAGTCGACT GGAA TCACTTCCGGTATCT
GACTATAGACGAACCC GGAA ACACTGGTACATACT
GACAGGATAGCA GGAA ATAAGACGTTCGTAGTTCT
AGTGACC GGAA GCAAGTTAGTGCCGCTGGTTCGTC
AGGA GGAA GTTCGGCTATGCCCCCTGCATGCAGTC
GGAGGAATATACTGAAC GGAA GTTGACTG
ATAGTATCCC GGAA GTCTCCACAACTATCCG
AGC GGAA ATTGCGTTATTCCAGGTAGTTCCCGG
TATGAACC GGAA AATGCCTGCGCGTCGG
CCGGATAGTAAGAACATTC GGAA ACTAGTA
C GGAA CACATCCGCATTAGCCAGAACATCCT
C GGAA GTTGGAAATAACTAAAGTATCAAGAC
CA GGAA GACTTTCTAGGTAATAATACAGTGTC
CACATTGACCGTGACAAA GGAA ATGATACT
CAAAGCAAGC GGAA GTGTTAGCAAAGGATA
CGAGATGGGTAATATCC GGAA GTGTACCGCC
CTGGC GGAA ATGAATATGAGATGGGGTCCAC
CAATCATCA GGAA GGAGAAACCAGTC
ATTTCCAATTGGC GGAA GAGGAAAAGTGAATGCTT
CTACC GGAA GAGCGGCCAGCTAAATGCCGGG
TGGTGTAT GGAA GTAAATCGTATACTTTGTG
GACTATAGTTCA GGAA GTGGATTCTGAAACTCTCT
TGATCCG AGAA TGTTACGTTACGCCACATCCGGGTC

$c_{38}a_{36}a_{36}C_{52}C_{64}$ $G_{100}G_{100}A_{100}A_{100}$ $G_{64}T_{56}A_{40}a_{32}t_{32}$
$t_{29}g_{32}t_{27}g_{32}a_{28}$ $a_{28}a_{24}g_{28}g_{24}a_{28}$

Figure 4 TEL consensus DNA binding site. Nucleotide sequences resulting from six rounds of amplification and selection using bacterially expressed GST-ETS (i.e. 138 C-terminal aminoacids of TEL) are shown from 5' to 3'. Sequences are aligned at canonical ETS recognition element GGAA. Consensus sequence is shown at bottom. The percent abundance of most prevalent flanking nucleotides at each position is shown. Underlined are additional ETS binding elements in sequences.

DNA binding activity of TEL is modulated by intramolecular interactions

To study the DNA binding activity of TEL, GST-ETS and GST-TEL fusion proteins were expressed in *E.* coli (figure 5a). Double-stranded oligomers containing the TEL consensus DNA-binding sequence (CCGGAAGT) or a mutant sequence (CCCCAAGT) were used in electrophoretic mobility shift assays (EMSA) to analyze the DNA-binding activities of GST-ETS and GST-TEL. The GST-ETS protein shifted the consensus oligomer (lane 5), and addition of α-TEL antiserum led to a supershifted complex (lane 6)(figure 5b). Interestingly, the intensity of the signal increased dramatically. No shift was observed with the mutant oligomer (lane 4). These observations confirm the ability of the TEL ETS domain to bind the consensus TEL DNA-binding sequence. By contrast, the GST-TEL protein failed to bind to the consensus sequence probe (lane 8), but addition of α-TEL serum led to a weak specific signal (lane 9) and an a-specific signal (lanes 3, 6 and 9). Oligomers incubated with GST protein alone did not shift. These results suggest that binding of the antiserum to the C-terminus of the GST-TEL fusion proteins increases their affinity for the consensus oligomer. The intensities of the signals in lanes 6 and 9 differed, suggesting that GST-ETS and GST-TEL have different affinities for the oligomer. Thus, GST-TEL may contain sequences that negatively interfere with the proteins affinity for or availability to the consensus oligonucleotide.

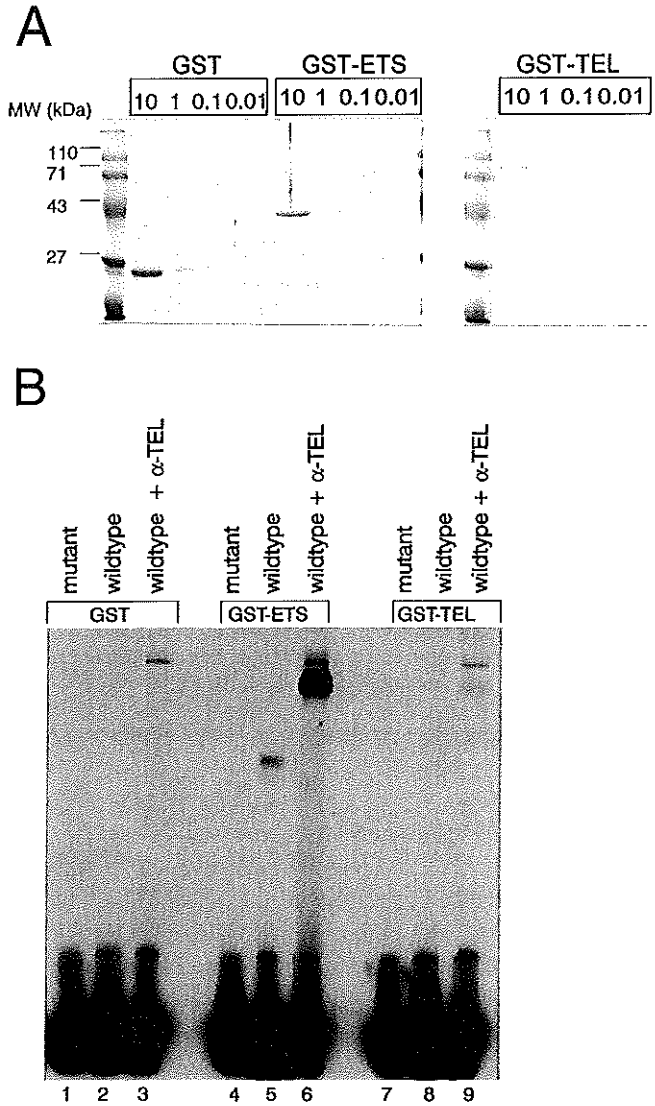


Figure 5 DNA binding analysis of bacterially expressed GST-TEL fusion proteins. A Eluates of bacterially expressed GST, GST-ETS (i.e. 138 C-terminal aminoacids) and GST-TEL fusion proteins were analyzed on a 10% SDS-polyacrylamide gel and visualized by Coomassie Brilliant Blue staining. B 0.1 µl of GST and GST-ETS or 1 µl of GST-TEL fusion protein eluates were used in a bandshift assay with radiolabeled TEL consensus or mutant probe. Complexed and free probe were separated on a 4% polyacrylamide gel in 0.25 x TBE and visualized by using autoradiography.

MN1 contributes transactivating sequences to MN1-TEL

Both translocation partners MN1 and TEL contribute features to MN1-TEL, that are necessary for transformation of NIH3T3 cells. We wondered whether addition of MN1 (including its proline- and glutamine-rich regions) to the ETS domain of TEL would influence the transcriptional activity of TEL. Initial transient transfection studies in NIH3T3 cells, using a luciferase reporter construct containing 10 concatemerized TEL recognition sequences, cloned upstream of the SV40 minimal promoter in pGL2 (Promega) showed that both TEL and MN1-TEL are transcriptionally silent in this promoter/cell type context (data not shown). Because the Moloney sarcoma virus LTR is regulated by ETS-1 (Gunther *et al.*, 1990), we reasoned that it could also be a target of transcription regulation by TEL and we used this promoter in our luciferase reporter construct (pMSVluc). Co-transfection of pMSVluc with increasing amounts of CMV promoter driven TEL cDNA construct resulted in a minimal (i.e., 4-fold) activation of luciferase expression (figure 6a). However, the MN1-TEL type I and type II fusion proteins induced luciferase activity in a dose-dependent manner (up to 18-fold). Cotransfection of the MN1-containing construct with pMSVluc also led to dose-dependent induction of luciferase expression. These results showed that MN1 contributes transactivating sequences to TEL and that MN1 itself may regulate transcription. In addition, transactivation by MN1-TEL was dependent on a functional ETS domain of TEL; MN1-TEL I DBDM and MN1-TEL II DBDM failed to induce expression of luciferase.

We used the VP16-TEL expression construct as a positive control for TEL-mediated activation of pMSVluc. As shown in figure 6a, VP16-TEL strongly induced the expression of luciferase. Because the DNA binding mutant VP16-TEL-DBDM is expressed in the cytoplasm (figure 2t), we have no formal proof that transactivation induced by VP16-TEL is dependent on the ETS domain.

Distinct domains in MN1 mediate the transactivating capacity of MN1-TEL

To determine whether the HLH domain of TEL is involved in transcription control of the MSV LTR and to map the sequences in MN1 that transactivate the MSV LTR, we tested a HLH deletion mutant, TELΔ53-116, and several MN1-TEL I deletion mutants in transient transcription assays. No difference in transactivation activity of the MSV LTR was observed between TEL and TELΔ53-116, indicating that the HLH domain of TEL does not influence TELs inability to transactivate the MSV LTR (figure 6b). Deletion of almost the entire MN1 moiety of MN1-TEL (MN1-TELΔ18-1123) abolished the transactivating capacity of the fusion protein. The MN1-TELΔ12-228 and MN1-TELΔ692-1123 constructs induced expression of the luciferase gene, indicating that sequences spanning the glutamine-stretches are necessary for the transactivating potential of MN1-TEL. Because MN1-TELΔ12-228 transactivated the MSV LTR but failed to transform NIH3T3 cells, sequences within the first 228 amino acids of MN1-TEL seem to be essential for its transforming activity. A mutant containing this domain (MN1-TELΔ229-1223) moderately induced luciferase gene expression, indicating that these

sequences possess weak transactivation activity, which coincides with increased number of microscopic colonies induced by this molecule. Therefore, the transactivating sequences of MN1 can be divided into two subdomains, the first of which is essential for transformation.

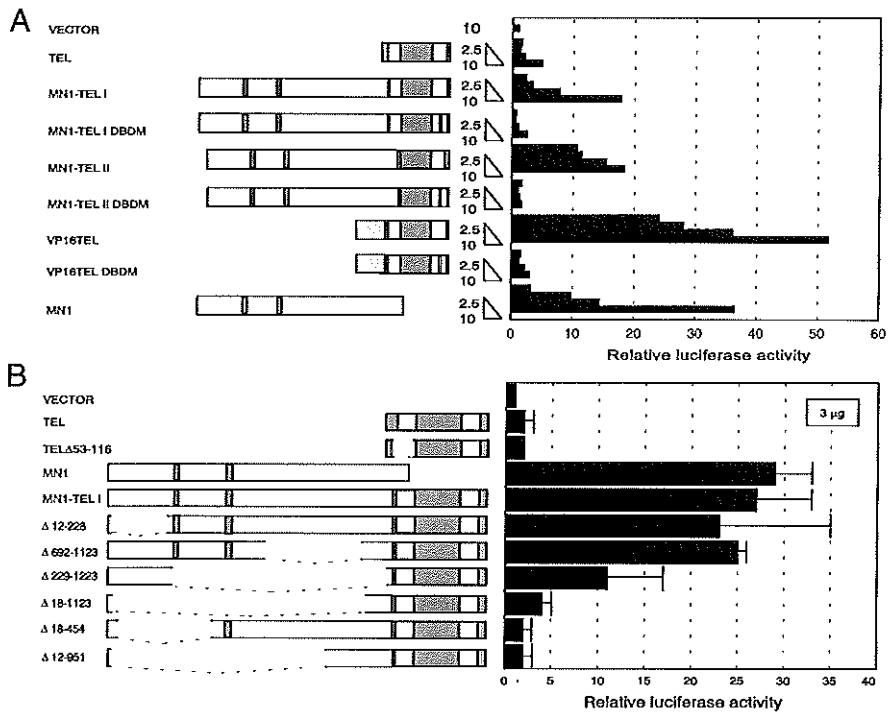


Figure 6 MN1 contributes transcription activating sequences to TEL. **A** Transient transcription experiments were performed using increasing amounts of CMV promoter driven TEL, MN1, MN1-TEL and VP16-TEL activator constructs (2,5 to 10 µg), as well as their respective ETS DNA binding domain mutants, with 1µg of pMSVluc. Luciferase assays were performed 24 hours after removal of calcium phosphate precipitate. Induction of luciferase (normalized to a secreted alkaline phosphatase control) are relative to value of empty vector. **B** Transient transcription experiments using 3 µg of CMV promoter driven activator constructs were performed to analyze whether HLH domain in TEL and which domains in the MN1 moiety of MN1-TEL I mediate transactivation of MSV LTR. Normalized luciferase values relative to empty vector are shown. Mean values of two experiments are shown. Each transfection was performed *in triplo*.

The HLH domain is inactive in MN1-TEL and does not engage in homotypic interaction

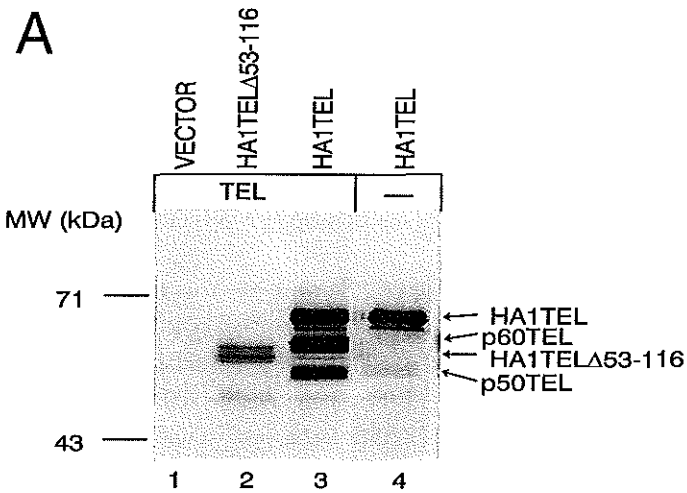
The HLH domain of TEL has sequence homology with known protein interaction domains in c-MYC and MyoD (Seth & Papas, 1990). This domain was recently shown to define a specific protein interaction interface, that mediates oligomerization of TEL (Jousset *et al.*, 1997). This function also mediates oligomerization of TEL-ABL and TEL-PDGFβR fusion proteins, which is essential for the activation of their tyrosine kinase activity (Carroll *et al.*, 1996; Golub *et al.*, 1996). Although in MN1-TEL type I the junction occurs 5' of the HLH domain, the fusion in MN-TEL type II occurs within the HLH domain, suggesting that the HLH domain could be functionally unimportant or inactive in MN1-TEL type I, and it would therefore play no role in MN1-TELs transforming activity.

We used the HA1-specific monoclonal antibody 12CA5 to immunoprecipitate complexes from HeLa cells cotransfected with expression plasmids encoding TEL and HA1TEL or HA1TELΔ53-116. Immunoprecipitated complexes using MoAb 12CA5 were separated on a 10% SDS-polyacrylamide gel and electroblotted. By using Western analysis with α-TEL serum 32, a doublet of 67 kD ($p67^{HA1TEL}$; lane 3) and 58 kD ($p58^{HA1TELΔ53-116}$; lane 2) was recognized (figure 7a). The identities of $p67^{HA1TEL}$ and $p58^{HA1TELΔ53-116}$ were confirmed by subsequent Western analysis with monoclonal antibody 12CA5 (data not shown). Furthermore, only $p67^{HA1TEL}$ coprecipitated TEL (a doublet of 60 kD and a doublet of 50 kD; lane 3), detected with α-TEL antiserum. To analyze whether HA1TEL co-immunoprecipitated endogenous TEL from HeLa cells, a similar experiment was performed in which only HA1TEL was transfected. Proteins of 60 and 50 kD were detected by α-TEL serum (lane 4). These observations show that we can visualize the HLH-mediated homotypic TEL interaction. Our results further suggest that there may be multiple modified forms of two distinct TEL proteins. This was recently confirmed by Bernard and coworkers (Poirel *et al.*, 1997).

We then performed a similar cotransfection experiment to study whether the HLH domain in MN1-TEL type I is functional. Therefore COS-1 cells were (co)transfected with either HA1TEL or HA1TELΔ53-116 in the presence of MN1-TEL I, MN1-TEL II or MN1-TEL I Δ229-1223. Cell were labeled with ^{3}H-leucine, and HA1-tagged proteins were immunoprecipitated with 12CA5. Immunocomplexes were separated on a 8% SDS-polyacrylamide gel. Proteins were visualized by autoradiography. COS-1 cells only expressed endogenous $p50^{TEL}$ which was coprecipitated with transfected human HA1-tagged TEL (figure 7b; lanes 3, 6, 10, 14, 15). In COS-1 cells co-transfected with HA1TEL and MN1-TEL I no protein of the expected size of MN1-TEL I (200 kD) was immunoprecipitated with 12CA5 (lane 6). However, in a subsequent immunoprecipitation with monoclonal 2F2 the presence of MN1-TEL I in these cells was verified, since a protein of the expected size of 200 kD was precipitated (lane 7). Similarly, HA1TEL did not coprecipitate MN1-TEL II (lane 10). These results indicate that although the HLH domain is present in MN1-TEL I, it does not physically interact with HA1TEL or with endogenous simian TEL, possibly due to steric hindrance by the bulky MN1 moiety of the fusion protein. To test this hypothesis, we analyzed if a substantial deletion of MN1 sequences would allow interaction with HA1TEL and repeated the experiment

by co-transfection of HA1TEL and MN1-TEL IΔ229-1223. Endogenous $p50^{TEL}$ and a protein of the expected size of MN1-TEL IΔ229-1223 (approximately 75 kD) were coprecipitated with HA1TEL (lane 14). Similarly, MN1 specific MoAb 2F2 precipitated MN1-TEL IΔ229-1223, $p50^{TEL}$ and HA1TEL (lane 15). As expected, HA1TELΔ53-116 did not co-precipitate MN1-TEL IΔ229-1223 (lane 16). In addition, endogenous $p50^{TEL}$ did not co-precipitate with MN1-TEL IΔ229-1229 using MoAb 2F2 (lane 17), suggesting that the affinity of this deletion mutant for TEL is low. Overall, these results indicate that possible regulation of TEL function via the HLH domain has been eliminated in the MN1-TEL fusion protein.

Figure 7 TELs Helix-Loop-Helix oligomerization domain is non functional in MN1-TEL. A HeLa cells were transiently transfected with expression plasmids encoding HA1TELΔ53-116, HA1TEL and TEL as indicated above the lanes. Proteins were immunoprecipitated with MoAb 12CA5. Complexes were separated on a 10% SDS-polyacrylamide gel and electroblotted. Proteins were visualized by using Western analysis with α-TEL antiserum. HA1TELΔ53-116 and HA1TEL and co-precipitating proteins are indicated by arrows on the right. A molecular weight standard is indicated. B COS-1 cells were (co)transfected with expression plasmids encoding HA1TEL or HA1TELΔ53-116 and MN1-TEL I, MN1-TEL II and MN1-TEL IΔ229-1223 as indicated above the lanes. Following metabolic labeling with ^{3}H-leucine, proteins were immunoprecipitated using MoAb 12CA5, followed by immunoprecipitation with MN1 specific MoAb 2F2, and analyzed on a 8% SDS-polyacrylamide gel. HA1TEL, HA1TELΔ53-116 and co-precipitating proteins are indicated by arrows on the right. A molecular weight standard is indicated.


A
VECTOR
HA1TELΔ53-116
HA1TEL
HA1TEL
TEL
—
MW (kDa)
71
43
HA1TEL
p60TEL
HA1TELΔ53-116
p50TEL
1
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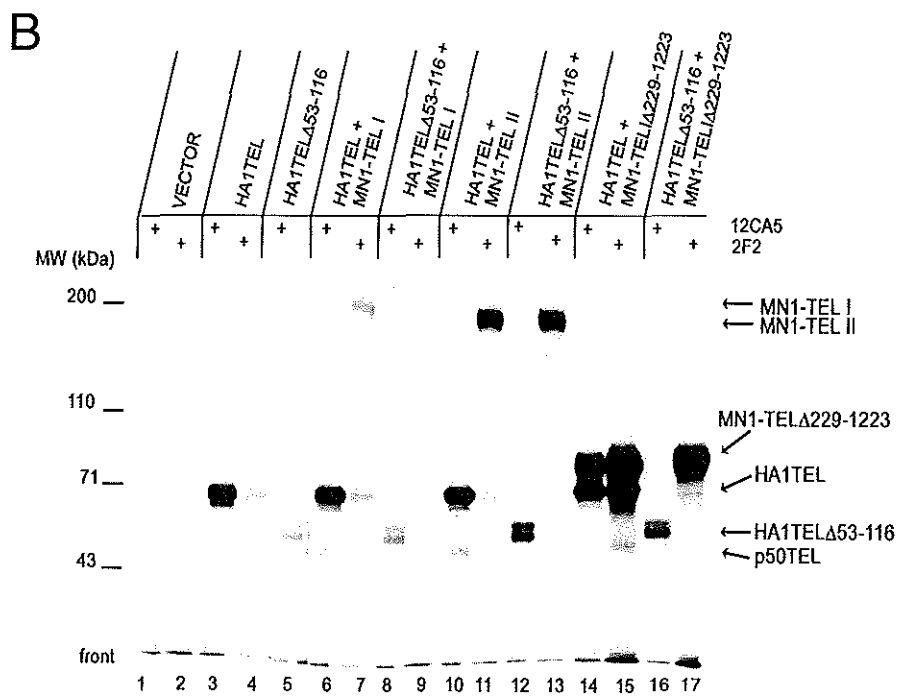
4


B
VECTOR
HA1TEL
HA1TELΔ53-116
HA1TEL + MN1-TEL I
HA1TELΔ53-116 + MN1-TEL I
HA1TEL + MN1-TEL II
HA1TELΔ53-116 + MN1-TEL II
HA1TEL + MN1-TELΔ229-1223
HA1TELΔ53-116 + MN1-TELΔ229-1223
12CA5
2F2
MW (kDa)
200
110
71
43
MN1-TEL I
MN1-TEL II
MN1-TELΔ229-1223
HA1TEL
HA1TELΔ53-116
p50TEL
front
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Discussion

Our results demonstrate that MN1-TEL, which is associated with cases of t(12;22)(p13;q11) human myeloid leukemia (Buijs *et al.*, 1995), has transforming and transactivating functions. Only NIH3T3 cells infected with a MN1-TEL-containing retrovirus displayed anchorage-independent growth in soft agar. MN1-TEL, however, has a weak transforming activity that can only be shown in a specific NIH3T3 subline that is sensitive to transformation by ETS factors (May *et al.*, 1993b). Standard NIH3T3 cells could not be transformed by MN1-TEL , in contrast to activated RAS (data not shown).

Deletion of N-terminal sequences (aminoacids 12-228) from MN1 was sufficient to abolish the transforming capacity of MN1-TEL. This domain was not sufficient to render MN1-TEL transforming, because deletion of aminoacids 229-1223 resulted in a decrease of colonies in size as well as in number. It is interesting to correlate the transactivation activity of MN1-TEL mutants with their transforming activity in NIH3T3 cells. Deletion of aminoacids 12-228 from MN1-TEL had a minor impact on its transactivation of the MSV-LTR and fusion of these sequences alone to TEL confirmed their moderate transactivation activity. The strongest transactivating sequences of MN1 are comprised within aminoacids 228 and 692. These sequences contain two glutamine stretches and proline-rich sequences, that can both function as transactivation sequences (Gerber *et al.*, 1994). Glutamine stretches can form β-sheets that mediate protein-protein interaction by functioning as a polar zipper (Perutz *et al.*, 1994; Stott *et al.*, 1995). Glutamine stretches in SP1 have been demonstrated to interact with the basal transcription machinery (Gill *et al.*, 1994). Interestingly, the presence of this strong transactivation domain in MN1-TEL is not sufficient for transformation, because the transformation potential of MN1-TEL was completely abolished or greatly reduced by deletion of aminoacids 12-228 or 692-1123, respectively. This observation is further supported by the VP16-TEL protein that strongly transactivates the MSV-LTR but failed to transform NIH3T3 cells. We conclude that MN1 does not contribute to the transforming properties of MN1-TEL solely through addition of strong transactivating sequences. Our experiments identify the first 228 amino acids of MN1 as a functionally distinct domain. Because the domain appears to contain mild transactivating sequences, it remains entirely possible that it influences expression of genes crucial for transformation. Alternatively, the domain may interact with cellular factors that directly or indirectly interfere with cell cycle control, not involving transcription regulation. Further studies are needed to distinguish between the different possibilities. Likewise, a domain between aminoacids 692-1123 also seems to be important for the transforming potential of MN1-TEL. The finding that the transforming activity was greatly reduced, suggests that this domain, although it does not seem to contribute to transactivation of the MSV LTR, may be important for protein-protein interaction, and in that way may contribute to transformation. Also in EWS-FLI1, sequences were mapped that are important for transformation, but have no function in transactivation (Lessnick *et al.*, 1995).

The finding that the transforming potential of MN1-TEL-DBDM mutant is impaired, strongly suggests that interference with expression of TEL target genes is a crucial aspect of MN1-TELs transforming activity. The fact that mutation of the ETS domain impaired, but not completely eliminated, the transforming potential of MN1-TEL may indicate that the molecule may still weakly interact with DNA via factors that normally cooperate in binding TEL to its cognate sequences as has been found for other ETS factors (Giese *et al.*, 1995; Watson *et al.*, 1997). Therefore the study of this fusion protein will provide insight in subversion of the TEL pathway as an oncogenic event, which is fundamentally different from all other characterized translocations involving TEL. In those cases, the HLH domain of TEL is employed for the oncogenic activation of pathways controlled by the different fusion partners, such as PDGFβR, ABL or AML1. In addition, MN1-TEL-DBDM fails to transactivate the MSV-LTR, suggesting that activation of this promoter by MN1-TEL occurs via a TEL binding site. We determined the consensus binding site of TEL, CCGGAAGT, and found it to be similar to binding sites determined for other ETS family members (Wasylyk *et al.*, 1993) containing the ETS core motif GGAA. In *in vitro* binding studies most ETS family proteins are promiscuous, and it has been suggested that their *in vivo* specificity derives from the context of the binding site and interaction with neighboring transcription factors (Wasylyk *et al.*, 1993). This is also suggested by the difference in transforming activity between the VP16-TEL and VP16-FLI1 proteins. Although TEL and FLI1 bind to very similar sequences *in vitro*, VP16-FLI1 must activate transformation specific target genes in NIH3T3 cells (Lessnick *et al.*, 1995), that are not activated by VP16-TEL. The MSV-LTR contains several ETS sites that, on the basis of their sequence, may also bind TEL including the one between the TATA box and CAAT box that was shown to bind ETS-1 (Gunther *et al.*, 1990). Presently, we are using deletion analysis of the MSV-LTR to determine which site(s) confer(s) MN1-TEL responsiveness. By contrast, a minimal promoter preceded by a ten-fold concatamerized TEL binding site could not be transactivated by TEL or MN1-TEL. This observation is again in accordance with the notion that *in vivo* binding of TEL may need cooperation of other factors that bind in close vicinity and interact with TEL.

The *in vitro* DNA binding activity of full length TEL is inhibited. It can be activated by either deletion of N-terminal sequences (Δ1-314) or by incubation of the protein with C-terminal TEL antibody. Because the two modifications synergize, a mechanism of intramolecular interaction could be responsible for inhibited DNA binding activity of TEL. One could argue that fusion of TEL to GST could be the reason for its inability to bind DNA. However, bandshift analysis using extracts of TEL transfected cells never produced a TEL specific bandshift, indicating that overexpressed TEL did not bind our consensus nucleotide either (A.B. and G.G.; unpublished observations). ETS-1 also has low affinity for its recognition site and it was recently shown that the DNA binding activity of ETS-1 is regulated by intramolecular interaction; short α-helical sequences, situated N-terminal and C-terminal of the ETS domain interact, preventing the ETS domain from binding DNA (Jonsen *et al.*, 1996; Petersen *et al.*, 1995). Sequence comparison between TEL and ETS-1 did not identify

homologous α-helical sequences in TEL, thus at present we cannot exclude alternative explanations for the DNA binding behavior of TEL. More detailed deletion analysis of TEL is needed to pinpoint the involved sequences which would confirm a possible intramolecular regulation of DNA binding activity.

Endogenous TEL was predominantly localized in the nuclei of NIH3T3 and HeLa cells, although some protein was detected in the cytoplasm. Higher expressed, virally transduced TEL could be found in either the nucleus or the cytoplasm or concurrently in both compartments. This may indicate that the subcellular distribution of TEL is regulated and may be functionally relevant. Overexpression of TEL may amplify a natural difference in the cellular distribution of TEL. In this respect it is interesting that, like its closely related ETS family member YAN, TEL contains several consensus MAPK phosphorylation sites. The subcellular localization of YAN was reported to be regulated by RAS dependent phosphorylation (Rebay & Rubin, 1995), which may be similar in TEL. Another possibility is that the two TEL proteins ($p50^{TEL}$ and $p60^{TEL}$) translated from the TEL mRNA (Poirel *et al.*, 1997), have a different subcellular localization or their subcellular localization is regulated in a different manner. $p50^{TEL}$ is missing the first putative MAPK site, that is present in $p60^{TEL}$. Similar to what was suggested above, phosphorylation of this MAPK site may have an important role in the subcellular localization of $p60^{TEL}$. Alternatively, overexpression of TEL may account for the artificial cytoplasmic accumulation of TEL. A careful analysis of the cellular localization of TEL mutants with activating and inactivating MAPK-site mutations will be pursued to address these issues.

Mutation of arginine residues 396 and 399 within the ETS domain of TEL restricted TEL DBDM and VP16-TEL DBDM to the cytoplasm; these residues are required for DNA-interaction (Kodandapani *et al.*, 1996). At present, it is not clear whether mutation of the two arginine residues targets the NLS of TEL or leads to aberrant protein folding that prevents its NLS mediated transfer to the nucleus. In this respect, it will be interesting to analyze the subcellular localization of TEL-B, which lacks the ETS domain, including its alleged NLS, because of alternative splicing (Ringold *et al.*, 1996). If TEL-B localizes to the cytoplasm, it may act as a dominant negative molecule through HLH-mediated interaction with TEL. MN1-TEL-DBDM mutants were found in the nucleus, suggesting that the nuclear localization of MN1-TEL is mediated by an NLS within MN1. Indeed, sequence analysis identified a candidate NLS (RRPR) between residues 240 and 244 of MN1. Studies are in progress to analyze whether this candidate NLS is functional. Analysis of the subcellular localization of MN1-TEL deletion mutants proved to be an essential control for our transformation and transactivating studies. Mutants such as MN1-TELΔ18-454 and MN1-TELΔ12-951 were non-informative to functionally map domains, since they appeared to be expressed exclusively in the cytoplasm.

We confirmed that TEL interacts with itself via the HLH domain (Jousset *et al.*, 1997). In contrast, no heteromerization between MN1-TEL and TEL was observed, suggesting that the HLH oligomerization domain in t(12;22) MN1-TEL is non-functional. This inactivation may be of functional importance, since it is conceivable that MN1 inhibits interaction of TEL with a protein, that normally regulates its transcription activity. By deletion of the bulk of MN1

sequences (Δ229-1223) in MN1-TEL, the interaction with TEL via the HLH domain was restored. The simplest interpretation of these results is that the bulky MN1 moiety of the protein sterically hinders oligomerization via the HLH domain. Alternatively, MN1 sequences between 229 and 1223 may specifically bind to the HLH domain of TEL, making it unavailable for interaction with TEL. We find this latter explanation less plausible because we never found interaction between TEL and MN1 using co-precipitation analysis (data not shown). The observation that MN1-TEL IΔ229-1223 does not coprecipitate endogenous simian $p50^{TEL}$, unless HA1TEL is present, suggests that the affinity of the mutant for $p50^{TEL}$ is low. Furthermore, it suggests that HA1TEL mediates the formation of at least a trimer of MN1-TELΔ229-1223, $p50^{TEL}$ and HA1TEL via its HLH domain.

In conclusion, our data demonstrate that TEL is a proto-oncogene that can be activated by fusion to the transcription regulator MN1 as a result of the t(12;22)(p13;q11) rearrangement. The resulting MN1-TEL chimeric protein has both transforming and transactivating properties. Unlike other TEL-containing fusion genes, MN1-TEL requires the ETS DNA-binding domain (rather than the HLH oligomerization domain) to cause oncogenic transformation. The diversity of the domains in MN1-TEL that contribute to its transforming and transactivating functions is similar to that seen with EWS-FLI1.

Acknowledgements

Dr. Judith Boer for assisting with confocal microscopy, Dr Richard Ashmun for FACS analysis, Dr Linda Shapiro for providing the pGL2 luciferase vectors, Dr. Marcel Koken for providing α-PML antibodies and Amy Frazier for editing of the manuscript. Peggy Burdick and Charlette Hill for secretarial assistance These studies were supported by Cancer Center CORE Grant CA-21765 and by the Associated Lebanese Syrian American Charities (ALSAC) of St. Jude Children's Research Hospital.

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Chapter 3. Chromosomal translocation (12;21)(p13;q22)

Chapter 3.1.

TEL/AML1 fusion resulting from a cryptic t(12;21) is the most common genetic lesion in pediatric ALL and defines a subgroup of patients with an excellent prognosis. (1995). *Leukemia* **9**: 1985-1989.

LEADING ARTICLE

TEL/AML1 fusion resulting from a cryptic t(12;21) is the most common genetic lesion in pediatric ALL and defines a subgroup of patients with an excellent prognosis

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The t(12;21)(p13;q22) is identified by routine cytogenetics in less than 0.05% of pediatric acute lymphoblastic leukemia (ALL) patients. This translocation encodes a TEL/AML-1 chimeric product comprising the helix–loop–helix domain of TEL, a member of the ETS-like family of transcription factors, fused to AML-1, the DNA-binding subunit of the AML-1/CBFβ transcription factor complex. Both TEL and AML-1 are involved in several myeloid leukemia-associated translocations with AML-1/CBFβ being altered in 20–30% of *de novo* acute myeloid leukemia (AML) cases. We now demonstrate that a *TEL/AML1* chimeric transcript encoded by a cryptic t(12;21) is observed in 22% of pediatric ALL, making it the most common genetic lesion in these patients. Moreover, *TEL/AML1* expression defined a distinct subgroup of patients characterized by an age between 1 and 10 years, B lineage immunophenotype, non-hyperdiploid DNA content and an excellent prognosis. These data demonstrate that molecular diagnostic approaches are invaluable in identifying clinically distinct subgroups, and that the AML1/CBFβ transcription complex is the most frequent target of chromosomal rearrangements in human leukemia.

Keywords: acute lymphoblastic leukemia; AML-1; TEL; translocations

Introduction

The cytogenetic identification of leukemia-associated chromosomal translocations has helped identify patient subgroups with defined clinical features and therapeutic responses.[1] Moreover, cloning of the genes involved in these translocations has provided insights into the mechanism of leukemogenesis and has led to the development of molecular approaches for the diagnosis and monitoring of a patient's response to therapy.[2]

The most common translocations in pediatric ALL, t(9;22), t(1;19), and t(4;11), have each been shown to identify a subgroup of patients with distinct clinical features; however, overall these translocations are detected in less than 15% of patients.[1] The majority of the remaining cases of ALL have either normal karyotypes, non-recurrent structural chromosomal abnormalities, or rare recurrent translocations.[3] Recent data suggests that the low frequency of these latter cytogenetic lesions may be due, in part, to the inherent difficulty in cytogenetically identifying translocations involving some chromosomes.

Deletions and translocations of the short arm of chromosome 12 are detected in greater than 10% of pediatric ALL cases; however, specific recurrent translocations involving this locus are exceedingly rare.[3,4] For example, the t(6;12), t(7;12), t(8;12), t(12;17), t(12;18), or t(12;21), each occur in less than 0.05% of patients.[3] Recently, one of these translocations, the t(12;21), was cloned and shown to fuse the helix–loop–helix (HLH) domain of TEL to the DNA-binding and transactivation domains of AML-1.[5,6] AML-1 is the DNA-binding component of the AML-1/CBFβ transcription factor complex, which is the most frequent target of myeloid-associated translocations including, the t(8;21), t(3;21), and inv(16).[7,8] TEL is a member of the ETS-like family of transcription factors and was originally cloned as a TEL/platelet-derived growth factor receptor β (PDGFRβ) fusion encoded by the t(5;12) that is observed in a subset of cases of chronic myelomonocytic leukemia.[9] TEL is also involved in several other rare translocations including t(9;12), t(10;12), and t(12;22).[10–12]

Although the t(12;21) is only rarely identified by cytogenetics in pediatric ALL, fluorescence *in situ* hybridization (FISH) analysis indicates that cytogenetics may underestimate its prevalence.[13–17] Moreover, recent work has demonstrated molecular evidence of a *TEL/AML1* fusion gene in a subset of B progenitor ALLs, indicating that this cryptic translocation may be a more frequent event.[13,17] To investigate the frequency of *TEL* rearrangements, we have analyzed 160 cases of newly diagnosed ALL by Southern blot and reverse transcriptase-polymerase chain reaction (RT-PCR) assays. We now show that *TEL* is rearranged in 24% of ALL cases, with greater than 90% of these cases (22% of the total) expressing a *TEL/AML1* chimeric mRNA. Moreover, none of these cases had cytogenetic evidence of the t(12;21), demonstrating that routine cytogenetics is unable to accurately identify this genetic lesion. Furthermore, our data suggests that cases expressing *TEL/AML1* constitute a uniform subgroup of patients with an excellent prognosis.

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Leading Articles are stringently selected prime articles reporting major advances in the field of leukemia, lymphoma or myeloma (4% of total manuscripts published per year). Selection is based on a score of 4 or more on a scale of 5, with regards to novelty, quality of methodology, and biological significance.

Materials and methods

Patient samples

Ficoll–Hypaque-enriched leukemic blasts were obtained from bone marrow aspirates of 160 children with newly diagnosed ALL and 50 pediatric cases of AML. These patients were treated at St Jude Children's Research Hospital (SJCRH) between 1984 and 1994. A portion of each sample was analyzed for immunophenotypic markers and cytogenetics, and the remainder was cryopreserved. Immunophenotyping and cytogenetic analysis were performed as previously described.[18,19] Although cases analyzed were selected based on the availability of cryopreserved material, no significant differences in clinical features or outcome were observed between analyzed and unanalyzed patients treated during the same period at SJCRH. Thus, this selected patient cohort does not appear to represent a biased sample. Written informed consent was obtained from patients or their legal guardians and all studies were approved by our Institutional Review Board.

Southern blot analysis

Genomic DNA was Southern blotted as previously described.[20] Briefly, high molecular weight DNA (5–10 μg) was digested with either *Bam*HI, *Hin*dIII, or *Eco*RI restriction endonucleases, separated by electrophoresis in 0.8% agarose gels, and transferred to nylon membranes (Oncor, Gaithersburg, MD, USA). Membranes were then sequentially hybridized with α^{32}P-dCTP-labeled partial *TEL* cDNAs including a 5′ 260-bp *Xho*I fragment (pXX), a 538-bp central *Xho*I/*Sac*I fragment (pXS), and a more 3′ 466-bp *Sac*I/*Bam*HI fragment (pSB) (Figure 1a). After high stringency washes, membranes were analyzed by autoradiography.

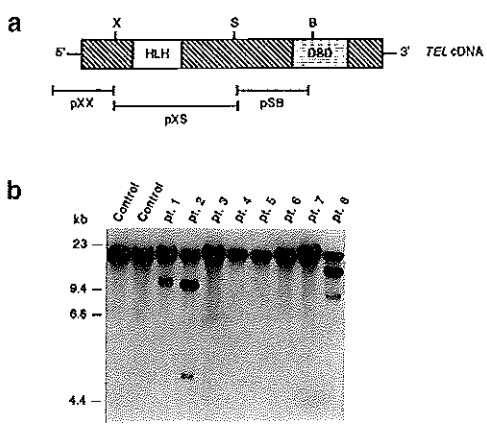

Figure 1 Schematic of *TEL* cDNA and Southern blot analysis of *Bam*HI-digested genomic DNA from pediatric patients with ALL. (a) The helix–loop–helix (HLH) and DNA-binding domain (D8D) of *TEL*, and the positions of the restriction sites for the endonucleases *Xho*I (X), *Sac*I (S), and *Bam*HI (B) are indicated, as are the positions of partial cDNA probes pXX, pXS, and pSB. (b) *Bam*HI-digests were probed with the pSB cDNA probe and the washed membranes were exposed to XAR-5 film for 24 h. Patients (pt.) 1–8 are children with ALL whereas the controls are the leukemic cell lines RS4;11 and HL-60.

Reverse transcriptase-polymerase chain reaction (RT-PCR) assay

RNA was extracted and RT-PCR performed as previously described.[21] Samples were analyzed for the presence of both the der(21)t(12;21)-encoded *TEL/AML1* and the der(12)t(12;21)-encoded *AML1/TEL* fusion products. The sequence of amplification primers for the *TEL/AML1* product are the previously described set,[6] 5′TEL (5′CGT GGA TTT CAA ACA GTC CA 3′), 3′AML1 (5′CAT TGC CAG CCA TCA CAG TGA C 3′). The primers for the reciprocal *AML1/TEL* product are 5′AML1 (5′ATT TTC AGG AGG AAG CGA TG 3′), and 3′TEL (5′GAT TTC ATC TGG GGT TTT CAT A 3′). After amplification, the PCR products were separated by electrophoresis, transferred to nylon membranes and hybridized with either an *AML1* probe (5′CCA TCT GGA ACA TCC CCT 3′), or a *TEL* probe (5′CGC CAC TAC TAC AAA CTA AAC 3′), which are specific for the *TEL/AML1* and *AML1/TEL* products, respectively. Samples negative for the der(21) *TEL/AML1* product were reamplified using a more 5′ *TEL* oligonucleotide, 5′ex3 TEL (5′GAT GAC GTA GCC CAG TGG CTC 3′) and the 3′*AML1* oligonucleotide and blotted with the *AML1* probe.

DNA sequence analysis

PCR products were cloned into the plasmid vector pCRII (Invitrogen, San Diego, CA, USA), and sequenced by the dideoxynucleotide chain termination method modified for use with double-stranded DNA templates (US Biochemical, Cleveland, OH, USA).

Northern blot analysis

Total RNA was isolated, electrophoretically separated in a 1.2% formaldehyde/MOPS (3-(N-morpholino)propanesulfonic acid)-containing gel,[22] transferred to nitrocellulose and probed with the pSB *TEL* cDNA probe labeled with ^{32}P-dCTP. The washed membranes were analyzed by autoradiography.

Statistical analysis

The distributions of clinical and biologic features for patients with or without expression of the *TEL/AML1* chimeric transcript were compared by the two-tailed Fisher exact test for categorical data and by the Wilcoxon test for continuous data. Life-table estimates of event-free survival (EFS) were derived by the method of Kaplan and Meier,[23] and compared using the logrank test stratified by treatment regimens and WBC count at diagnosis.[24] Failure to achieve remission was considered an event at time zero.

Results

To investigate the possibility of *TEL* rearrangements, Southern blots of *Bam*HI, *Eco*RI, or *Hin*dIII digested DNA from pediatric ALL cases were sequentially hybridized with partial *TEL* cDNA probes. As shown in Figure 1, *TEL* rearrangements were detected in a high frequency of cases using a 466-bp *Sac*I/*Bam*HI (pSB) *TEL* cDNA probe on *Bam*HI digests. This probe encodes the central portion of TEL, including the N-terminal third of the DNA-binding domain. By contrast, only

rare cases were identified with rearrangements that extended outside of this area. Moreover, each of these latter cases still had rearrangements that were detected with this probe/restriction endonuclease combination (data not shown). Accordingly, we used the *TEL* pSB probe to hybridize Southern blots of *Bam*HI-digested DNA from 160 ALL and 50 AML cases to determine the frequency of *TEL* rearrangements in pediatric acute leukemia. *TEL* rearrangements were detected in 24% (38/160) of ALL cases, but not in any of the 50 AML cases analyzed. Similarly, *TEL* was not rearranged in samples from 19 non-leukemic control individuals, demonstrating a lack of detectable polymorphisms with this restriction endonuclease/probe combination.

Most of the ALL cases with *TEL* rearrangements had two altered *TEL Bam*HI fragments, consistent with detection of both reciprocal products from a *TEL* translocation. In addition, a 21-kb germ-line *TEL* fragment was detected in most cases, suggesting retention of a non-rearranged *TEL* allele (Figure 1 and see below).

The location of rearrangements within *TEL* were identical to those described for the t(12;21) translocation, which encodes a chimeric *TEL/AML1* product.[5,6] To investigate whether the observed rearrangements were the result of a cryptic t(12;21), RT-PCR analysis was performed for detection of the der(21)t(12;21)-encoded *TEL/AML1* and the der(12)t(12;21)-encoded *AML1/TEL* chimeric mRNAs (Figure 2). RT-PCR analysis revealed the presence of the der(21)t(12;21) *TEL/AML1* transcript in 35 of 38 cases. By contrast, the reciprocal der(12) *AML1/TEL* fusion product was detected in only 29 cases, all of which also expressed *TEL/AML1*. Sequence analysis of *TEL/AML1* PCR products demonstrated fusion points identical to those previously published,[5,6] with the exception of a single case in which the *TEL* breakpoint occurred at a more 5′ site that corresponded to the site of fusion described for the t(5;12)-encoded *TEL/PDGFRβ* product.[9]

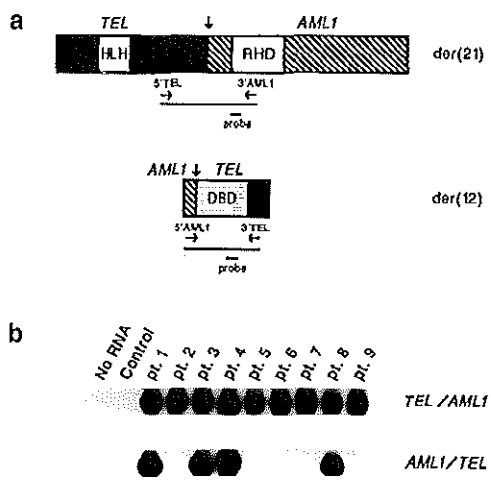

Figure 2 Schematic of the der(21)t(12;21)-encoded *TEL/AML1*, der(12)t(12;21)-encoded *AML1/TEL*, oligonucleotide primers and probes used for RT-PCR analysis, and the results of RT-PCR analysis of patients with leukemias containing *TEL* gene rearrangements. (a) The helix–loop–helix (HLH) and DNA-binding domain (DBD) of TEL, and the DNA-binding *runt* homology domain (RHD) of AML-1 are indicated. The fusion points are shown with arrows. (b) RT-PCR products were size fractionated by electrophoresis, transferred to nylon membranes, and hybridized with the AML1 (*TEL/AML1*) or TEL (*AML1/TEL*) probes.

Although 35 cases had molecular evidence of the *TEL/AML1* fusion, none of the cases had a cytogenetically detectable t(12;21). However, 12/18 cases with other cytogenetic abnormalities of chromosome 12p including deletions, dicentric chromosomes, or other 12p translocations, expressed the fusion transcript, suggesting that the frequency of this cryptic translocation may be even higher in the subgroup of patients with cytogenetic abnormalities of chromosome 12, band p12–13.

Previous published data on a limited number of patients implied that cases expressing the *TEL/AML1* fusion transcripts frequently have deletions of the non-rearranged *TEL* allele.[5,6] Our Southern blot analysis, however, demonstrated definitive loss of the non-translocated *TEL* allele in only seven out of 35 of the *TEL/AML1*-expressing cases (data not shown). Moreover, Northern blot analysis performed on three *TEL/AML1* containing cases that had greater than 95% blasts and retention of a non-rearranged *TEL* allele, revealed expression of a normal sized *TEL* mRNA. However, direct sequence analysis of *TEL* was not performed and thus the presence of inactivating point mutations or microdeletions in the non-rearranged allele cannot be excluded.

The 160 patients analyzed were treated on one of three consecutive front-line ALL protocols at SJCRH, and had a median follow-up of 30 months (range, 5 to 123 months).[25–27] As described above, 35 of these 160 cases expressed the *TEL/AML1* transcript. Analysis of the clinical features of these patients demonstrated that *TEL/AML1* expression identified a uniform patient subgroup characterized by an age between 1 and 10 years (100%), and leukemic blasts with a B precursor immunophenotype (100%), and nonhyperdiploid DNA content (DNA index less than 1.16 or greater than 1.60) (100%). Among the 126 B lineage cases that were analyzed, the 35 *TEL/AML1*-expressing cases appeared to have a better survival than the 91 cases that lacked evidence of this fusion product, with 5-year event-free survival (± s.e.) estimates of 92 ± 8% *vs* 70 ± 10% ($P = 0.14$ by stratified logrank test).

Discussion

Although abnormalities of 12p13 occur often in ALL, the t(12;21)(p13;q22), which encodes a *TEL/AML1* chimeric product, is rarely seen by cytogenetic analysis. Recently, however, the use of molecular approaches to detect this translocation have suggested that its true frequency may be underestimated by routine cytogenetics.[13–17] We now show that the fusion of the *TEL* and *AML1* genes, as a result of a cryptic t(12;21), is the most common genetic lesion in pediatric ALL, occurring in 22% of patients. By comparison, the most common cytogenetically detected translocation, the t(1;19)(q23;p13), occurs in approximately 5% of ALL patients. Moreover, this translocation was unique to ALL cases and was not seen in any of 50 childhood cases of AML. In addition, our data also suggests that expression of *TEL/AML1* defines a uniform subgroup of ALL patients age 1 to 10 years who have B precursor, nonhyperdiploid leukemic lymphoblasts. Taken together, these data indicate that cytogenetic analysis is inadequate to accurately identify this large, but apparently distinct subgroup of patients.

Analysis for the presence of t(12;21)-derived fusion products

in cases with *TEL* rearrangements demonstrated the nearly uniform expression of the der(21)t(12;21)-encoded *TEL/AML1* transcript (35 of 38 cases), whereas the reciprocal *AML1/TEL* mRNA was detected in only a subset of patients (29 of 38 cases). These data suggest that the TEL/AML-1 product is important in establishing the transformed phenotype in these cases. This product consists of the HLH-domain of TEL fused to almost the complete coding region of AML-1. Although both genes are the frequent targets of other leukemia-associated translocations, the structure of this product suggests a primary role for altered AML-1 activity in leukemogenesis in these cases.

AML-1 is a member of a family of transcription factors with homology to the *Drosophila* gene *runt*, and encodes the DNA-binding subunit of AML1/CBFβ transcription factor (Figure 3).[28–31] AML-1/CBFβ has been shown to regulate expression of a variety of hematopoietic-specific genes, including the cytokines IL-3 and GM-CSF, the CSF-1 receptor, and the myeloid specific gene myeloperoxidase.[31–33] Moreover, AML-1/CBFβ is the most common target of AML-associated translocations with the AML-1 subunit involved in the formation of fusion products with ETO and EVI1 in the t(8;21) and t(3;21),[7] respectively, and the CBFβ subunit involved in the formation of a fusion product with the smooth muscle myosin heavy chain, MYH11, in the inv(16)[8] (Figure 3). Several of these chimeric products have been demonstrated to interfere with normal AML-1-dependent transcription, suggesting that transformation results in part, from a dominant negative repression of normal AML-1-target gene expression.[34] The TEL/AML-1 product may also function in leukemogenesis by abnormally regulating AML-1 target genes in lymphoid cells. Several mechanistically different possibilities for this deregulation could be envisioned: (1) Fusion of the TEL-HLH domain to AML-1 may prevent AML-1 from binding to DNA and activating its normal target genes. (2) The TEL/AML-1 fusion protein may still bind to the regulatory region of AML-1 target genes, but the chimeric protein may interfere with normal transcriptional activity. (3) The TEL-HLH domain could also alter the DNA-binding specificity of AML-1, leading to the expression or repression of genes not normally regulated by AML-1. Irrespective of the ultimate transforming mechanism, these data now demonstrate that alteration of the AML-1/CBFβ complex occurs at a high frequency in not only myeloid leukemias, but also in lymphoid leukemias, and thus implicate the AML-1/CBFβ transcription complex as the most frequent target of chromosomal rearrangements in human leukemia.

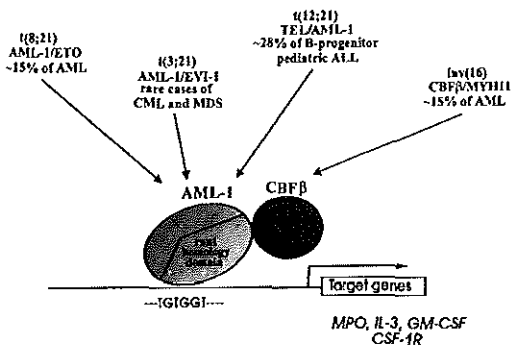

Figure 3 Schematic of the AML-1/CBFβ transcription factor complex and the leukemia-associated chromosomal rearrangements that affect this complex.

Direct alterations of cellular processes regulated by the normal TEL encoded product are also likely to contribute to transformation in these cases. Either reciprocal t(12;21)-encoded chimeric product may directly interfere with the function of normal TEL. In addition, the moderate frequency of deletion of the non-rearranged *TEL* allele suggests that loss of normal TEL may also contribute to leukemia progression in a subset of patients.

Three cases were identified that contained *TEL* rearrangements but lacked expression of *TEL/AML1* mRNA. These findings may have resulted from a t(12;21) with alternative breakpoints that result in a chimeric product that could not be amplified by our oligonucleotide primers. Alternatively, in these cases *TEL* may be fused to genes other than *AML1* or may be partially deleted. Further studies are underway to clarify the molecular basis of the lesions in these cases.

To date, the only subset of B lineage ALL cases with a favorable prognosis has been the hyperdiploid group.[25,35] Our analysis of outcome data from patients treated on three sequential total therapy ALL protocols suggests that patients with leukemic blasts expressing *TEL/AML1* identifies a second subset of B lineage cases that have favorable clinical features. However, in this patient cohort, which spans over 10 years and represents patients treated on a variety of intensive multiagent rotational chemotherapy protocols, the differences in EFS between this group and other B lineage cases was not statistically significant. Nevertheless, the apparent improved prognosis of cases with *TEL/AML1* expression suggests that the clinical significance of this molecular lesion should be evaluated further, by analyzing a large group of patients receiving uniform treatment.

Acknowledgements

We thank Margaret Griffith, W Paul Conn, W Kent Williams, and Anita Curcio-Brint for excellent technical assistance, Mary Heim for assistance in obtaining clinical data, and Susan Vallance for editorial assistance. This work was supported, in part, by Cancer Center Core grant CA-21765 and Leukemia Program Project Grant CA-20180 from the National Institute of Health, and by the American Lebanese Syrian Associated Charities (ALSAC) of St Jude Children's Research Hospital.

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Chapter 3.2.

Subcellular localization of the TEL-AML1 fusion protein, associated with childhood pre-B acute lymphoblastic leukemia; data supporting a tumor suppressor model for TEL. *submitted*

Subcellular localization of the TEL-AML1 fusion protein, associated with childhood pre-B acute lymphoblastic leukemia; data supporting a tumor suppressor model for TEL.

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Abstract

The t(12;21)(p13;q22) occurs in 25% of cases of childhood pre-B acute lymphoblastic leukemia and results in the fusion of *TEL* on 12p13 to *AML1* on 21q22. This translocation is often accompanied by deletion of the second *TEL* allele. In a few cases the deletion occurred intragenic, suggesting that *TEL* is a potential tumor suppressor gene. Recent data indicate that TEL-AML1 acts as a dominant repressor of AML1 dependent transcription regulation. By using immunofluorescence analysis, we show that exogenously expressed TEL-AML1 localizes to the cytoplasm of retrovirally transduced and transiently transfected cells. The cytoplasmic localization of the fusion protein is dependent on the helix-loop-helix (HLH) domain in the TEL moiety. Although endogenous TEL is a nuclear protein, expression of TEL-AML1 causes TEL to colocalize to the cytoplasm. This colocalization seems to be the result of physical interaction between the HLH domains of TEL and TEL-AML1. However, in human B cell lines, expressing TEL-AML1, the chimeric protein localized to the nucleus. Furthermore, overexpression of TEL in fibroblasts resulted in the induction of cell death. Our data support the hypothesis, that diminishing of the effective dosage of nuclear TEL protein provides a proliferative advantage to t(12;21) leukemic cells in childhood pre-B ALL.

Introduction

Cytogenetic studies demonstrate abnormalities of the short arm of chromosome 12 in 10 % of lymphoid and myeloid leukemias (Raimondi *et al.*, 1986). The cryptic t(12;21)(p13;q22) is identified in fewer than 0.05% of cases of childhood acute lymphoblastic leukemia (ALL) (Raimondi, 1993). Molecular analysis of the breakpoint revealed that the translocation results in the fusion of *TEL*, a member of the ETS family of transcription factors, and *AML1*, the human homolog of the *Drosophila* pair-rule *RUNT* gene (Golub *et al.*, 1995; Romana *et al.*, 1995a). TEL was first reported as a result of its involvement in t(5;12) in chronic myelomonocytic leukemia (Golub *et al.*, 1994), in which its N-terminus, including a helix-loop-helix (HLH) protein interaction domain, was fused to the PDGFβR. TEL has also been found to be fused to ABL in t(9;12) and MN1 in t(12;22) (Buijs *et al.*, 1995; Papadopoulos *et al.*, 1995). The *AML1* gene was identified through its involvement in t(8;21), which is associated with 15% of cases of acute myelogenous leukemia (Miyoshi *et al.*, 1991). In addition, AML1 is important in hematopoiesis, as mice homozygous for a mutated *AML1* allele, died at midgestation because of defective fetal liver hematopoiesis (Okuda *et al.*, 1996).

The TEL-AML1 transcript derives from chromosome 21q- and encodes a fusion protein that contains N-terminal TEL sequences, including its HLH domain, linked to the RUNT DNA-binding domain and transactivating sequences of AML1. Using Southern blot and reverse transcriptase-polymerase chain reaction analyses, we and others have shown that t(12;21) occurs in 16% to 25% of cases of childhood ALL (McLean *et al.*, 1996; Romana *et al.*, 1995b; Shurtleff *et al.*, 1995), making it the most frequent translocation in pediatric ALL. This chromosomal abnormality defines a subtype of pediatric ALL with a B lineage immunophenotype and nonhyperdiploid DNA content that is found in patients who are 1 to 10 years of age. A retrospective study of 188 cases of childhood pre-B ALL revealed that t(12;21) is a favorable prognostic indicator for this subgroup of patients (Rubnitz *et al.*, 1997).

About 5% of cases of childhood ALL have cytogenetically detectable deletions in the 12p13 region (Raimondi *et al.*, 1991). In addition, loss of heterozygosity of the *TEL* locus has been observed in 15% to 26% of cases of pediatric ALL and in 27% to 40% of patients with pre-B ALL (Cavé *et al.*, 1995; Stegmaier *et al.*, 1995; Takeuchi *et al.*, 1996). T(12;21) is frequently associated with deletion of the second *TEL* allele (Golub *et al.*, 1995; Raynaud *et al.*, 1996; Romana *et al.*, 1995a; Wlodarska *et al.*, 1996). This deletion is a secondary event in pre-B ALL (Kim *et al.*, 1996; Raynaud *et al.*, 1996; Wlodarska *et al.*, 1996). In one case, the deletion was intragenic in a subclone of leukemic blasts. Such a deletion may correlate with a proliferative advantage for these cells, in turn suggesting that *TEL* may be a tumor suppressor gene. However, mutations in the coding region of *TEL* are rare in childhood ALL (Stegmaier *et al.*, 1996), thereby calling into question the validity of this hypothesis.

Little is known about the biochemical mechanism by which TEL-ALL contributes to leukemogenesis. Recent *in vitro* studies of TEL-AML1 suggested that it may act as a dominant negative repressor of AML1-dependent regulation of transcription (Hiebert *et al.*, 1996). In light of its alleged nuclear localization, it was hypothesized that TEL-AML1, through the HLH domain of TEL, recruits repressors for AML1-dependent activation of transcription.

In contrast to these previous studies, we used immunofluorescence analyses to show that TEL-AML1 is located in the cytoplasm of cells that express the fusion protein after retroviral transduction or transient transfection. The cytoplasmic localization of TEL-AML1 is dependent on the HLH domain, which mediates both the homotypic association with TEL-AML1 and heterotypic association with TEL. In transfected cells, TEL-AML1 relocates endogenous TEL from the nucleus to the cytoplasm, presumably due to a physical interaction of TEL and TEL-AML1. However, in human pre-B cell lines, that express TEL-AML1, the chimeric protein was localized to the nucleus. In addition, retroviral transduction of TEL cDNA in murine fibroblasts resulted in cell death, of which the onset was accelerated by a serine to alanine mutation at a putative phosphoacceptor site at serine residue 22. Cell death was not induced by a mutant lacking the HLH domain. We feel that our data support the hypothesis that TEL has tumor suppressor activity.

Materials and Methods

Cell lines

Mouse fibroblast NIH3T3 cell line, human cervical epithelial cell line HeLa, lung carcinoma cell line C33A, simian kidney cell line COS-1 and kidney carcinoma cell line 293T were maintained in DMEM +10% FBS. Human B cell lines REH, SUP-B28, UoC-B1 and Nalm-6 were maintained in RPMI + 10 % FBS.

Constructs

Various TEL and TEL-AML1 cDNA inserts were cloned into the CMV promoter driven expression vector pSCTOP (Fornerod *et al.*, 1995) and into the retroviral expression vector pSRαMSVTKCD8. The latter allows the selection of transduced cells based on the expression of CD8 (Hirai *et al.*, 1995). Influenza virus hemagglutinin-tagged proteins (e.g., HA1TEL) were generated by the in-frame cloning of three HA1 epitopes 5' of the ATG codon of TEL. To generate mutants lacking the HLH domain (TELΔ53-116), we deleted the 192 bp *Fsp*I-*Xmn*I fragment (corresponding to amino acids 53 through 116) from the TEL cDNA construct. The TEL$^{22S\rightarrow 22A}$ construct was generated through site-directed mutagenesis in bacteriophage M13 according to manufacturer's recommendations (Biorad, Hercules, CA) by using the oligomer 5'-ACACCTCCAGAGGCCCCACTGCCGAG-3', in which codon 22 of TEL was mutated from AGC (Ser) to GCC (Ala), thereby eliminating a putative MAPK phosphorylation site. The TEL-OCT6 fusion construct was generated by

cloning the 1219bp *Eco*RI-*Ssp*I TEL cDNA fragment into the *Sma*I site of the OCT6 deletion clone, N229 (Meijer *et al.*, 1992). This cloning resulted in the in-frame fusion of the first 366 amino acids of TEL to the last 219 amino acids of OCT6 which include its POU homeo DNA binding domain. Figure 1 provides schematic diagrams of the constructs used in our experiments.

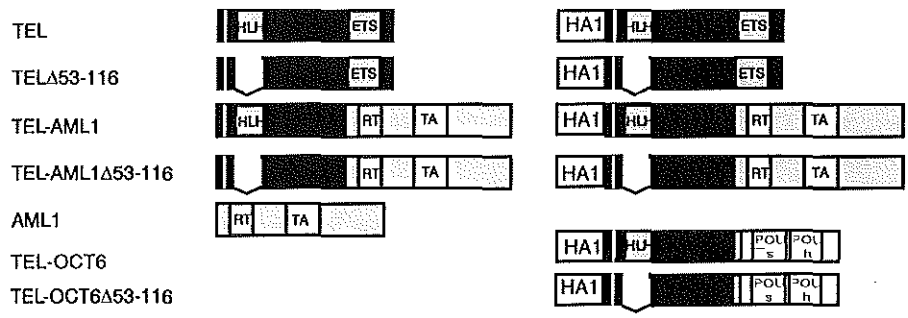

Figure 1 Schematic representation of the various constructs used in these studies. See Materials and Methods for cloning details. HLH, helix-loop-helix domain, ETS, ETS DNA-binding domain, RT, RUNT DNA-binding domain, TA, transactivating sequences, POUs, POU specific domain, POUh, POU-homeo domain. Bright line in TEL indicates serine residue 22

Retroviral transduction

Retroviruses containing TEL, TELΔ53-116, TEL$^{22S\rightarrow 22A}$, TEL-AML1, TEL-AML1Δ53-116, HA1TEL-AML1, HA1TEL-AML1Δ53-116 and RAS were generated by using calcium phosphate precipitation of $3x10^6$ 293T cells in a 10 cm diameter dish cotransfected with 10 μg of the appropriate pSRαMSVTKCD8-based plasmid and 10 μg of the replication-defective helper plasmid pSV-ψ-E-MLV (Muller *et al.*, 1991). After 20 hours, the precipitate was removed and virus-containing supernatant was harvested for 42 hours at 4-8 hour intervals. The supernatants were filtered over a 0.45 μm gauze filters. We then overlaid $2x10^5$ NIH3T3 fibroblasts, which were plated in a 10 cm diameter dish, with 1.5 ml of high-titer supernatant that contained 6 μg/ml polybrene. The virus-containing supernatant was replaced with fresh medium 3 hours later, and CD8-expressing cells were collected by using fluorescence-activated cell sorting (FACS) 60 hours after infection. These cells were then tested for anchorage-independent growth in soft agar and for protein expression of the transduced cDNAs using immunofluorescence(see following).

Antibodies

A synthetic peptide containing the 10 C-terminal amino acids of TEL (Golub *et al.*, 1994) was conjugated to keyhole limpet hemocyanin and injected into New Zealand White rabbits (Rockland, Gilbertsville, Pa) according to standard techniques (Harlow & Lane, 1988). Immunopurified C-terminal α-TEL antibodies were obtained by passing serum 32 over a C-

terminal TEL peptide-coupled Affi-Gel 10 column (Biorad, Hercules, CA). N-terminal TEL antiserum was kindly provided by Dr. P. Marynen, Leuven, Belgium. The α-AML1 serum has been described previously (Meyers *et al.*, 1993). MoAb 12CA5, recognizing an influenza hemagglutinin tag has been described before (Wilson *et al.*, 1984).

Immunofluorescence analysis

We seeded $1.5x10^5$ cells from each CD8-expressing, retrovirally transduced NIH3T3 population on microscope slides. In addition, $1.5x10^5$ C33A, COS-1, NIH3T3, or HeLa cells were seeded on microscope slides and transfected with 10 μg of the various pSCTOP-based plasmids by using calcium phosphate precipitation (Graham & Eb, 1973). After 24 hours, the precipitate was removed. Cytospin preparations of human B cell lines were generated. Cells were fixed in 3% paraformaldehyde for 15 min and permeabilized with 0.2% Triton in PBS for 10 min. The cells were incubated with immunopurified α-AML1 (diluted 1:100 in PBS/1%BSA), C-terminal α-TEL (diluted 1:1250 in PBS/1%BSA), N-terminal α-TEL (diluted 1:1500 in PBS/1% BSA) or the monoclonal antibody 12CA5 (2 μg/ml PBS/1%BSA), as described (Fornerod *et al.*, 1995). α-TEL antibodies were competed with bacterially expressed GST-TEL fusion protein for 1 hour at room temperature (Buijs et al., manuscript in preparation). Images were obtained by using confocal microscopy (BioRad MRC1000 Laser Scanning confocal microscope) or conventional microscopy.

Immunoprecipitation and Western analysis

By using calcium phosphate precipitation, $1.5x10^5$ HeLa cells were seeded in a 6 cm dish and transfected with 10 μg of the pSCTOP-based expression vector containing TEL, TEL-AML1, HA1TEL, HA1TELΔ53-116, HA1TEL-AML1, or HA1TEL-AML1Δ53-116. After 16 hours, the precipitates were removed, and 36 hours later, the cells were metabolically labeled by using 100 μCi of a ^{35}S-methionine-cysteine *in vivo* labeling mix (DuPont NEN, Wilmington, DE) in 1.4 ml of methionine-cysteine-free DMEM supplemented with 8% dialyzed FBS. Immunoprecipitations with the anti-HA1 monoclonal antibody 12CA5 were performed as described (Fornerod *et al.*, 1996). Immunecomplexes were separated on a 10% SDS-PAGE gel and electroblotted onto PVDF membrane (Millipore, Bedford, MA). To control for efficient transfer, gels were analyzed after blotting by autoradiography for residual protein. Blots were blocked overnight in PBS/1% BSA, then incubated for 3 hours with affinity purified α-TEL (diluted 1:1000 in PBS/1%BSA) or 12CA5 (1 μg/ml). Bound antibody was visualized with an alkalinephosphatase-conjugated secondary antibody and colorimetry.

RNA isolation and RT-PCR analysis

RNA isolation and RT-PCR analysis have been performed as been described (Shurtleff *et al.*, 1995).

Results

Exogenous TEL-AML1 is a cytoplasmic fusion protein

To address whether the t(12;21)-specific TEL-AML1 fusion gene could transform NIH3T3 fibroblasts, we first generated replication-defective retroviruses that contained either TEL, TELΔ53-116, HA1TEL, HA1TELΔ 53-116, AML1B, TEL-AML1, HA1TEL-AML1, TEL-AML1Δ53-116, HA1TEL-AML1Δ53-116, or activated RAS (figure 1). Only cells transduced with the retrovirus containing activated RAS showed significant colony formation in soft agar (data not shown). This observation suggested that, except for activated RAS, non of the transduced viruses displayed transforming activity in this assays.

Concomitantly with the soft agar assays, aliquots of the transduced cells were tested for expression of exogenous protein by immunofluorescence analysis. In contrast to what was suggested by previous cell fractionation studies in which TEL-AML1 was found to be associated with the nuclear and nuclearmatrix fractions (Hiebert *et al.*, 1996), we found TEL-AML1 and HA1TEL-AML1 proteins solely in the cytoplasm of cells transduced with the respective constructs, using α-AML1 antibodies and 12CA5 respectively (figure 2a, b). Cytoplasmic localization of these products was dependent on the presence of the HLH domain of TEL, because proteins lacking amino acids 53 through 116 (which span the HLH domain) localized to the nucleus (figure 2c, d). Exogenous AML1B was predominantly found in nucleus (figure 2e). Retrovirally transduced TEL, expressed at a high level was present in the cytoplasm, nucleus, or both subcellular compartments, whereas TEL expressed at low level was predominantly nuclear (figure 2f). TELΔ53-116 was equally present in the cytoplasm and the nucleus (figure 2g).

To verify the cytoplasmic localization of the fusion proteins also in cell lines not transducible by ecotropic retroviruses, we studied the cellular distribution of TEL-AML1 and TEL-AML1Δ53-116 in transiently transfected C33A, COS-1, and HeLa cell lines. Immunofluorescence analysis of all three cells lines localized HA1TEL-AML1 in the cytoplasm of the vast majority of the transfected cells and HA1TEL-AML1Δ53-116 in the nuclei of the cells (figure 3 a,c). In a small percentage of transfected cells HA1TEL-AML1 was detected in the nucleus, when expressed at low levels (figure 3a). This localization was independent of the presence of the N-terminal HA1 tag (data not shown).

To verify that the behavior of TEL is a function of the HLH domain, we linked the HLH domain to a heterologous DNA binding domain. We expressed the HA1TEL-OCT6 and HA1TEL-OCT6Δ53-116 fusion cDNAs by transient transfection, in which the ETS domain of TEL was replaced by the POU-homeo domain of transcription factor OCT6 (Meijer *et al.*, 1990). HA1TEL-OCT6 also localized to the cytoplasm of transfected HeLa cells, despite the presence of the OCT6 nuclear localization signal (NLS)(figure 3b). By contrast, the HLH-deletion mutant HA1TEL-OCT6Δ53-116, like TEL-AML1Δ53-116, was expressed in the nucleus (figure 3d).

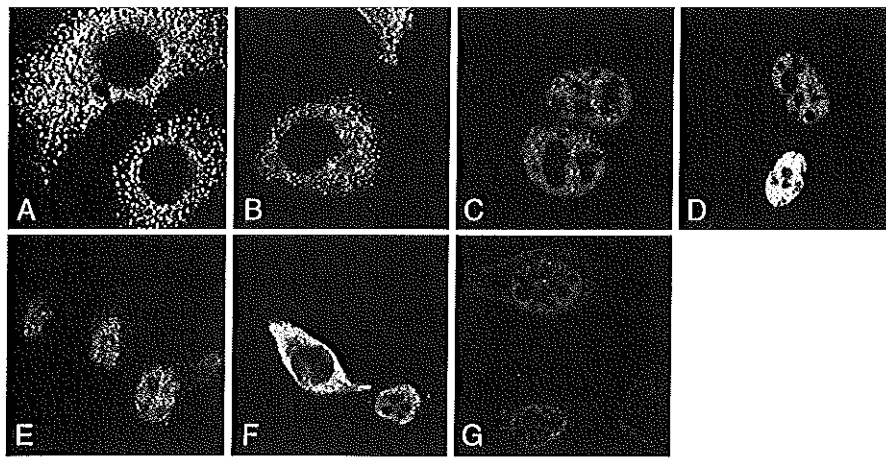

Figure 2 Subcellular localization of proteins expressed by retrovirally transduced cDNAs. Images represent NIH3T3 cells retrovirally transduced with TEL-AML1 (A), HA1TEL-AML1 (B), TEL-AML1Δ53-116 (C), HA1TEL-AML1Δ53-116 (D), AML1-B (E), TEL (F) and TELΔ53-116 (G) cDNAs. Proteins were detected with immunopurified α-AML1 antibodies (A,C and E), immunopurified α-TEL antibodies (F,G), and HA1-specific MoAb 12CA5 (B and D). The presence of the first antibody was visualized by a FITC-labelled second antibody. Images were obtained by using confocal microscopy.

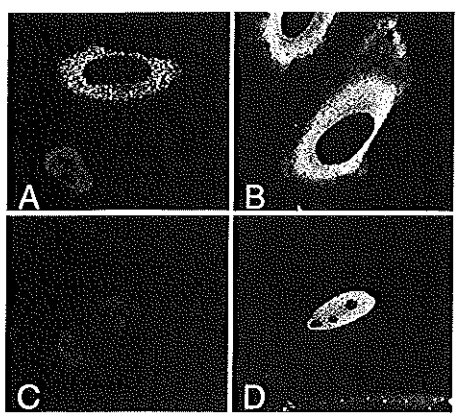

Figure 3 Subcellular distribution of TEL-AML1, TEL-AML1Δ53-116, TEL-OCT6 and TEL-OCT6Δ53-116. Indirect immunofluorescence of HeLa cells transfected with HA1TEL-AML1 (A), HA1TEL-AML1Δ53-116 (B), HA1TEL-OCT6 (C) and HA1TEL-OCT6Δ53-116 (D). HA1 tagged proteins were detected with MoAb 12CA5. The presence of first antibody was visualized with a FITC-labeled second antibody. Images were obtained by using confocal microscopy.

Colocalization of HA1TEL-AML1 and endogenous TEL in the cytoplasm requires the HLH domain

Because the localization of TEL-AML1 to the cytoplasm depended on the presence of the HLH domain of TEL, we determined the localization of endogenous TEL in HeLa cells transiently transfected with HA1TEL-AML1 or HA1TEL-AML1Δ53-116. Endogenous TEL localized predominantly to the nucleus in untransfected cells (figure 4a ,c, d). By contrast, endogenous TEL was detected in the cytoplasm of cells transfected with HA1TEL-AML1 (figure 4a,e), but when transfected with HA1TEL-AML1Δ53-116, endogenous TEL and exogenous HA1TEL-AML1Δ53-116 colocalized in the nucleus (figure 4 d,h). In the few cells that expressed HA1TEL-AML1 in the nucleus, TEL localized in the nucleus as well (figure 4 c,g). To verify that the signal obtained with immunopurified α-TEL antibodies was derived from endogenous TEL, the immunofluorescence signal could be competed by addition of bacterially expressed GST-TEL protein in cells transfected with HA1TEL-AML1(figure 4 b,f). These data suggest that TEL-AML1 relocates TEL from the nucleus to the cytoplasm, dependent on the HLH domain.

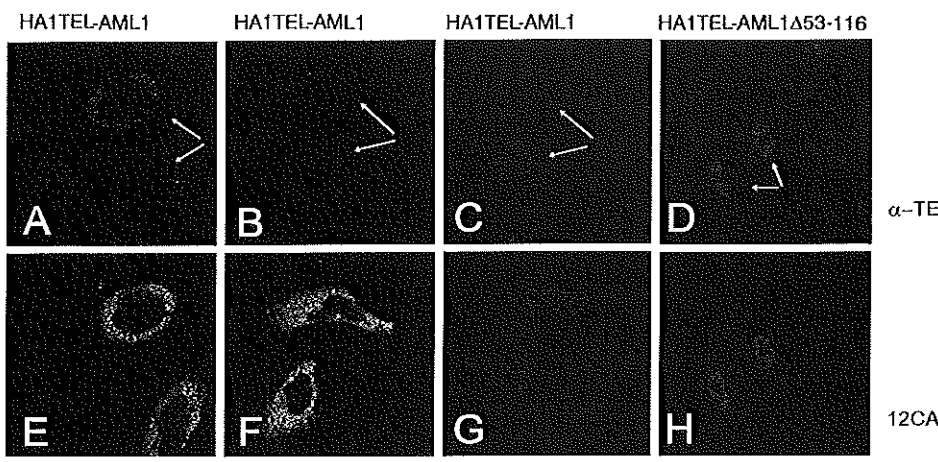

Figure 4 Cytoplasmic colocalization of endogenous TEL and exogenous TEL-AML1. HeLa cells were transiently transfected with HA1TEL-AML1 (**A,B,C,E,F,and G**) or HA1TEL-AML1Δ53-116 (**D** and **H**). Proteins were detected by indirect immunofluorescence in a double immunostaining using α-TEL antibodies (upper panel) and HA1 specific MoAb 12CA5 (lower panel). Anti-TEL signal was competed using bacterially expressed GST-TEL protein (**B**). Proteins were visualized using FITC- or Texas-Red-conjugated second antibodies. Arrows indicate transfected cells. Images were obtained by using confocal microscopy. Signals of **A, B, C, D** and **G** have been electronically amplified.

TEL-AML1 localizes to the nucleus in leukemic t(12;21) pre B cell lines

Because exogenous TEL-AML1 localized to the cytoplasm, we wished to address whether the localization was due to overexpression of the protein or whether it reflected its localization in leukemic cells. By using immunofluorescence analysis we studied the localization of TEL-AML1 in pre B-cell lines, which carry t(12;21) and have been demonstrated to express a TEL-AML1 chimeric transcript (Kim *et al.*, 1996). First, we performed RT-PCR analysis to confirm the presence of TEL-AML1, AML1-TEL and TEL transcripts in the leukemic cell lines. Figure 5 (right upper panel) demonstrates that the TEL-AML1 chimeric cDNA (1164 bp) was amplified from both REH and SUP-B28, but not from HL60, Nalm-6 and UoC-B1. However, no wildtype TEL cDNA product (1007 bp) could be amplified from REH and SUP-B28, whereas it was amplified from HL60, Nalm-6 and UoC-B1 (figure 5 upper left panel). We also amplified the reciprocal AML1-TEL cDNA product SUP-B28 (380 bp) (figure 5; lower left panel) As a control a 364 bp c-ABL cDNA was amplified from all cell lines, demonstrating the integrity of the mRNAs (figure 5; right lower panel).

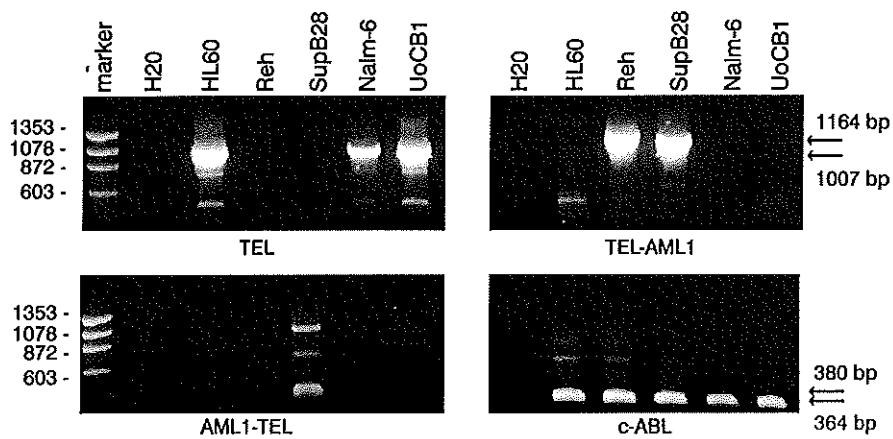

Figure 5. RT-PCR analysis of TEL-AML1, AML1-TEL and TEL mRNA in human pre-B cell lines. mRNA transcripts, specific for TEL (upper left panel), TEL-AML1 (upper right panel), AML1-TEL (lower left panel) and c-ABL (lower right panel) were amplified and run on a 2 % agarose gel. Arrows indicate the specific products.

By using immunofluorescence analysis, TEL-AML1 was localized to the nucleus in pre B-cell lines REH using N-terminal TEL antiserum (figure 6a). To verify that the signal was indeed derived from TEL-AML1, we showed that addition of bacterially expressed GST-TEL protein to the incubation of the TEL antibody abolished the signal (figure 6b). No signal could be obtained using C-terminal TEL antibodies, confirming the absence of TEL in this cell line (figure 6c). Similar results were obtained for SUP-B28 (data not shown). It is interesting to note that, despite the presence of AML1-TEL mRNA, the reciprocal AML1-TEL protein

could not be detected in SUP-B28 using C-terminal TEL antibodies, suggesting that it is not translated or rapidly degraded (data not shown). As a control, we studied the localization of TEL in human B-cell line UoC-B1 using either N-terminal or C-terminal TEL antibodies. With both antibodies TEL could be localized to the nucleus (figure 6d, e). Some signal is localized to the cytoplasm of REH and UoC-B1 cells using N-terminal TEL antibodies (figure 6a, d). Because the same signal was obtained when the samples were not incubated with the primary antibody, it is likely that the signal is due to aspecific binding of the secondary goat anti-rabbit IgG antibodies. This conclusion is further supported by the observation that competition with GST-TEL did not eliminate this signal (figure 6b). These results indicate that in leukemic pre-B cell lines carrying t(12;21), TEL-AML1 is predominantly nuclear.

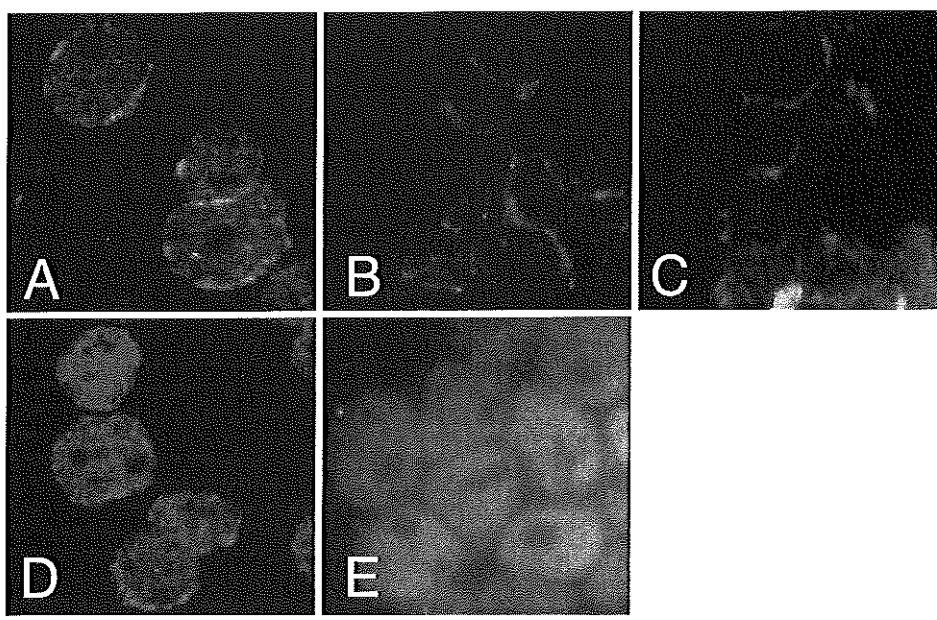

Figure 6. Subcellular distribution of TEL-AML1 and TEL in human pre B-cell lines. Proteins were detected by using indirect immunofluorescence analysis of cytospin preparations of human pre-B cell lines REH and UoC-B1. (A) TEL-AML1 was detected in the nucleus of REH cells by indirect immunofluorescence analysis using N-terminal antibodies. (B) Anti-TEL signal was competed using bacterially expressed GST-TEL protein. (C) No TEL specific signal could be obtained by using C-terminal TEL antibodies. (D,E) TEL was detected in the nucleus of UoC-B1 cells using N-terminal (D) or C-terminal TEL antibodies (E). Proteins were visualized using FITC- (A, B, C, E) or Texas-Red-conjugated second antibodies (D). Images were obtained by using confocal microscopy (A, B, D) or conventional microscopy (C and E).

TEL-AML1 and TEL co-immunoprecipitate through interaction of their HLH domains

To address whether TEL and TEL-AML1 interact via their HLH domains we used HeLa cells that expressed TEL or TEL-AML1 and the HA1-tagged version of the these molecules. By immunoprecipitating the HA1-tagged proteins from these cells with MoAb 12CA5, we analyzed whether the untagged molecules would co-precipitate. To this end we transfected HeLa cells with TEL or TEL-AML1 in the presence of HA1TEL, HA1TELΔ53-116, HA1TEL-AML1, or HA1TEL-AML1Δ53-116. The cells were then labeled with ^{35}S-methionine/cysteine, and the HA1-tagged proteins were precipitated with 12CA5. After SDS-PAGE, separated proteins were electroblotted onto nylon membranes and identified by Western analysis.

Figure 7a shows a Western blot of 12CA5 immunoprecipitates from cells co-expressing endogenous (lanes 1-7) and exogenous (lanes 1-5) TEL together with the different HA1-tagged molecules after probing with the C-terminal TEL antibody. The antibody recognized HA1-tagged TEL proteins HA1TELΔ53-116 (lane 2) and HA1TEL (top band in lanes 3 and 6), and co-precipitated untagged TEL proteins of 60 and 50 kDa (lanes 3,5,6 and 7). The fact that two TEL proteins are co-precipitated coincides well with data from Bernard and coworkers, who showed that two TEL proteins are translated from a single mRNA by alternative initiation codon usages (Poirel *et al.*, 1997). The untagged TEL products were not present in co-precipitations with HLH deletion mutants of HA1TEL and HA1TEL-AML1 (lanes 2 and 4). The data show that co-precipitation of TEL with HA1TEL and HA1TEL-AML1 is dependent on the presence of the HLH interaction domain.

Figures 7b and 7c show a similar analysis for TEL-AML1 and HA1-tagged TEL and TEL-AML1 proteins. After co-transfection, cells were metabolically labeled with ^{35}S-methionine/cysteine, HA1-tagged proteins were immunoprecipitated with 12CA5, separated on SDS-PAGE and electroblotted onto nylon filters. Figure 7b shows the Western blot probed with 12CA5, whereas labeled proteins that remained in the gel after blotting are shown in figure 7c. Figure 7b identified the different HA1-tagged proteins that are labeled with an asterix in the autoradiogram of the immunoprecipitates in figure 7c. This allowed us to deduce that $p96^{TEL-AML1}$ co-precipitated with HA1TEL (lanes 3) and HA1TEL-AML1 (lanes 5), but not with the HLH deletion mutant of TEL (lanes 2). This conclusion can not be drawn for the HLH deletion mutant from TEL-AML1, because HA1TEL-AML1Δ53-116 and $p96^{TEL-AML1}$ are of the same size. These data show that TEL-AML1 interacts with itself and we infer, but can not conclude from this experiment, that this interaction is mediated by the HLH domain.

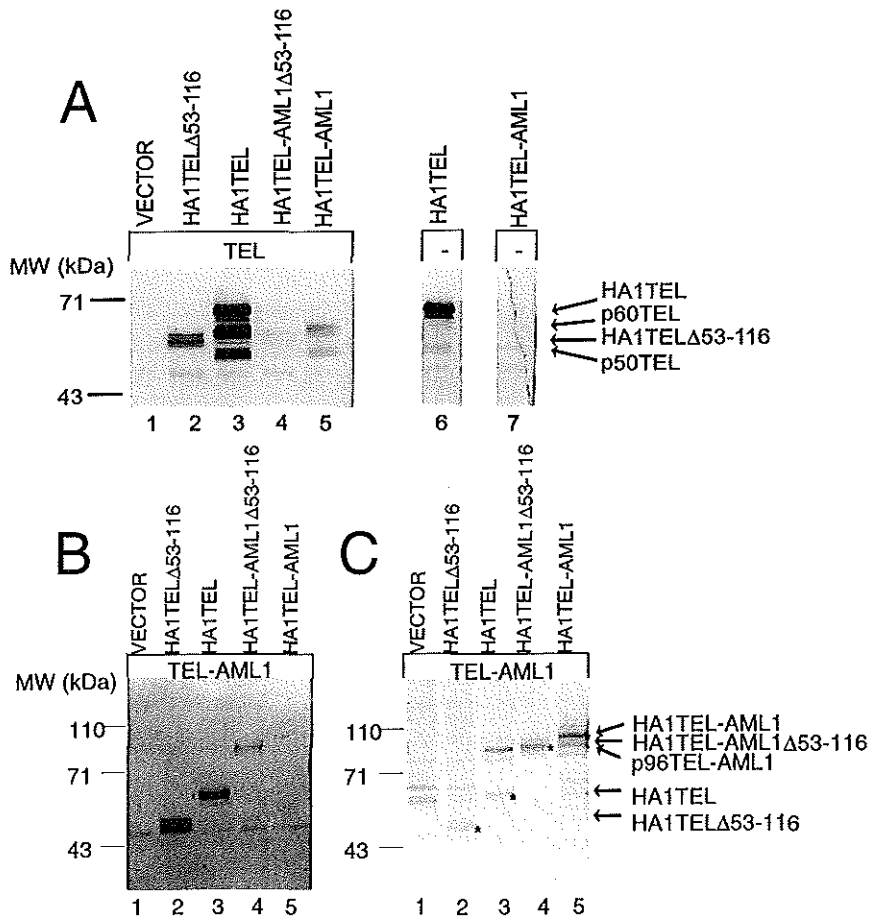

Figure 7 TEL and TEL-AML1 interact via the HLH domain of TEL. (**A**) HeLa cells were (co)transfected with expression plasmids encoding TEL, HA1TELΔ53-116, HA1TEL, HA1TEL-AML1Δ53-116 or HA1TEL-AML1 as indicated above the lanes. Proteins were immunoprecipitated with MoAb 12CA5, separated on a 10% SDS-polyacrylamide gel and electroblotted. Proteins on the Western blots were visualized using C-terminal α-TEL antiserum. HA1TELΔ53-116, HA1TEL and co-precipitating proteins are indicated with arrows on the right. (**B,C**) HeLa cells were transfected with expression plasmids encoding TEL-AML1, together with HA1TELΔ53-116, HA1TEL, HA1TEL-AML1Δ53-116 or HA1TEL-AML1. Following metabolic labeling with ^{35}S-methionine/cysteine, proteins were immunoprecipitated with MoAb 12CA5, separated on a 10% SDS-polyacrylamide gel and electroblotted. Proteins on the Western blot were visualized using MoAb 12CA5 (**B**). Residual protein in the gel after blotting was visualized by autoradiography (**C**). HA1TELΔ53-116, HA1TEL, HA1TEL-AML1Δ53-116, HA1TEL-AML1 are indicated with asterisk. Proteins are indicated with arrows on the right. Molecular masses of standard proteins are indicated (kDa).

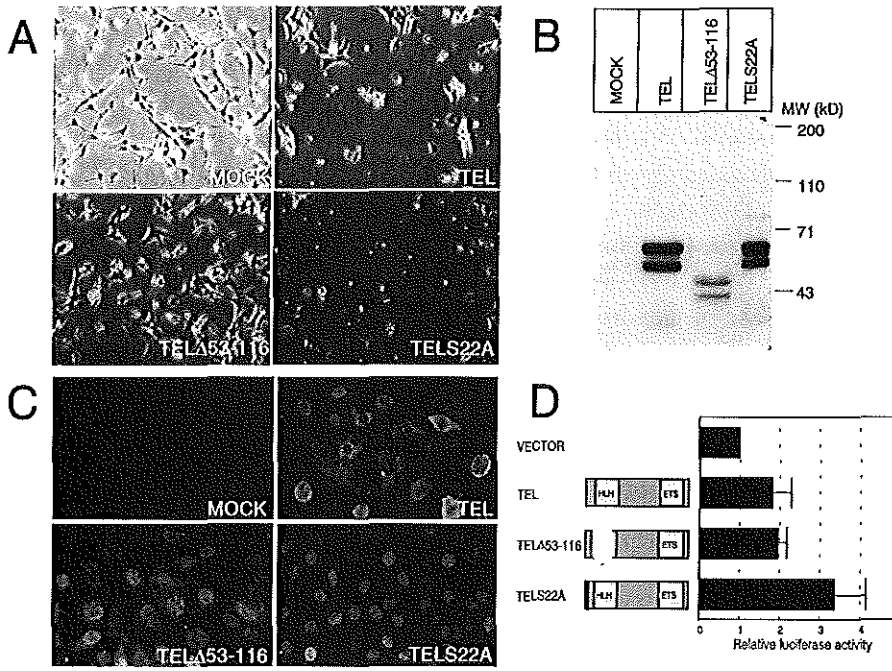

Figure 8 Induction of cell death by retroviral transduction of TEL into NIH3T3 cells. (A) Morphologic appearance of 2 x 10^5 NIH3T3 cells plated on plastic 16 hours after selection for CD8 that are transduced with mock, TEL, TELΔ53-116 or TEL^{S22A} cDNAs expressing retroviruses. (B) Analysis of protein expression of infected cells at the time of CD8 selection. Proteins were separated on a 10% SDS-polyacrylamide gel and electroblotted. Proteins were visualized by using α-TEL antiserum in Western analysis. (C) Indirect immunofluorescence analysis of NIH3T3 cells that are transduced with mock, TEL, TELΔ53-116 and TEL^{S22A} cDNAs expressing retroviruses using C-terminal α-TEL antibodies. Images were obtained by conventional microscopy. (D) Transient transcription experiments in NIH3T3 cells were performed using 3 μg of CMV driven TEL, TELΔ53-116 and TEL^{S22A} activator constructs with 1μg of pMSVluc reporter. Induction of luciferase (normalized to a secreted alkaline phosphatase control) are relative to empty vector (see Buijs et al., submitted for publication).

Overexpression of TEL induces cell death

At present, the biological role of TEL is still elusive. We observed that retroviral transduction of TEL into NIH3T3 fibroblasts resulted in dramatic cell death 24 to 30 hours after selection of CD8-positive, transduced cells (figure 8a). Induction of cell death was dependent on the presence of the HLH domain, because cells transduced with TELΔ53-116 did not die. Furthermore, cells transduced with a TEL mutant in which serine residue 22, a putative phosphoacceptor of a consensus MAPK phosphorylation sequence, had been eliminated, died even more rapidly (within 12 hours after sorting). This phenomenon was observed in at least three independent experiments. Figure 8b demonstrates the expression of exogenous TEL proteins in infected cells, analyzed by Western analysis with C-terminal

α-TEL antibodies at the time of cell sorting. Approximately equal amounts of the proteins were expressed, indicating that the difference between the observed phenotypes is not due to different expression levels of the proteins. These data directly or indirectly link TEL overexpression to the induction of cell death. Elimination of a putative MAPK phosphorylation site in TEL by mutation of serine residue 22, seems to enhance this activity. This observation opens the possibility that TEL normally has a positive regulatory role in induction of cell death.

To further analyze this phenomenon, the subcellular distribution of virally transduced TEL, TELΔ53-116 and TEL^{S22A} was analyzed by indirect immunofluorescence analysis. Figure 8c demonstrates that C-terminal TEL antibodies detected TEL^{S22A} exclusively in the nucleus of infected NIH3T3 cells, whereas TEL was distributed in the cytoplasm, the nucleus or in both compartments and TELΔ53-116 was predominantly nuclear, suggesting that TEL function is in part regulated by localization.

We recently observed that the MSV LTR is transcriptionally activated by MN1-TEL, dependent on the ETS domain of TEL, but luciferase expression is hardly induced by TEL (Buijs et al., submitted for publication). To determine whether deletion of the HLH domain or mutating S22 to A22 altered TELs transactivation activity of the MSV LTR, we tested the TEL mutants in transient transcription assays. Co-transfection of the reporter pMSVluc with CMV promoter driven TEL, TELΔ53-116 or TEL^{S22A} demonstrated that the transactivation activity of TEL was not changed by deleting the HLH domain. However, an increase in transcription activity was detected for the TEL^{S22A} mutant (figure 8d). These observations suggest that TEL transcriptional activity may be regulated by serine/threonine kinases.

Discussion

We showed that t(12;21) TEL-AML1, which is associated with 25% of the cases of childhood pre-B acute lymphoblastic leukemia (McLean *et al.*, 1996; Romana *et al.*, 1995b; Shurtleff *et al.*, 1995), is predominantly located in the cytoplasm of transiently transfected or stable, virally transduced cells. This cytoplasmic localization is dependent on an intact HLH protein-protein interaction domain. We observed the same phenomenon in cells transfected with a TEL-OCT6 construct, in which the ETS domain of TEL was replaced by the POU-homeo domain of OCT6. Perhaps TEL-AML1 and TEL-OCT6 remain in the cytoplasm because the HLH domain may mask the nuclear localization signals of the RUNT domain of AML1 or of the POU-homeo domain of OCT6. However, other explanations are equally plausible. Ghysdael and coworkers reported that TEL forms oligomers using the HLH domain as an interface (Jousset *et al.*, 1997). We demonstrated by using immunoprecipitation that TEL-AML1 interacts with itself and with TEL, confirming the observations of Golub and coworkers who used *in vitro* transcribed/translated TEL-AML1 (McLean *et al.*, 1996 1133). It is therefore possible that homotypic or heterotypic TEL-AML1 and TEL oligomers form insoluble cytoplasmic protein complexes that can not be processed by the nuclear import machinery. This possibility is supported by the observation that deletion of the oligomerization domain in TEL-AML1 and TEL-OCT6 prevents complex formation and causes the proteins to localize to the nucleus. For the same reason one would expect TELΔ53-116 to localize exclusively to the nucleus, which is not the case. The observation is not necessarily in contradiction with the above, since it can not be ruled out that the HLH mutant TEL protein may have different folding properties that partly shield its NLS from recognition.

Our observations are in contradiction with a recently published study, in which TEL-AML1, expressed in transient transfection experiments, was found to reside solely in the nucleus, based on results of cell fractionation and subsequent Western analysis (Hiebert *et al.*, 1996). This apparent discrepancy may be attributed to the different experimental approaches used for localization. It is entirely possible that, if oligomerized TEL-AML1 is insoluble, that aggregates residing in the cytoplasm would cofractionate with nuclear and nuclear matrix preparations in the procedure used by Hiebert and coworkers. Because TEL-AML1Δ53-116 localized to the nucleus in transient transfection experiments we are convinced that our immunolocalization of TEL-AML1 in the cytoplasm is not an artifact of the detection system. Furthermore, transfected TEL-AML1 and endogenous TEL colocalize in the cytoplasm, whereas in cells transfected with a TEL-AML1 cDNA construct that lacks the HLH domain, TEL resides in the nucleus.

Most importantly, TEL-AML1 in transfected cells seems to sequester endogenous TEL in the cytoplasm, thereby diminishing the concentration of nuclear TEL. Recently, Kim et al. described pre-B cell lines that were derived from leukemic blasts of patients with t(12;21) ALL (Kim *et al.*, 1996). By using immunofluorescence analysis, we demonstrated

that in contrast to exogenous TEL-AML1, endogenous TEL-AML1 localized to the nucleus of pre B-cell lines REH and SUP-B28. In these cell lines no endogenous TEL was present. Although TEL-AML1 localized predominantly to the nucleus in the B cells, we can not exclude that small amounts of TEL-AML1 are expressed in the cytoplasm.

Our data suggest that one of the consequences of t(12;21) is an association of TEL-AML1 and TEL via the HLH domain. Therefore, TEL-AML1 will reduce the effective concentration of TEL in the nucleus. In this respect, it is noteworthy that t(12;21) is frequently associated with deletion of the second *TEL* allele (Raynaud *et al.*, 1996; Wlodarska *et al.*, 1996). These deletions have been found to occur intragenically and as a secondary event in the pre-B ALL (Kim *et al.*, 1996; Raynaud *et al.*, 1996; Wlodarska *et al.*, 1996). Elimination of TEL protein seems to confer a proliferative advantage to these cells, leading to clonal selection. Thus, reduced TEL concentrations may already provide a partial growth advantage, which could be one of the growth promoting aspects of TEL-AML1 expression. Similarly, TEL/TEL-AML1 oligomers may still be able to bind to the cognate TEL DNA binding sites, adding to the partial inactivation of normal TEL activity. In addition, TEL-AML1 may interfere with AML1 dependent transcription regulation, representing the second growth promoting aspect of the fusion protein.

Our observation may invalidate the interpretation that TEL-AML1 has a dominant negative effect on AML1-B and AML2 dependent transcription of the TCRβ enhancer containing reporter plasmids in transient transfection assays by competition for the binding sites (Hiebert *et al.*, 1996; Meyers *et al.*, 1996). Although it cannot be excluded that small amounts of TEL-AML1 in transfected cells enter the nucleus, where it may inhibit AML1 dependent transcription, as suggested, the large amount of cytoplasmic TEL-AML1 in these cells may trap its dimerization partner, CBFβ. This would make it unavailable to nuclear AML1B or AML2, hence providing an interpretation for the observed dominant negative effect of TEL-AML1 in these experiments. Therefore to prove the dominant negative role of TEL-AML1 on AML1/CBFβ transcription regulation, the transient expression experiments should be repeated in a cell line in which the expression of TEL-AML1 can be regulated to a level where the protein is still nuclear. Provided that TEL-AML1 functions as a repressor then TEL/TEL-AML1 oligomerization could impair TEL-AML1s alleged repressing activity. Deletion of the second *TEL* allele would potentiate the repressing activity of TEL-AML1.

In concordance with the finding of Bernard and coworkers (Poirel *et al.*, 1997), both endogenous and exogenous TEL were present in two forms of 60 kDa and 50 kD. $p60^{TEL}$ is translated from the first ATG, whereas $p50^{TEL}$ is initiated from the ATG at codon 43 of the TEL ORF. It needs to be noted that the first ATG in the ORF of TEL, in contrast to the second, is in a suboptimal Kozak initiation sequence (Kozak, 1989). Addition of an HA1-tag to TEL with a model Kozak initiation sequence apparently suppresses initiation of translation from TEL ATG 1 and 43 codons since HA1TEL is expressed as a single doublet of 67 kDa.

Overexpression of TEL in NIH3T3 cells induced rapid cell death. The induction of cell death was dependent on the HLH domain of TEL and was dramatically accelerated by mutation of the consensus MAPK phosphorylation site at serine residue 22, suggesting that TEL activity may be regulated by a MAPK pathway mediated phosphorylation. Similar observations have been made in *Drosophila*. Transgenic flies expressing YAN cDNA, in which all eight MAPK phosphorylation consensus sequences had been mutated, exhibited a dramatic increase in cell death in the eye imaginal disc (Rebay & Rubin, 1995), suggesting that cells continuously prevented from responding to inductive signals may eventually opt to die. The 22S→22A mutation in TEL also influences its subcellular distribution, opening the possibility that TEL function is in part regulated by localization, as has been demonstrated for YAN (Rebay & Rubin, 1995). Furthermore, the transactivating activity of TEL was increased by mutating the putative phosphoacceptor site, suggesting that TELs transcription activity is regulated by phosphorylation. At present it is not known whether $p60^{TEL}$ and $p50^{TEL}$ have similar functions or whether their activities are similarly regulated. It could be of significance that the MAPK site at aminoacid 22 is absent from $p50^{TEL}$, suggesting that its putative regulatory function concerns $p60^{TEL}$. The present observations for TEL are reminiscent of those of *Drosophila* ETS factor POINTED, which encodes two proteins, P1 and P2. Transcription of the *POINTED* gene is initiated from two distinct promoters (Klämbt, 1993). These proteins have a common C-terminus, which includes the ETS domain, but have different N-terminal sequences. In addition, P2 contains a single MAPK phosphorylation site, which P1 lacks. When tested in transient transcription assays, P1 was a significantly stronger activator of transcription than was P2 (O'Neill *et al.*, 1994). Further, activation of the RAS/ERK2 pathway increased P2-associated effects on transcription but did not affect P1-related activity. Mutation of the phosphoacceptor residue in the MAPK consensus site of P2 abolished its ability to activate transcription.

Although the nature of TEL induced cell death is unclear at present, TEL may be directly or indirectly involved in regulation of cell death in pre B-cells. Ablation of TEL function may therefore inhibit cell death and thus contribute to an excess of pre B-cells, providing a rationale for TELs alleged tumor suppressor activity. Experiments to investigate whether overexpression of TEL-AML1 in growth factor dependent B-cells prevent induction of apoptosis are in progress. Other ALL-associated translocations containing N-terminal TEL may display similar effects as t(12;21) TEL-AML1. For example, t(9;12) TEL-ABL is found in the cytoplasm and contains the HLH domain of TEL. We predict that the effective concentration of nuclear TEL will be reduced by oligomerization with TEL-ABL. Further, in one case of t(9:12) TEL-ABL deletion of the second *TEL* allele has been reported (Golub *et al.*, 1996).

In conclusion, our studies suggest that TEL-AML1 may have a dual role in leukemogenesis: it may tether TEL activity and influence AML1/CBFβ-regulated expression of hematopoietic genes.

Acknowledgements

The authors wish to thank Dr. Dick Ashmun and coworkers for FACS analysis, Drs. Judith Boer and Victor Garcia for assisting with confocal microscopy, Dr. Scott Hiebert for providing α-AML1 antibodies, Dr. Peter Marynen for providing N-terminal TEL antiserum, Dr. Santos for providing the activated RAS cDNA, Peggy Burdick and Charlette Hill for secretarial assistance and Amy Frazier for editing of the manuscript. These studies were supported by Cancer Center CORE Grant CA-21765 and by the Associated Lebanese Syrian American Charities (ALSAC) of St. Jude Children's Research Hospital.

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Chapter 4. Discussion

Entities of t(12;22) associated leukemia

In the 1970s t(12;22)(p13;q11) has been described as a chromosomal aberration associated with CML (Engel *et al.*, 1977; van der Blij-Philipsen *et al.*, 1977; Verma & Dosik, 1979). These observations led to the hypothesis that it may represent an alternative Philadelphia translocation, involving the *BCR* gene on chromosome 22q11. However, Hagemeijer et al. reported t(12;22) as a sole chromosomal aberration in a case of AML FAB subtype M1 (Hagemeijer *et al.*, 1981). The translocation seems not to be associated with a specific FAB subtype, because it has been detected in acute myeloid leukemias of FAB classifications M2, M4, M7 as well as in MDS (Mitelman, 1991). The basis of classifying t(12;22) as a variant Philadelphia translocation in CML was further undermined by the finding that *MN1* rather than *BCR* was disrupted by the aberration (chapter 2.1).

Since t(12;22) has been identified as the sole chromosomal abnormality in some leukemic samples, it has been argued that it could be the primary event in leukemogenesis (Hu *et al.*, 1996). The incidence of t(12;22) as a sole recurrent translocation is low. It is more frequent in leukemic cells with a karyotype that is dominated by complex rearrangements (Mitelman, 1991). However, cases have been described in which t(12;22) was only detected at relapse, suggesting that the translocation is involved in progression of the disease (chapter 2.1) (Buijs *et al.*, 1995). The generation of the growth factor dependent cell line UCSD/AML1, that was established from leukemic cells of a patient with multilineage AML in relapse, support this notion. While only a subset of leukemic cells in the bone marrow of the patient at relapse carried the translocation, it was present in all cells of the cell line (Oval *et al.*, 1990). These cells responded to a broad range of growth factors. This suggests that selection for this cell population may have occurred *in vitro* in the presence of growth factors, which may reflect clonal selection of cells carrying the translocation by a proliferative advantage *in vivo*. In conclusion, t(12;22) represents a distinct chromosomal rearrangement, that does not identify a specific leukemogenic FAB phenotype. The fusion protein seems to be involved in progression of the disease.

Putative function of MN1-TEL

The characterization of t(12;22) enabled us to initiate a series of experiments to unravel the molecular mechanism by which the MN1-TEL fusion protein may contribute to leukemogenesis. We demonstrated that MN1-TEL transformed a specific subline of NIH3T3 fibroblasts, using a retroviral transfer system (chapter 2.2). However, it had a weak transforming potential, since, unlike activated RAS, it did not transform standard NIH3T3 fibroblasts. Apparently, MN1-TEL needs additional mutations to transform.

The transforming potential of MN1-TEL was found to depend on a functional ETS domain, suggesting that the fusion protein aberrantly regulates transcription of TEL target genes, leading to malignant transformation. Indeed, unlike TEL, MN1-TEL was found to

transactivate transcription of a luciferase reporter gene driven by the Moloney sarcoma virus LTR in transient transcription assays. Likewise, MN1 transactivated the MSV LTR. Transcription activation of a reporter construct by MN1 has also demonstrated in yeast and HeLa cells when linked to a GAL4 DNA binding domain. Furthermore, MN1 could induce expression of a reporter construct under the control of the HSV TK promoter in NIH3T3 cells, but not in hepatoma cell line Hep3B (Molijn and Zwarthoff; manuscript in preparation). This may reflect a tissue specific function of MN1 in transcription regulation.

Transcription factors typically contain distinct domains for DNA binding and activation of target genes (Triezenberg, 1995). At present, it is unknown whether MN1 can bind DNA. However, it contains proline-rich sequences and glutamine stretches, characteristic for transcription activators that act from a proximal promoter position in conjunction with an enhancer element (Gerber *et al.*, 1994). Glutamine stretches form β-sheets that can function as oligomerization domains (Perutz *et al.*, 1994; Stott *et al.*, 1995). The human transcription factor SP1, that has two glutamine rich domains, enhanced transcription of a reporter gene by interaction of SP1 molecules bound to GC-rich recognition sites 1.8 kb apart. This interaction, that mediates DNA bending, was dependent on the glutamine-rich sequences (Su *et al.*, 1991). Furthermore, SP1 bound the *Drosophila* TAX, a component of TFIID of the transcription initiation complex, via its glutamine sequences (Gill *et al.*, 1994). This interaction is thought to stabilize the transcription/initiation complex. Likewise, MN1 sequences in MN1-TEL may contribute to aberrant transcription activation of TEL target genes by influencing the stabilization of the basal transcription complex. Alternatively, MN1-TEL may result in the aberrant transactivation of genes normally regulated by MN1 and an ETS factor.

VP16-TEL, a synthetic fusion protein that contains the acidic transactivating domain of HSV1, also transactivated the MSV LTR. However, it did not transform murine fibroblasts. Furthermore, a domain within the first 228 aminoacids of MN1-TEL appeared to be essential for transformation, while deletion of this domain did not influence its capacity to transactivate the MSV LTR. Based on these two observations, it seems that MN1 does not render MN1-TEL transforming by simple addition of transactivating sequences to TEL. We postulate that a domain within the first 228 aminoacids of MN1-TEL mediates specific protein-protein interactions that are important for the transcription activation of target genes that initiate transformation pathways. The observation that the first 228 aminoacids of MN1, when linked to TEL, have some transforming potential, supports this hypothesis.

At present only two chromosomal translocations, t(16;21) FUS/CHOP-ERG and t(12;22) MN1-TEL, have been documented in human leukemia, in which the DNA binding domain of an ETS factor is present in a chimeric protein, (Buijs *et al.*, 1995; Prasad *et al.*, 1994; Shimizu *et al.*, 1993). Our data indicate that subversion of normal transcription regulation by ETS domain containing fusion proteins may contribute to the pathogenesis of leukemia, in analogy to some tumors of neuroectodermal origin (chapter 1.4).

Aberrant localization of MN1-TEL

Oncogenic mutation of proteins has been found to result in altered subcellular localization that may be of functional importance for leukemogenesis. For example, c-ABL, a non-receptor tyrosine kinase, that is mainly expressed in the nucleus, is located in the cytoplasm as a result of its fusion with BCR by the Philadelphia translocation (Dhut *et al.*, 1990; Van Etten *et al.*, 1989). BCR-ABL associates with F-actin fibers, which is essential for malignant transformation (McWhirter & Wang, 1993).

Altered distribution within a subcellular compartment has also been observed for proteins involved in chromosomal translocations, that are associated with leukemia. Acute promyelocytic leukemia (APL) is characterized by t(15;17) that results in a PML-RARα fusion protein (1991; de Thé *et al.*, 1990; Goddard *et al.*, 1991; Kakizuka *et al.*, 1991). PML is localized in nuclear bodies or PML oncogenic domains (PODs), a macromolecular structure, of which the function is unknown (Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994). In leukemic cell lines expressing PML-RARα these nuclear bodies are disrupted and the functional dimerization partner of RARα, RXR, is redirected to aberrant structures in the nucleus. Treatment of these cells with retinoic acid (RA) induces a relocation of RXR to PODs.

A distinct speckled pattern has been described for E2A-PBX1, associated with t(1;19) in pre B-ALL (Kamps *et al.*, 1990; Nourse *et al.*, 1990). When transfected into NIH3T3 cells, E2A-PBX1 was concentrated in spherical domains denoted chimeric oncoprotein domains (CODs). Deletion of an N-terminal transcription activation domain in E2A, essential for transformation by E2A-PBX1, or transfection of HOXB7, a homeodomain protein capable of enhancing DNA binding by PBX1 and E2A-PBX1, impaired COD formation (LeBrun *et al.*, 1996). At present, it is not known whether overexpression of HOXB7 reduces the transforming capacity of E2A-PBX1. These data indicate that aberrant localization of fusion proteins in nuclear bodies may also be of functional importance for leukemogenesis.

Do TEL and ETS-1 have a common DNA binding mechanism?

The transforming and transactivating potentials of MN1-TEL were found to be dependent on a functional ETS domain. Data presented in chapter 2.2 indicated that the ETS domain of TEL binds a canonical GGAA sequence, CCGGAAGT. This sequence closely resembles the recognition sequences for ETS-1 in the MSV LTR and for ELK1 and ERG in the E74 promoter (Wasylyk *et al.*, 1993). As mentioned in chapter 1.1, DNA binding by ETS-1 is regulated by an intramolecular mechanism involving two regions: an α-helix upstream of the ETS domain and the C-terminus. DNA allosterically induces unfolding of the helix and transiently relieves inhibition. Interaction with a partner protein may stabilize the protein/DNA complex (Petersen *et al.*, 1995). Graf and coworkers (Lim *et al.*, 1992) presented a model that the inhibitory domain and the C-terminus interact, thereby preventing DNA binding via the ETS domain (figure 1; upper panel). Graves and coworkers modified this model and suggested that interaction with the DNA results in a uncoupling of the inhibitory domains, followed by a

conformational change of the ETS domain (Jonsen *et al.*, 1996). Seth et al. demonstrated that DNA binding of ETS-1 to ETS binding sites was stabilized by addition of ETS-1 specific monoclonal antibodies (Seth *et al.*, 1993). Preliminary data suggest that a similar mechanism may regulate TEL DNA binding. Addition of antibody directed against the last ten aminoacids of TEL resulted in an intensified supershifted signal, suggesting that binding of the antibody may interfere with an intramolecular interaction, involving sequences in the C-terminus of TEL that mediate a DNA binding regulatory mechanism (figure 1; middle panel). Furthermore, deletion of sequences N-terminal of the ETS domain abolishes the intramolecular interaction and thereby interferes with the regulation of ETS/DNA interaction (figure 1; lower panel). This mechanism may be common to a subgroup of ETS factors in which the ETS domain is located in the C-terminus of the protein. An interesting aspect is its relation with MN1-TEL. Additional studies may reveal whether in MN1-TEL the DNA binding inhibition is still present or not.

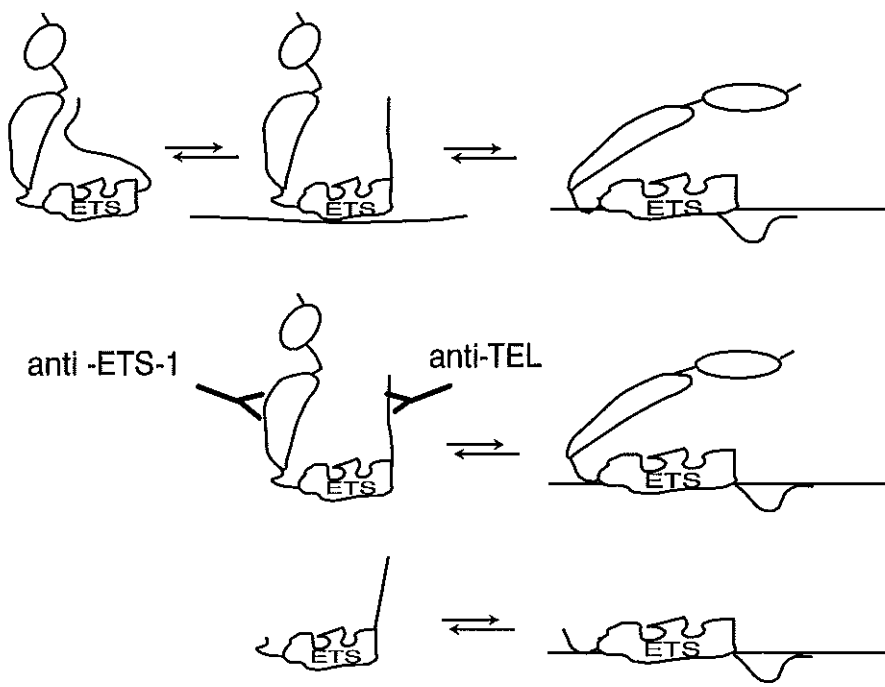

Figure 1. *Model of repression of DNA binding by ETS factors.* Sequences in the C-terminus of ETS factor interact with the inhibitory domain, preventing DNA binding via the ETS domain. DNA interaction is accompanied with uncoupling of the inhibitory domains, which allosterically alters the conformation of the ETS domain (upper panel). Interaction with heterologous factors or antibodies (middle panel), or deletion of one of the domains (lower panel) interferes with this intramolecular DNA binding regulatory mechanism

Clinical significance of t(12;21)

Molecular characterization of t(12;21)(p13;q22) made it possible to increase the level of detection of the translocation in childhood ALL from 0.05%, using standard cytogenetic techniques, to 25% (500x) by using molecular genetic and molecular cytogenetic techniques (chapter 3.1). This increase in sensitivity of diagnosis has important clinical implications. Retrospective analysis of children with ALL treated with high-risk treatment protocols, revealed that cases with TEL-AML1 t(12;21) do significantly better than those with germline *TEL*, making this translocation the first example of a specific genetic abnormality associated with a favorable prognosis in childhood ALL. Preliminary data of a study with patients treated with antimetabolite-based therapy revealed that patients with *TEL* rearrangements also do significantly better than those with germline *TEL* (Rubnitz *et al.*, 1996). This indicates that t(12;21) patients may be treated less aggressively without loss of therapeutic efficacy, reducing the toxicity, effects on growth and development, and treatment induced secondary acute myeloid leukemia. However, preliminary data of other retrospective studies indicate that less aggressive treatment results in less favorable outcome (Chambost *et al.*, 1996). Therefore, it will be necessary to perform protocol driven prospective studies to resolve this discrepancy.

How may TEL-AML1 contribute to leukemogenesis?

The AML1/CBFβ transcription complex is targeted by t(8;21) and t(3;21), and inv(16), associated with 30% of myeloid leukemias (figure 2). The rearrangements result in AML-ETO, AML1-EVI1 and CBFβ-MYH11 fusion genes, respectively (Erickson *et al.*, 1992; Liu *et al.*, 1993; Mitani *et al.*, 1994; Miyoshi *et al.*, 1993; Nisson *et al.*, 1992; Sacchi *et al.*, 1994). AML1-ETO retains the ability to interact with the enhancer core DNA sequence and has been shown to negatively interfere with AML1-dependent and AML2-dependent transactivation (Meyers *et al.*, 1993; 1995; 1996).

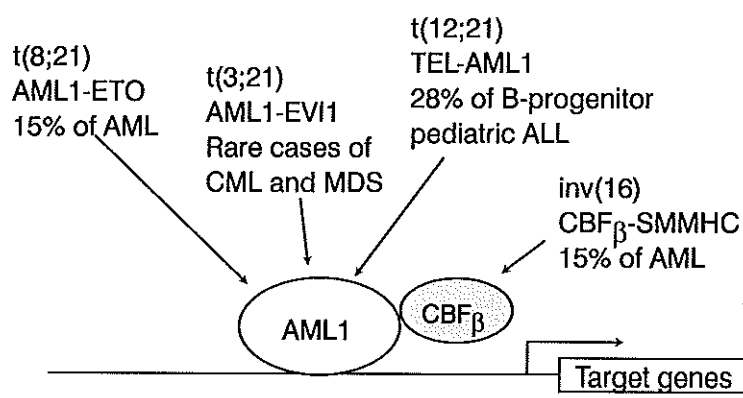

Figure 2. *Schematic representation of chromosomal aberrations affecting the AML1/CBFβ transcription complex* (courtesy to Dr. J.R. Downing)

The crucial role of the AML1/CBFβ transcription complex during hematopoiesis was further shown by gene knockout experiments in mice. The complex appeared to be essential for establishment of fetal liver hematopoiesis during embryonic development as demonstrated in mice nullizygous for *AML1* or the gene encoding the CBFβ subunit (Okuda *et al.*, 1996a; Wang *et al.*, 1996). Interestingly, mice carrying AML1-ETO or CBFβ-MYH11, generated by homologous recombination using the knock-in strategy, displayed a phenotype similar to the knockout mice (Castilla *et al.*, 1996; Okuda *et al.*, 1996b; Yergeau *et al.*, 1996). These data are consistent with the dominant negative inhibitory function of both fusion proteins over the AML1/CBFβ transcription factor complex.

Hiebert and collaborators discovered that TEL-AML1 negatively interfered with AML1 dependent transcription of the TCRβ enhancer in transient transcription assays (Hiebert *et al.*, 1996), suggesting that TEL-AML1 may have a dominant negative inhibitory function in the nucleus as well. However, immunofluorescence analysis revealed that TEL-AML1 is located in the cytoplasm of virally transduced murine fibroblasts, despite the fact that the nuclear localization signal (NLS) in the RUNT domain of AML1 is present in the chimeric protein (chapter 3.2). A mutant version of this protein lacking the HLH domain localized to the nucleus. A similar subcellular localization was observed for a synthetic fusion protein in which the ETS domain of TEL had been replaced by the POU domain of OCT6. Several explanations are plausible for this phenomenon. TEL-AML1 or TEL-OCT6 may form homotypic complexes or heterotypic complexes with TEL via the HLH domain, preventing nuclear transfer. The HLH domain in TEL-AML1 and TEL-OCT6 may mask the NLS in the RUNT domain or POU homeodomain, respectively. The observation that highly expressed TEL-AML1 is a cytoplasmic protein opens the possibility that it, unlike AML1-ETO, may not act as a dominant negative repressor of AML1 dependent transcription in the nucleus. In this respec, it is interesting to note that Tanaka et al. reported that AML1-ETO and AML1-EVI1 accumulate CBFβ more efficient in the nucleus than AML1 (Tanaka *et al.*, 1996). Our observation suggest that TEL-AML1 may sequestrate factors, such as CBFβ, in the cytoplasm. This would provide a distinct mechanism for dominant interference. However, in human pre-B cell lines carrying t(12;21), TEL-AML1 was found to be localized to the nucleus, suggesting the cytolasmic localization of TEL-AML1 in transient transfection experiments is due to overexpression of the chimeric protein. This observation argues for the hypothesis that TEL-AML1 is a dominant negative repressor of AML1 dependent transcription. Nevertheless, our observations of overexpressed protein may reflect the biology of the chimeric protein *in vivo*. TEL-AML1 could associated with TEL in the nucleus and thereby hamper nuclear TEL activity. Thus, TEL-AML1 may have a dual effect; it could diminish the effective concentration of both AML1/CBFβ and TEL in the nucleus. This biochemical mechanism would support the hypothesis, until now based on genetic data, that progression of the leukemia is dependent on the dosage of TEL (Kim *et al.*, 1996; Raynaud *et al.*, 1996; Wlodarska *et al.*, 1996). Expression of TEL-AML1 would already diminish TELs nuclear function, which is completely ablated by inactivation/deletion of the second *TEL* allele, selected for during progression of the disease.

At present the physiological function of TEL in hematopoiesis is not known. One possibility is that TEL may be involved in regulation of differentiation or growth of precursor cells. Alternatively, TEL may be involved in the regulation of cell death of pre-B cells, since overexpression of TEL in fibroblasts induced cell death (chapter 3.2). Ablation of TEL function may therefore have a reverse effect which would lead to an excess of pre-B cells. Fusion proteins, such as BCR-ABL, E2A-PBX1 and E2A-HLF, associated with 10% of pre-B ALL (figure 2), were found to affect cell survival, making cells resistant to apoptosis induced by a variety of agents (Cortez *et al.*, 1996; Dedera *et al.*, 1993; Inaba *et al.*, 1996; Roger *et al.*, 1996). They account for a considerable percentage of aberrations in pre-B ALL, indicating that interference with apoptotic pathways, necessary for maintenance of a balanced B-cell population, is a common step in development of B-cell leukemia.

HLH domain of TEL defines a specific oligomerization domain

The HLH domain is present in a subset of ETS factors (see chapter 1; figure 1). It has been termed the pointed domain (Klämbt, 1993), or the HLH domain, based on its weak sequence homology with HLH dimerization domain present in transcription factors such as c-Myc and MyoD (Seth & Papas, 1990). The HLH domain of TEL has been demonstrated to be a homotypic oligomerization domain (Jousset *et al.*, 1997), essential for the oncogenic properties of TEL-PDGFβR, TEL-ABL (Carroll *et al.*, 1996; Golub *et al.*, 1996), and probably for TEL-AML (chapter 3.2). However, the domain is non-functional in MN1-TEL (chapter 2.2). The physiological function of oligomerization of TEL is still elusive. Interestingly, *Drosophila* POINTED P1, which lacks the HLH domain, is a constitutive transcription activator. Transcriptional activation of its splice variant P2, which includes this domain, is regulated by RAS mediated phosphorylation (O'Neill *et al.*, 1994). It remains to be determined whether this difference in transcription activation is mediated through phosphorylation-controlled oligomerization with a regulatory factor, and whether it reflects a mechanism for regulation of transcription control by ETS factors in general. MN1 may contribute to the activation of TEL by substituting for an activating partner, or by preventing interaction with a negative regulatory factor.

Future directions

MN1-TEL

Transcription regulation of the MSV LTR in NIH3T3 cells by MN1, TEL and MN1-TEL will be characterized in more detail. Deletion analysis of the MSV LTR will determine which enhancer/promoter sequences and putative cooperating factors are essential for the transcription activation by MN1 and MN1-TEL in this cell type.

To extend the evidence for MN1-TELs involvement in leukemogenesis, cell line U937T (Boer and Grosveld, manuscript in preparation), a derivative of the human monoblast cell line U937 (Sundström & Nilsson, 1976) that expresses the tetracycline responsive chimeric transactivator TET-VP16 (Gossen & Bujard, 1992) will be used. Combined treatment with transforming growth factor (TGF)β and 1,25 dihydroxyvitamin D3 was

demonstrated to induce monocytic maturation of U937 cells (Testa *et al.*, 1993). With this system the influence of tetracycline dependent expression of the TEL, MN1 and MN1-TEL cDNAs on the proliferation or maturation potential of these cells will be analyzed.

Potential target genes of TEL will be characterized by representational difference analysis of mRNAs in MN1-TEL transformed fibroblasts and U937T cells expressing MN1-TEL, with the aim to unravel the transcription cascade controlled by TEL. This system has been used successfully to identify genes transcribed in response to the EWS-FLI-1 chimeric protein, associated with Ewing's sarcoma (Braun *et al.*, 1995).

The human monocytic cell lines, UCSD/AML1 and MUTZ-3, carrying a t(12;22)(p13;q11) (Hu *et al.*, 1996; Oval *et al.*, 1990), represent very useful model systems for MN1-TEL leukemogenesis. They can be used to characterize additional genetic mutations, as well as to elucidate the pathophysiology of MN1-TEL. Furthermore, they can be used for studies on monocytic activation as well as *in vitro* analyses of therapeutic agents.

In vivo leukemogenesis of MN1-TEL will be investigated by generating MN1-TEL transgenic mice which express the fusion protein from the human cathepsin G gene promoter. This promoter was successfully used to direct expression of a PML-RARα cDNA to early myeloid precursor cells in transgenic mice (Grisolano *et al.*, 1995). Alternatively, MN1-TEL chimeric mice will be generated using a "knock-in" strategy, using homologous recombination of the human TEL cDNA into the mouse *MN1* gene. This approach has the advantage that expression of the fusion gene is driven from the *MN1* promoter. It has successfully been used to induce myeloid leukemia by MLL-AF9, associated with t(9;11)(p22;q23) in AML (Corral *et al.*, 1996), and has been applied to generate t(6;9) DEK-CAN chimeric mice (van Deursen and Grosveld, personal communication). Demonstration of the *in vivo* oncogenicity of the MN1-TEL chimera would establish its role as an oncogene. If expression of MN1-TEL predisposes mice to develop myeloid leukemia, this would strongly indicate an intrinsic role of the protein in disruption of normal growth regulation of myeloid cells and would provide a rationale for its myeloid specific role in human malignancy. Dependent of the phenotype of the mice, induction of leukemogenesis may be accelerated by infection of the mice with murine leukemia virus (Haupt *et al.*, 1991; van Lohuizen *et al.*, 1991). This method has the advantage that cooperating oncogenes can be detected.

TEL-AML1

Clinical trials will start to determine treatment protocols aimed at maintaining therapeutic efficacy but to reduce toxicity and the risk of secondary myeloid leukemia. Furthermore, pre-B cell lines generated from blasts of patients with ALL that express TEL-AML1 will be used to determine the role of TEL-AML1 in leukemogenesis (Kim *et al.*, 1996). The effects of chemotherapeutic agents used for treatment in t(12;21) ALL can be studied with these cell lines.

To analyze whether TEL-AML1 interferes with apoptosis of the B-cell population *in vitro*, growth factor dependent B-cell lines will be generated that express TEL-AML1. Inhibition of apoptosis and growth characteristics of cell lines will be analyzed upon withdrawal of the growth factor.

To determine the oncogenic potential of TEL-AML1 *in vivo*, B-cell specific transgenic TEL-AML1 mice will be generated by expression of the chimeric protein from a lymphoid-specific Eμ based expression vector (van Lohuizen *et al.*, 1991). These mice will be used to determine whether TEL-AML1 is involved in deregulation of apoptosis in the B cell population. The same vector will be used to generate B-cell specific transgenic TEL mice. It will be very interesting to analyze the role of TEL in FAS and CD40 dependent regulation of B-cell proliferation and apoptosis (Rathmell *et al.*, 1996).

We generated transgenic mice that express the cytoplasmic TEL DNA binding mutant from the lymphoid-specific Eμ based expression vector (Buijs and Grosveld, unpublished results). These mice will allow us to study the phenotypic consequences of TEL inactivation by its sequestration in the cytoplasm. We will study the proliferation and maturation of lymphoid cells and assess whether these mice are predisposed to lymphoid malignancies and thus confirm TELs possible role as a tumor suppressor.

TEL involvement in other 12p13 aberrations in leukemia and other malignancies

As discussed in chapter 1.5, *TEL* is a frequent target of chromosomal aberrations in human leukemia. Additional chromosome 12p13 aberrations associated with human leukemia should be analyzed for TEL involvement. TEL may also be involved in other malignancies, since deletions of the short arm of chromosome 12 are observed in 10-20% of patients with non-small cell lung carcinoma (Sato *et al.*, 1994; Tsuchiya *et al.*, 1992) and frequent LOH of region 12p13 was observed in both non-small cell lung carcinoma and breast carcinoma (Spirin *et al.*, 1996; Takeuchi *et al.*, 1996).These data at least provide additional evidence for the presence of one or more tumor suppressor genes at this locus. Further analysis will show if *TEL* is one of these.

Clinical significance of molecular diagnosis of chromosomal translocations in childhood ALL

To date, more that 70% of children with ALL treated with current chemotherapeutical regiments will become long-term survivors (Rivera *et al.*, 1993). Current risk-based protocols emphasize intensified treatment for patients predicted to be at high risk of relapse, whereas less toxic therapy is reserved for lower-risk patients.

Clinical features with recognized prognostic value include the initial leukocyte count, age at diagnosis, sex, presence of a mediastinal mass, degree of organomegaly, and early response to treatment (Rubnitz *et al.*, 1997). Patient age and leukocyte count at diagnosis provide the most important prognostic information. Age 1 to 10 years and a leukocyte count of less than 50,000/mm^3 are recommended as standard-risk criteria, whereas other combinations of these features are considered to be high-risk.

Genetic abnormalities have also been useful in defining prognostically important patient subgroups. Hyperdiploidy (i.e. >50 chromosomes per cell or DNA index of 1.16) is associated with favorable outcome. By contrast, specific chromosomal translocations, such as

t(9;22), t(1;19) and t(4;11) (figure 3), confer a poor prognosis. Taken together, these genetically defined risk-groups account for only 35% of childhood ALL patients (20% with hyperdiploidy and 15% with adverse translocations) (Pui, 1995; Raimondi, 1993).

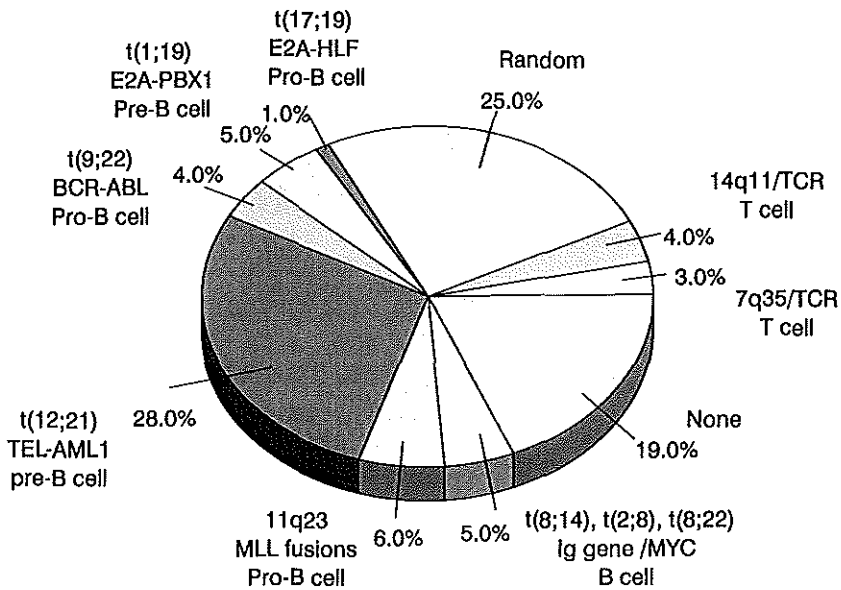

Figure 3. *Distribution of translocation-generated fusion genes among commonly recognized immunologic subtypes of ALL in children and young adults* (courtesy to Dr. A.T. Look).

The discovery that the cryptic t(12;21) has a favorable prognostic outcome and occurs in >25% of patients with pediatric ALL, is extremely important for risk-directed chemotherapy. For instance, a patient with a white blood count of >50,000 /mm^3 with a cryptic t(12;21), who would have been considered to be of high-risk one year ago, will now be regarded to be of moderate to low risk. T(12;21) is the first example of a specific genetic abnormality to be associated with a favorable prognosis in childhood ALL. However, approximately 40% of the cases with ALL still lack risk-identifying genetic features. Most of these cases have non-recurring chromosomal abnormalities or rare recurring translocations. Molecular characterization of these alterations may lead to the identification of additional clinically important genetic risk groups.

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Summary

Leukemias and lymphomas, malignancies derived from the hematopoietic system, are frequently associated with specific chromosomal aberrations, such as translocations and deletions. Characterization of these aberrations resulted in the isolation of affected genes. Investigations with these genes contributed to the understanding of the function of their protein products in physiology and pathology.

Aberrations on the short (p) arm of chromosome 12 can be detected in 2 % of myeloid leukemia and 10 % of acute lymphoblastic leukemia (ALL) in children. These aberrations consist of both chromosomal translocations as well as deletions, involving band p, suggesting that this region may contain one or more (tumor suppressor) genes.

The aim of the investigations described in this thesis was to characterize translocation (12;22)(p13;q11), associated with myeloid leukemia. The following questions needed to be answered: 1) Is t(12;22)(p13;q11) a variant Philadelphia translocation, resulting in fusion of the *BCR* gene on chromosome 22q with a gene on chromosome 12p? 2) Is this gene on chromosome 12p also the target of other translocations in this chromosomal region? 3) Is the gene on chromosome 12p13 a tumor suppressor gene, because of the fact that this region is frequently deleted in childhood ALL? 4) Via which biochemical mechanism contributes t(12;22)(p13;q11) to leukemogenesis?

Because the gene on chromosome 12p13 appeared to be a new member of the family of ETS transcription factors, an introduction about the functions of ETS factors in the development of organs and organisms, hematopoiesis and malignancies is preceding the description of the experimental work (**chapter 1**).

In **chapter 2.1** we describe that t(12;22)(p13;q11) results in fusion of the *MN1* gene, not the *BCR* gene, on chromosome 22q11 with the *TEL (ETV6)* gene on chromosome 12p13. *MN1* was recently isolated because of its involvement in t(4;22) in a cell line derived from a multiple meningioma. The MN1 protein contains two homopolymeric glutamine chains and many proline residues. These are two properties that suggest that MN1 may be involved with transcription regulation. *TEL* was independently isolated by three other groups by characterization of chromosomal translocations t(5;12)(q33;p13), t(9;12)(q34;p13), t(12;21)(q22;p13). TEL contains a protein-protein interaction domain and an ETS DNA binding domain. T(12;22) results in two reciprocal fusion transcripts, because of difference in the localization of the breakpoints in *TEL* in different patients. We postulated that the MN1-TEL may be involved in the ontogeny or progression of the leukemia by aberrant transcription regulation of genes that are normally controlled by TEL, because MN1-TEL contains the major part of MN1 and the DNA binding ETS domain of TEL.

Experiments described in **chapter 2.2** suggests that this hypothesis seems to be valid. We demonstrated that TEL binds to a canonical GGAA sequence. We determined the TEL consensus recognition sequences as CCGGAAGT. Binding of TEL to the consensus sequences was confirmed by bandshift analysis. These studies suggested that the DNA binding of TEL is negatively regulated by intramolecular interactions in the polypeptide. In transient transcription assays we demonstrated that MN1-TEL is a transcription regulator of the

Moloney sarcoma virus LTR, dependent on the ETS domain in the fusion protein. Furthermore, MN1-TEL, but not MN1 or TEL, transformed murine fibroblasts. Sequences within the first 228 aminoacids of MN1 are crucial for transformation by MN1-TEL. Furthermore, the ETS domain of TEL mediates transformation by MN1-TEL. Indirect immunofluorescence revealed that MN1 TEL locates to nuclear speckles characteristic for the fusion protein. Our data suggest that MN1-TEL is involved in leukemogenesis.

In the meantime it was clear that *TEL* was involved in many translocations (summarized in **chapter 1.5**). One of them is t(12;21)(p13;q22), that results in expression of a TEL-AML1 chimeric product. T(12;21) is a cryptic translocation that can be detected in 0.05% of childhood ALL by cytogenetic analysis. Preliminary data suggested that t(12;21) could be more frequent. In **chapter 3.1** we describe that TEL-AML1 occurs in 25% of childhood ALL, when analyzed by Southern analysis and reverse transcriptase-polymerase chain reaction (RT-PCR), which is an increase of 500x in sensitivity of diagnosis. Retrospective analysis revealed that TEL-AML1 defines a subgroup in childhood ALL that is characterized by an age of onset of 1 to 10 years, a non-hyperdiploid DNA index, a B-cell immunephenotype and a good prognosis. It may be possible to use t(12;21) as a genetic marker and a positive prognostic indicator for outcome of the disease. We observed that exogenous TEL-AML1, in contrast what had been suggested, localized to the cytoplasm of transfected cells, and not to the nucleus (**chapter 3.2**). Cytoplasm localization was dependent on the helix-loop-helix oligomerization domain in the TEL moiety. Endogenous TEL colocalized with transfected TEL-AML1 in the cytoplasm. However, in human pre B-cells carrying t(12;21) TEL-AML1 localized to the nucleus, suggesting the cytoplasmic localization of TEL-AML1 in transfected cells is due to overexpression of the chimeric protein. Therefore, our observations are important for the correct interpretation of the role of TEL-AML1 in transient transactivation experiments. Furthermore, TEL-AML1 may still interfere with TEL dependent transcription regulation via association of the two molecules. This may suggest a biochemical mechanism, arguing for the hypothesis that loss of nuclear TEL function results in progression of the leukemia. We hypothesize that TEL is involved in the regulation of growth or differentiation of pre B-cells. Our observation that overexpression of TEL in fibroblast results in cell death, suggest that TEL-AML1 in t(12;21) may contribute to B-cell leukemogenesis by negatively influencing TELs alleged function in inducing apoptosis.

Samenvatting

Leukemiën en lymfomen, maligniteiten die uitgaan van het bloedvormende systeem, zijn vaak geassocieerd met specifieke chromosoom afwijkingen, zoals translocaties en deleties. Het karakterizeren van deze afwijkingen heeft geleid tot de isolatie van genen betrokken bij deze afwijkingen. Onderzoek aan deze genen heeft bijgedragen aan de kennis van hun eiwitprodukten in fysiologie en pathologie.

Afwijkingen van de korte (p) arm van chromosoom 12 zijn detekteerbaar in 2% van de myeloide leukemiën en in 10% van acute lymfoblastische leukemie bij kinderen. Deze afwijkingen bestaan zowel uit chromosoom translocaties als deleties, waarbij band p13 betrokken is. Deze observaties deden vermoeden dat in dit gebied één of wellicht meerdere genen liggen, die het doelwit zouden kunnen zijn van verschillende translocaties. De deleties suggereerden dat dit gebied ook één of meerdere tumor suppressor genen zou kunnen bevatten.

Het onderzoek beschreven in dit proefschrift had tot doel de chromosomale translocatie (12;22)(p13;q11) te karakteriseren, die geassocieerd is met myeloide leukemie. De volgende vragen moesten beantwoord worden: 1) Is t(12;22)(p13;q11) een variante Philadelphia translocatie die leidt tot de fusie van het *BCR* gen op chromosoom 22q met een gen op chromosoom 12p? 2) Is dit gen op chromosoom 12p ook het doelwit van andere translocaties in deze chromosomale regio? 3) Is het gen op chromosoom 12p13 wellicht een tumorsuppressor gen, omdat deze regio frequent gedeleteerd is in ALL bij kinderen? 4) Wat is het moleculaire mechanisme waarmee t(12;22)(p13;q11) bijdraagt aan het ontstaan van de leukemie?

Aangezien het gen op chromosoom 12p13 een nieuw familielid van de ETS transcriptie factoren bleek te zijn, wordt de beschrijving van de bevindingen van het experimentele werk voorafgegaan door een inleiding over de rol van ETS factoren bij de ontwikkeling van organen en organismen, de bloedvorming en maligniteiten **(hoofdstuk 1)**.

In **hoofdstuk 2.1** wordt beschreven dat t(12;22)(p13;q11) resulteert in de fusie van het *MN1* gen en niet het *BCR* gen, op chromosoom 22q11 met het *TEL* gen op chromosoom 12p13. MN1 was juist daarvoor geisoleerd omdat het betrokken bleek te zijn bij een t(4;22) in een cellijn geïsoleerd uit meningioom weefsel. Het MN1 eiwit bevat twee homopolymere glutamine ketens en is rijk aan prolines. Dit wijst erop dat het eiwit betrokken zou kunnen zijn bij regulatie van transcriptie van genen. Het *TEL* gen werd onafhankelijk door drie andere groepen ontdekt bij de karakterizering van chromosoom translocaties t(5;12)(q33;p13), t(9;12)(q34;p13), t(12;21)(q22;p13). Het TEL eiwit bevat een domein, dat mogelijk betrokken is bij interactie met andere eiwitten, en een ETS DNA bindend domein. T(12;22) resulteert in twee typen reciproke fusie transcripten als gevolg van het verschil in de lokatie van de breukpunten in *TEL* bij verschillende patiënten. Op grond van de struktuur van de MN1-TEL fusie-eiwitten, die een groot gedeelte van MN1 en het DNA bindende ETS domein van TEL bevatten, postuleerden we dat dit eiwit betrokken zou zijn bij het ontstaan of de progressie van de leukemie door afwijkende regulatie van genen, die normaal onder controle staan van TEL of verwante ETS factoren.

Experimenten, beschreven in **hoofdstuk 2.2** toonden aan dat deze hypothesis waarschijnlijk juist is. Door middel van amplificatie van DNA oligonucleotiden, die bonden aan het ETS domein van TEL, uit een verzameling van willekeurige oligomeren werd aangetoond dat TEL bindt aan een GGAA nucleotide sequentie. Op grond van vergelijking van omliggende sequenties werd de consensus herkenningssequentie van TEL gedefinieerd als CCGGAAGT. Binding van TEL aan deze sequentie werd bevestigd door bandshift assays. Daarnaast gaven deze studies aanleiding te veronderstellen, dat DNA binding van TEL negatief beïnvloed wordt door een intramoleculaire interactie in het TEL eiwit. In transcriptie studies werd aangetoond dat MN1 domeinen toevoegt aan TEL, waardoor MN1-TEL een sterke activator wordt van de Moloney sarcoma virus LTR. Activatie van de LTR bleek afhankelijk te zijn van het DNA bindend domein van TEL. Daarnaast bleek MN1-TEL, in tegenstelling tot TEL en MN1, in staat te zijn om muize fibroblasten te transformeren. Voor transformatie is een domein in de eerste 228 aminozuren van MN1 van cruciaal belang. Daarnaast draagt het ETS domein van TEL bij aan transformatie. Deze observaties maken het waarschijnlijk dat MN1-TEL betrokken is bij leukemogenese. MN1-TEL bleek gelokaliseerd te zijn in korrelige structuren in de kern. Op dit moment is de relevantie van de expressie van MN1-TEL in deze nucleaire structuren voor een mogelijke rol bij leukemogenese onbekend.

Inmiddels was duidelijk dat *TEL* betrokken was bij meerdere translocaties (samengevat in **hoofdstuk 1.5**), waaronder t(12;21)(p13;q22), die leidt tot expressie van een TEL-AML1 fusie produkt. T(12;21) is een cryptische translocatie die slechts in 0.05% van de gevallen van ALL bij kinderen met behulp van cytogenetica gediagnostiseerd kon worden. Er waren sterke aanwijzingen dat t(12;21) veel frequenter voor zou kunnen komen dan aanvankelijk werd gedacht. In **hoofdstuk 3.1** wordt beschreven dat de TEL-AML1 fusie voorkomt bij 25% van de ALL bij kinderen, hetgeen een 500-voudige toename in detectie gevoeligheid van deze chromosoom translocatie betekent. Daarnaast werd duidelijk dat TEL-AML1 specifiek is voor een groep van kinderen in een leeftijds catagorie van 1 to 10 jaar, waarvan de ALL gekenmerkt wordt door een B cel immunofenotype, een niet-hyperdiploide DNA index, en een uitermate gunstige prognose. Uitbreiding van het aantal onderzochte patienten stelde vast dat de translocatie mogelijkerwijs als een genetisch merker en als positieve prognostische indicator voor de uitkomst van de ziekte gebruikt kan worden. Onderzoek aan het moleculaire mechanisme waarmee TEL-AML1 aan leukemogenese bijdraagt, leidde tot de observatie dat getransfecteerd TEL-AML1, in tegenstelling tot wat verondersteld werd, in het cytoplasma van de cellen, en niet in de kern, to expressie komt (**hoofdstuk 3.2**). De localisatie van TEL-AML1 bleek af te hangen van het HLH domein in het fusie-eiwit. Evenals getransfecteerd TEL-AML1, bevond endogeen TEL zich in het cytoplasma van de cel. Deze observatie suggereerde dat een consequenties van t(12;21) zou kunnen zijn dat TEL uit de kern in het cytoplasma gehouden wordt. Dit zou de hypothese, dat verlies aan functioneel TEL bijdraagt aan progressie van de leukemie, gepostuleerd op grond van genetische studies, steunen. Echter in humane pre-B cellijnen die t(12;21) bevatten, bleek TEL-AML1 in de kern gelocaliseerd te zijn, hetgeen suggereert dat de cytoplasmatische localisatie van getransfecteerd TEL-AML1 berust op overexpressie van het fusie-eiwit. Dit

neemt echter niet weg dat TEL-AML1 door associatie met TEL bij kan dragen aan leukemogenese door functionele TEL activiteit in de kern negatief te beïnvloeden. Onze observaties zijn van belang voor de interpretatie van transactivatie experimenten met TEL-AML1 cDNA constructen. Daarnaast duiden ze op een mogelijk biochemisch mechanisme, wat de hypothese steunt dat verlies aan TEL functie leidt tot progressie van de leukemie. Enerzijds kunnen we veronderstellen dat TEL betrokken is bij de regulatie van groei en/of differentiatie van pre-B cellen. Anderzijds, gesteund door de observatie dat overexpressie van TEL in fibroblasten leidt tot celdood, kunnen we postuleren dat t(12;21) TEL-AML1 bijdraagt tot pre-B cel leukemie door remming van TELs apoptose inducerende functie te beïnvloeden.

Abbreviations

ALL	acute lymphoblastic leukemia
AML	acute myeloblastic leukemia
ANNL	acute non-lymphoblastic leukemia
APL	acute promyelocytic leukemia
bp	base pair
cALL	common ALL
cDNA	complementary deoxyribonucleic acid
C-terminus	carboxyl terminus
CML	chronic myelogenous leukemia
CMML	chronic myelomonocytic leukemia
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
ETS	E26 transformation-specific
FAB	French-American-British Cooperative Group
FISH	Fluorescence *in situ* hybridisation
GM-CSF	granulocyte macrophage colony stimulating factor
HLH	helix-loop-helix
HTH	helix-turn-helix
Il	interleukin
kb	kilo bases
kDa	kilo Dalton
LOH	loss of heterozygosity
LTR	long terminal repeat
MAPK	mitogen activated protein kinase
M-CSF	macrophage colony stimulating factor
MDS	myelodysplastic syndrome
MPD	myeloproliferative disorder
mRNA	messenger ribonucleic acid
MSV	Moloney sarcoma virus
NLS	nuclear localization signal
N-terminus	amino termimus
PCR	polymerase chain reaction
PDGFβR	β chain plateled derived growth factor receptor
RAR	retinoic acid receptor
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
SRE	serum response element
SRF	serum response factor

Epilogue

C. William Pollard, chairman of The ServiceMaster Company, wrote in his book, *The Soul of the Firm*: "It is not just what we are doing, but what we are becoming in the process that gives us our distinct value." At the end of this thesis which I hope to defend succesfully, so as to be granted the doctorate, I would like to acknowledge those people who supervised me, worked with me, and supported me to help me become who I am now.

First, I would like to thank my co-promotor, Dr. Gerard Grosveld. Thank you for your supervision, first when I was an undergraduate student, and later as a Ph.D. student. If there is someone who has had a great impact on my development in science, it is you. Thank you for trusting me and giving me the great opportunity to go with you to Memphis. I thank my two promotors, Profs. Bootsma and Hagemeijer, for their support and interest in the project, and for critically reading the manuscript, along with Profs. F. Grosveld and Löwenberg and Dr. Zwarthoff. Special thanks to Drs. Ellen Zwarthoff, as well as Ronald Lekanne Deprez, Peter Riegman and, last but not least, Anco Molijn, for their great collaboration during the years. Anco, you were more than just a paranimph. You became the very needed discussion partner, especially during the time you were in Memphis. I also want to thank Drs. Martine Roussel and Nathan Davis for their support, Dr. Dick Ashmun for FACS analysis and Dr. Jim Downing for being the link between the basic science and the clinic. Thank you for introducing me to Drs. Fred Behm, Jeff Rubnitz, Susana Raimondi and Sheila Shurtleff. For me, one of the most satisfying aspects of my work was that the cloning of a gene resulted in such a quick beneficial application in patient care.

I thank Drs. Maarten Fornerod, Judith Boer, Jan van Deursen, Sjozèf van Baal, and Edwin Mientjes for being superb colleagues and friends. Your work on the t(6;9) made it easier for me to start with functional analyses of t(12;22) and t(12;21). Thanks also to Drs. Robbert Rottier, Erik Bonten, Aarnoud van der Spoel, and Alessandra d'Azzo for their helpful suggestions; Drs. Dorien van der Plas and Marieke von Lindern for their supervision in the early years. I thank Saskia van Bezouw, Sarah Sherr, Alfred Vertegaal, Hema Thakar, Tony McMickle, and Jacqueline Bonten for their contributions to the project. Dr. Marella de Bruijn for keeping in touch and being a perfect paranimph. Mrs Rita Boucke, Peggy Burdick and Charlette Hill for secretarial assistance.

Special thanks to Dave and Phyllis Baseler, who welcomed us in Memphis and became our great Christian friends. Thank you very much for your care and support. I especially appreciate my in-laws for their interest in the work and in our life. My parents, sister and brother, who especially contributed to who I became.

Finally I thank my wife, Marleen, for her love and care, especially during the past year. Thank you for your willingness to go to Memphis, although it could have jeopardized your own career. Most importantly, thanks to God for taking care of our lives, keeping us and giving us a great son, George.

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