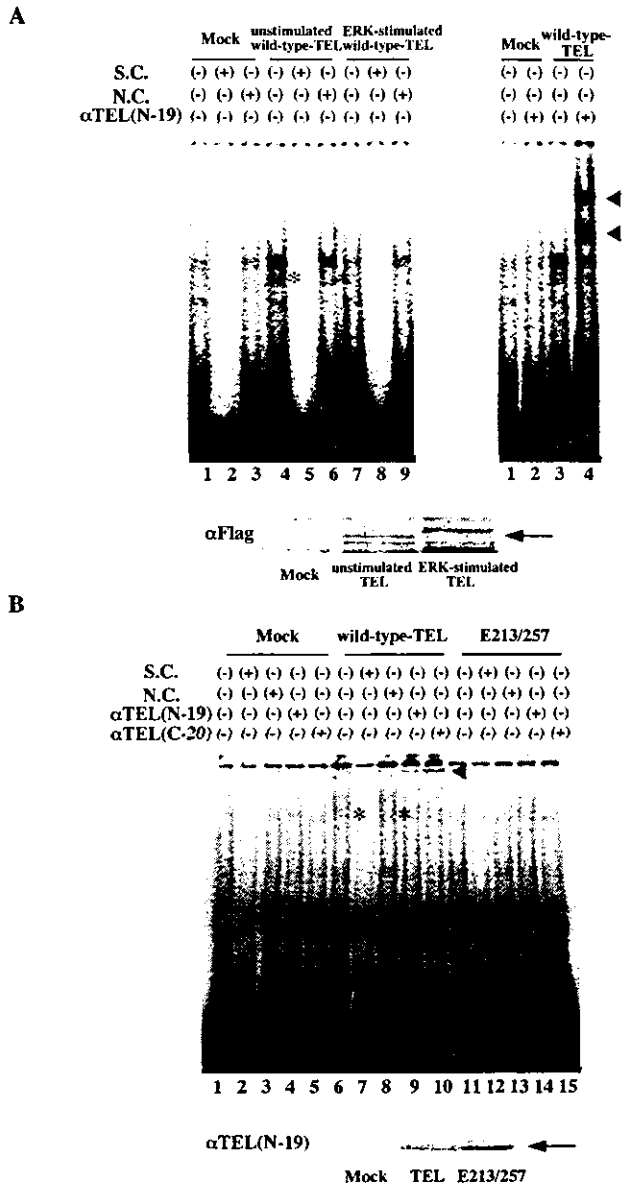

FIG. 6. (A) Hyperphosphorylated TEL does not bind to DNA. The top left panel shows results of EMSA carried out with the ^{32}P-labeled EBS probe (oligonucleotide A) and mock lysate (lanes 1 to 3), wild-type TEL-expressing COS-7 lysate without activated ERK (lanes 4 to 6), and wild-type TEL-expressing COS-7 lysate with activated ERK (lanes 7 to 9). A 300-fold molar excess of oligonucleotide A (S.C., lanes 2, 5, and 8) or oligonucleotide M (N.C., lanes 3, 6 and 9), which contains mutations in the EBS, was also added to the reaction mixtures. Asterisks indicate a specific DNA-TEL complex-derived band. The top right panel shows results obtained when anti-TEL antibody (lanes 2 and 4) was also added to the reaction mixtures. The supershifted bands are indicated with arrowheads. In the bottom panel, the expression of unstimulated or ERK-stimulated wild-type TEL protein is shown. An arrow indicates overexpressed wild-type TEL proteins. (B) The E213/257 mutant also does not bind to DNA. The top panel shows results obtained when EMSA was carried out with the ^{32}P-labeled EBS probe and mock (lanes 1 to 5), in vitro-translated wild-type TEL (lanes 6 to 10), or E213/257 (lanes 11 to 15) proteins. Asterisks indicate a specific DNA-TEL complex-derived band. A 300-fold molar excess of cold-specific competitor (S.C., lanes 2, 7, and 12) or nonspecific competitor (N.C., lanes 3, 8, and 13) was also added to the reaction mixtures. Two kinds of anti-TEL antibodies (N-19 for lanes 4, 9, and 14 and C-20 for lanes 5, 10, and 15) were added to the reaction mixtures. The supershifted bands are indicated with a solid arrowhead. In the bottom panel, the expression of in vitro-translated wild-type TEL or E213/257 protein is shown. An arrow indicates wild-type TEL or E213/257 mutant proteins.

and/or the E213/257 mutant in each of two independent clones. When stimulated with HMBA, the wild-type TEL-expressing cells showed an earlier onset and a higher incidence of benzidine positivity, while mock cells began to be benzidine positive on day 4 (Fig. 7B). Surprisingly, the E213/257-expressing cells completely lost their abilities to become benzidine positive. However, coexpression of wild-type TEL recovered E213/257 mutant-induced differentiation block. Thus, it is plausible that the dominant-negative form of TEL, namely, E213/257, blocks erythroid differentiation in MEL cells by repressing the propelling function of wild-type TEL.

It has also been demonstrated that the expression of TEL in Ras-transformed NIH 3T3 cells inhibits cell growth in liquid and soft agar cultures (6). To look at whether the E213/257 mutant modulates the growth of H-Ras-transformed NIH 3T3 cells, we infected these cells with recombinant retroviruses expressing wild-type TEL or the E213/257 mutant. Western analysis confirmed that wild-type TEL and the E213/257 mutant were expressed at similar levels (Fig. 7C). Interestingly, the E213/257 mutant cooperated with Ras to stimulate cell growth in both liquid and semisolid media, while wild-type TEL inhibited growth under both conditions (Fig. 7D and E). All of these data indicate growth-stimulating and transforming activities of the E213/257 mutant in the murine fibroblasts. It is plausible that hyperphosphorylated TEL might act as an inhibitory protein that blocks tumor-suppressive functions of nonhyperphosphorylated TEL.

Extracellular and intracellular signals regulate the phosphorylation status of endogenous TEL. We further analyzed the phosphorylation status of endogenous TEL proteins to clarify a physiological role of their ERK-dependent phosphorylation. We at first induced erythroid differentiation into parental MEL cells by treating them with HMBA. Upon treatment, ERK significantly became dephosphorylated, and thus inactivated, within 8 h, although expression levels of the protein were unchanged (Fig. 8A). In parallel with this result, phosphorylation levels of endogenous TEL proteins were markedly decreased within 1 day. Because hyperphosphorylated TEL blocks erythroid differentiation in MEL cells, dephosphorylation of TEL through the inactivation of ERK could play a role in HMBA-induced differentiation. This finding suggests that ERK could physiologically phosphorylate and thereby inactivate TEL in immature erythroid progenitors to maintain nondifferentiation status.

We next examined phosphorylation levels of endogenous TEL proteins in NIH 3T3 cells. ERK in H-Ras-transformed NIH 3T3 cells was markedly phosphorylated in comparison to that in nontransformed NIH 3T3 cells (Fig. 8B). Notably, the phosphorylation level of endogenous TEL proteins was higher in the former than in the latter. Moreover, the observation that endogenous TEL proteins showed a slight band shift in H-Ras-transformed NIH 3T3 cells supported the increased phosphorylation of TEL by ERK (Fig. 8C). These results indicate that

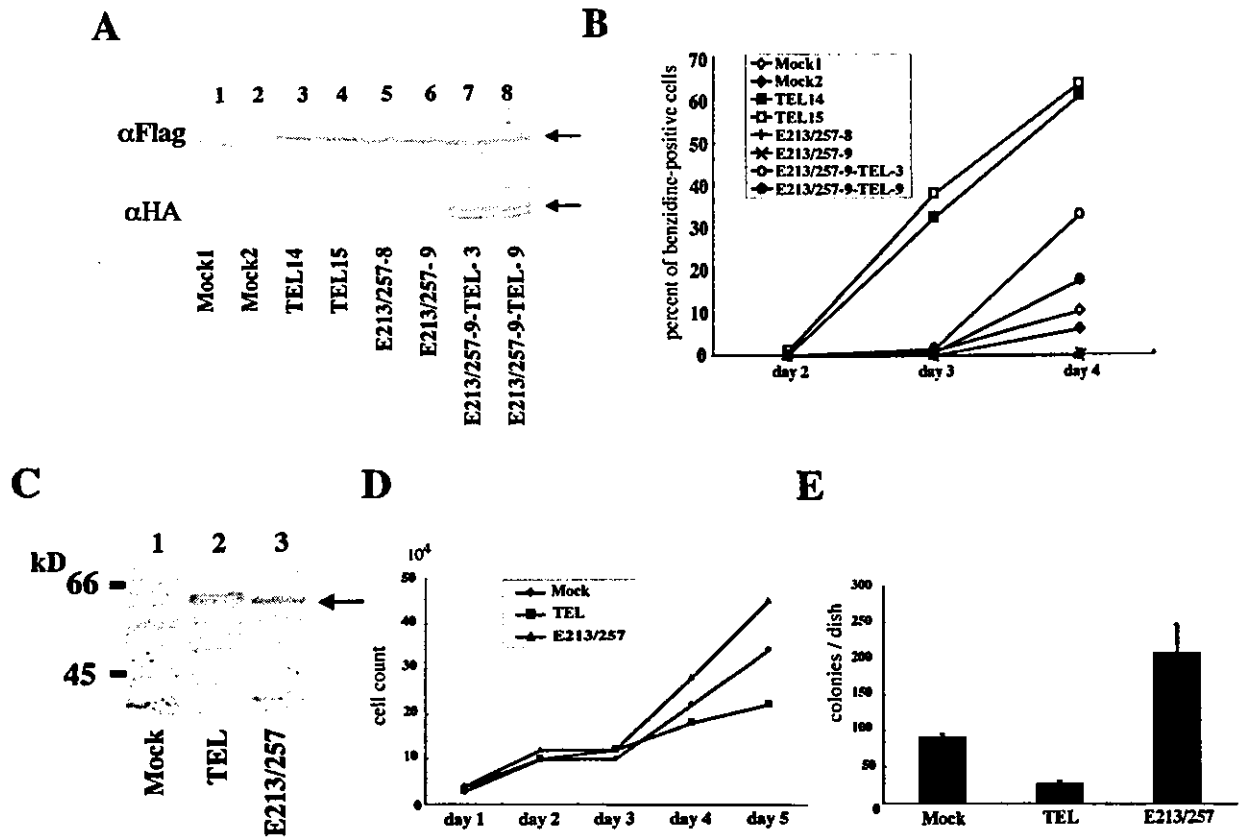

FIG. 7. (A) Expression of wild-type TEL or the E213/257 mutant in MEL clones. These clones were obtained as described in Materials and Methods. Arrows indicate overexpressed wild-type TEL or E213/257 mutant proteins. (B) The E213/257 mutant completely blocked erythroid differentiation in MEL cells after HMBA treatment, and coexpression of wild-type TEL relieved its differentiation block. Cell commitment to terminal differentiation was determined by benzidine staining, and percentages of benzidine-positive cells were calculated at different time points. (C) Expression of wild-type TEL or the E213/257 mutant in H-Ras-transformed NIH 3T3 cells. These H-Ras-transformed NIH 3T3 cells were obtained as described in Materials and Methods. An arrow indicates overexpressed wild-type TEL or E213/257 mutant proteins. (D) The E213/257 mutant stimulates the growth of H-Ras-transformed NIH 3T3 cells in liquid culture. After a total of 2×10^4 cells were plated in 24-well plates, cells were counted every 24 h for 5 days. (E) The E213/257 mutant also stimulates the growth of H-Ras-transformed NIH 3T3 cells in soft agar culture. Transformation assays were performed as described in Materials and Methods. Bars show means and standard deviations of colony counts in two independent experiments that were normalized to colony counts with 2×10^4 NIH 3T3 cells.

Ras/ERK pathways could mediate growth-stimulating signals partly through the inactivation of TEL by phosphorylation.

DISCUSSION

We demonstrated in this study that TEL is hyperphosphorylated in vivo with dependence on ERK activation. Because TEL is efficiently subjected to phosphorylation by ERK in vitro, TEL seems to be a direct target of ERK. According to the results of in vitro kinase assays, both Ser^{213} and Ser^{257} are inducible phosphorylation sites. TEL associates with the CD domain in ERK in vitro, suggesting that the interaction between TEL and ERK may be direct. Importantly, phosphorylation of TEL by overexpressed ERK or endogenous ERK activated by EGF treatment results in the diminishment of its *trans*-repressional effects on the natural EBS promoter. The E213/257 mutant loses its *trans*-repressional activities and functionally mimics hyperphosphorylated TEL, while the corresponding alanine mutant does not lose these activities even through the overexpression of ERK or the activation of endogenous ERK. We conclude that phosphorylation at both Ser^{213} and Ser^{257} is necessary to regulate TEL's molecular functions. Moreover, the glutamate mutant exerts a dominant-negative effect on TEL-mediated transcriptional repression. Therefore, ERK could be a physiologically important MAPK that induces the phosphorylation of TEL and thereby potentially modulates its functions.

Various ETS family transcription factors become phosphorylated by MAPKs and are thereby molecularly activated. The phosphorylating MAP kinases and phosphorylation sites differ among the molecules. TEL is a member of the ETS subfamily with ETS-1 and ETS-2, which possesses a highly conserved N-terminal HLH domain and a C-terminal ETS DNA-binding domain (7). Although the constitutive phosphorylation site Ser^{22} in a TEL molecule is equivalent to Thr^{38} in ETS-1 and Thr^{72} in ETS-2, which are phosphorylated by ERK (38), the ERK-inducible phosphorylation sites Ser^{213} and Ser^{257} are not conserved in ETS1 and ETS2. Moreover, TEL2, a protein that is highly structurally related to TEL (9, 27), also does not possess equivalent serine or threonine residues. Therefore,

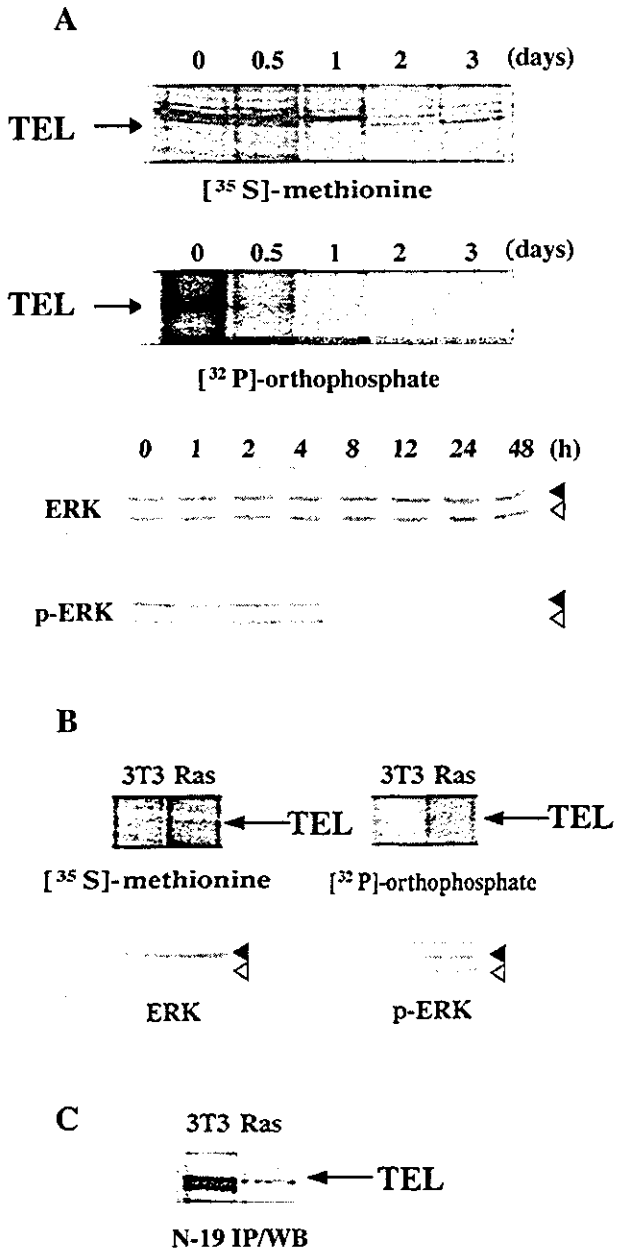

FIG. 8. (A) Dephosphorylation of endogenous TEL proteins during the course of erythroid differentiation in MEL cells. Parental MEL cells were induced into erythroid differentiation with 5 mM HMBA and subjected to metabolic labeling as described in the legend to Fig. 1C (top panel). Western analyses were performed with anti-ERK or anti-phosphorylated ERK antibody to detect total or phosphorylated ERK proteins (bottom panel). Arrows, solid arrowheads, and open arrowheads indicate endogenous TEL, ERK1, and ERK2 proteins, respectively. (B) Phosphorylation of endogenous TEL proteins through Ras/ERK pathways. Nontransformed or H-Ras-transformed NIH 3T3 clones were subjected to metabolic labeling as described for panel A (top panel). Western analyses were performed with anti-ERK or anti-phosphorylated ERK antibody to detect total or phosphorylated ERK proteins (bottom panel). Arrows, solid arrowheads, and open arrowheads indicate endogenous TEL, ERK1, and ERK2 proteins, respectively. (C) Western analysis with anti-TEL antibody (N-19) for immunoprecipitates with the same antibody from mock or H-Ras-transformed NIH 3T3 cells. An arrow indicates immunoprecipitated TEL.

TEL's regulation through ERK-induced phosphorylation appears to be highly characteristic of TEL in the subclass of the ETS transcription factors. Recently, we have reported that TEL also becomes phosphorylated at Ser^{257} by p38 but not by c-Jun NH_2-terminal kinase (1). Although it remains undetermined whether Ser^{213} is also phosphorylated by p38, phosphorylation by both ERK and p38 on the same serine residue in the internal domain is also a unique property of TEL. Both Ras and stress signaling pathways could converge on a transcription factor TEL in the nucleus under certain circumstances.

It has been reported that ERK physically associates with several transcription factors that it phosphorylates, including Elk-1, c-Myc, c-Jun, c-Fos, and AML1 (30). MAPK family members have a CD domain that lies just C terminal to the protein kinase catalytic core within a C-terminal extension shared by the MAPK family and binds to the D domains of substrates outside the phosphoacceptor site (14, 25). Acidic residues in the CD domain of MAPKs are thought to interact with a basic cluster in the D domains of their substrates. This docking reaction facilitates the phosphorylation of substrate phosphoacceptors by MAPK catalytic units by enhancing specificity between a substrate and a relevant MAP kinase. We have demonstrated a physical association between TEL and ERK depending on the CD domain in ERK. This finding may indicate that their interaction is direct. Because TEL is located in the nucleus, ERK that is activated and moves to the nucleus conceivably interacts with TEL. We could not find perfectly matched consensus sequences of the D domain, (R/K)X(R/K)X_{2-4}(L/I)X(L/I), around Ser^{213} and Ser^{257}. Further investigation should be carried out to identify an ERK-binding site in a TEL molecule. Alternatively, it is also possible that ERK indirectly associates with TEL and that unknown factors mediate the association.

Certain transcription factors, including members of the Forkhead family, are negatively regulated through phosphorylation, although its mechanisms are diverse (2, 15). Among the ETS family transcription factors, ETS-2 repressor factor, which exhibits strong transcriptional repressor activity on EBS promoters, becomes phosphorylated by ERK2 and cdc2/cyclin B kinase and loses its suppressive effects through export to the cytoplasm (20). TEL is like EBS-2 repressor factor in that phosphorylation by ERK causes a decrease in *trans*-repressional effects. We investigated possible mechanisms in the prevention of TEL's molecular functions through phosphorylation by using the glutamate mutant that contains substituted glutamates on both Ser^{213} and Ser^{257} and functionally mimics hyperphosphorylated TEL. It is conceivable that a loss of DNA binding to the EBS plays a fundamental role in interfering with transcriptional functions in hyperphosphorylated TEL. Although the identified phosphorylation sites reside outside the ETS DNA-binding domain, the ternary structure of the ETS domain might be changed through the phosphorylation. It is of note that hyperphosphorylated TEL works as a dominant-negative molecule over nonhyperphosphorylated TEL. Considering that the E213/257 mutant described above associates with nonhyperphosphorylated TEL, TEL could lose its transcriptional functions through interaction with a hyperphosphorylated form that does not bind to DNA.

MAPKs are important signal-transducing enzymes that are involved in cell survival regulation and adaptation upon chem-

ical and physical stresses. By and large, ERK and stress MAPKs such as p38 and c-Jun NH_2-terminal kinase mediate opposite signals for cell differentiation and proliferation (25). The activation of ERK is linked to cell survival, whereas that of stress kinases is related to apoptosis induction. We observed that endogenous TEL proteins in NIH 3T3 cells were phosphorylated by endogenous ERK activated through Ras signaling pathways. Moreover, the glutamate mutant mimicking hyperphosphorylated TEL stimulated the growth of Ras-transformed NIH 3T3 cells in liquid and soft agar cultures, in contrast to results obtained with wild-type TEL. Therefore, we conclude that activated ERK represses TEL's inhibitory effects on the natural EBS promoter and thus causes a loss of its tumor-suppressive functions. Because Ras/ERK pathways mediate growth-stimulating signals, this functional regulation of TEL is suitable for ERK's biological roles. On the other hand, some papers suggest that down-regulation of the Ras/ERK signaling pathway is essential for erythroid differentiation in various systems (23, 24, 37). We also showed that ERK was dephosphorylated and thus inactivated during the course of erythroid differentiation with HMBA in MEL cells. In parallel to this phenomenon, endogenous TEL proteins were found to be dephosphorylated upon HMBA treatment. Moreover, the glutamate mutant blocked erythroid differentiation in MEL cells, while wild-type TEL accelerated it. Therefore, the erythroid differentiation stimulator TEL appears to be positively regulated during differentiation through the functional loss of ERK and to play a role in the maturation of erythroid progenitors. All of these data indicate the physiological relevance of the ERK-mediated TEL's phosphorylation. In contrast, the functional significance of the p38-induced phosphorylation in physiological settings remains to be established.

In summary, ERK-induced TEL's phosphorylation results in a loss of its tumor-suppressive functions. Therefore, the functional inactivation of TEL through phosphorylation could be one step in the development and progression of human leukemias. Further studies of the functional regulation of leukemia-related transcription factors will provide some important clues to understanding complex mechanisms in leukemogenesis that have not yet been fully elucidated.

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Molecular mechanisms of leukemogenesis by AML1/EVI-1

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The AML1/EVI-1 chimeric gene is generated by the t(3;21)(q26;q22) translocation and plays a pivotal role in progression of hematopoietic stem cell malignancies such as chronic myelocytic leukemia and myelodysplastic syndrome. In AML1/EVI-1, an N-terminal half of AML1 including a runt homology domain is fused to the entire zinc-finger EVI-1 protein. AML1 is essential for hematopoietic cell development in fetal liver and its lineage-specific differentiation in adult. In contrast, EVI-1 is barely expressed in normal hematopoietic cells, but it is overexpressed in chronic myelocytic leukemia in blastic crisis and myelodysplastic syndrome-derived leukemia. There are at least four mechanisms identified in AML1/EVI-1 fusion protein that possibly lead into malignant transformation of hematopoietic stem cells. Firstly, AML1/EVI-1 exerts dominant-negative effects over AML1-induced transcriptional activation. Although target genes repressed by AML1/EVI-1 are still not known, binding competition to a specific DNA sequence and histone deacetylase recruitment through a co-repressor CtBP in EVI-1 part are conceivable underlying mechanisms for the dominant-negative effects. Secondly, AML1/EVI-1 interferes with TGFβ signaling and antagonizes the growth-inhibitory effects of TGFβ. The first zinc-finger domain of EVI-1 associates with Smad3, a TGFβ signal transducer, and represses its transcriptional activity by recruiting histone deacetylase through CtBP that interacts with EVI-1. Thirdly, AML1/EVI-1 blocks JNK activity and prevents stress-induced apoptosis. AML1/EVI-1 associates with JNK through the first zinc-finger domain of EVI-1 and disturbs the association between JNK and its substrates. Lastly, AML1/EVI-1 enhances AP-1 activity by activating the c-Fos promoter depending on the second zinc-finger domain of EVI-1, and promotes cell proliferation. All these functions cooperatively contribute to the malignant transformation of the hematopoietic stem cells by AML1/EVI-1.
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Introduction

In more than the past 10 years, most of the recurrent chromosomal abnormalities associated with specific subtypes of human leukemia have been molecularly characterized. These studies advanced our understanding in molecular mechanisms of leukemogenesis and provided deep insights for hematopoietic cell development. In particular, reciprocal translocations involving transcription factor-encoding genes play an important role in leukemogenesis through either inappropriate expression of a transcription factor or pathogenic generation of a chimeric transcription molecule. Hirai and his colleagues at the University of Tokyo have cloned several chimeric genes, including the AML1/EVI-1 gene generated by the t(3;21)(q26;q22) translocation (Mitani *et al.*, 1994), and established a number of unique molecular models for leukemogenesis induced by this molecule. In this short review, I will focus on the molecular mechanisms of leukemogenesis by AML1/EVI-1 chimeric protein, which have been clarified in his laboratory. I dedicate this review to late Dr Hisamaru Hirai who had been my reliable supervisor as well as helpful mentor, but regrettably passed away on August 23, 2003.

Cloning of the AML1/EVI-1 gene

We first established SKH1 leukemia cell line from a patient with chronic myelocytic leukemia (CML) in megakaryoblastic crisis acquiring the t(3;21)(q26;q22) translocation in addition to the t(9;22)(q34;q11) translocation. The AML1/EVI-1 fusion gene generated by the t(3;21)(q26;q22) translocation was cloned from this cell line 10 years ago. The t(3;21)(q26;q22) translocation fuses the AML1 gene on 21q22 and the EVI-1 gene on 3q26. The t(3;21) (q26;q22) translocation is occasionally observed in CML in blastic crisis or myelodysplastic (MDS)-derived leukemia. Since it is very rare that *de novo* acute leukemia carries the t(3;21)(q26;q22) translocation, the appearance of this chromosomal abnormality may trigger the transformation of chronic hematopoietic stem cell disorder into acute leukemic phase. Actually, treatment with antisense oligonucleotide complementary to the coding sequence of the AML1/EVI-1 junction markedly inhibits the growth of SKH1 cells that carry both the t(3;21) (q26;q22) and the t(9;22) (q34;q11) translocations, but does not inhibit the

growth of K562 cells that carry only the t(9;22)(q34;q11) translocation (Mitani *et al.*, 1995). Thus, the AML1/EVI-1 fusion protein could maintain proliferation capacity of the t(3;21)-carrying clones arising after blastic transformation of CML.

Structure of AML1/EVI-1

The AML1 (now referred to RUNX1) gene was originally isolated as a gene that is located at the translocation breakpoint of chromosome 21 in the t(8;21)(q22;q22) translocation specifically found in acute myeloblastic leukemia (AML) (M2 according to the French–American–British classification) (Miyoshi *et al.*, 1991). AML1 has a runt homology domain (RHD) at the N-terminus that is highly homologous to the product of Drosophila segmentation gene *runt* and α subunit of polyomavirus enhancer-binding protein 2 (PEBP2 or PAE2) (Daga *et al.*, 1992; Bae *et al.*, 1993; Ogawa *et al.*, 1993b) (Figure 1). The *AML1* gene is thought to be ubiquitously expressed in multiple hematopoietic lineages because human leukemia cell lines derived from myeloid, B and T lymphoid cells show normal AML1 transcripts (Miyoshi *et al.*, 1991). AML1 heterodimerizes with non-DNA-binding subunit PEBP2β to form an active DNA-binding complex and binds to a specific DNA consensus sequence named the PEBP2 site that contains R/TACCRAC through RHD (Ogawa *et al.*, 1993a, b). PEBP2β enhances the DNA-binding activity of AML1 and protects AML1 from ubiquitin-proteasome-mediated degradation (Huang *et al.*, 2001). The proline–serine–threonine (PST) domain at the C-terminus is required for transcriptional activation. AML1-mediated transcription depends on direct binding of transcriptional co-activators p300, CBP and P/CAF that have intrinsic histone acetyltransferase (HAT) activity (Kitabayashi *et al.*, 1998). AML1 is indispensable for expression of a number of hematopoietic lineage-specific genes, including the *myeloperoxidase* (MPO), *macrophage colony-stimulating factor* (M-CSF) *receptor*, *granulocyte-macrophage colony-stimulating factor* (GM-CSF), *interleukin-3* (IL-3), *T-cell receptor* (TCR) and *NP-3* genes. Gene knockout experiments show that null mutation in either AML1 (Okuda *et al.*, 1996) or PEBP2β (Sasaki *et al.*, 1996; Wang *et al.*, 1996a, b) is embryonic lethal at E12.5 due to complete lack of fetal liver hematopoiesis and lethal central nervous hemorrhages, although primitive yolk sac erythropoiesis appears normal. Using conditional gene inactivation strategy, AML1 is shown to play a role in T-cell development (Taniuchi *et al.*, 2002). Thus, AML1 is a key transcription factor for the hematopoietic cell development, proliferation and differentiation.

The EVI-1 (ecotropic viral integration site) gene was initially identified as a common locus of retrovirus integration in myeloid tumors in AKXD mouse (Morishita *et al.*, 1988). EVI-1 is a transcriptional regulator that possesses two Cys2His2-type zinc-finger domains and acidic domain (Morishita *et al.*, 1990). Expression of EVI-1 is barely detectable in healthy murine or human hematopoietic cells. However, EVI-1 is highly expressed in MDS or AML patients showing 3q26 abnormalities such as inv(3)(q21q26) or t(3;3)(q21;q26) (Morishita *et al.*, 1992). Even in the absence of evident 3q36 abnormalities, elevated expression of EVI-1 is reported in CML in blastic crisis and MDS-derived leukemia (Ogawa *et al.*, 1996a). These findings suggest a critical role for EVI-1 in malignant transformation of hematopoietic cells as a dominant oncogene.

In AML1/EVI-1 chimeric cDNA, an open reading frame of 4185 nucleotides encodes a 1395 amino-acid protein (Mitani *et al.*, 1994). The N-terminal portion of AML1 is abruptly interrupted at the end of RHD, followed by a 5′non-coding sequence of EVI-1 cDNA that is translated through the entire coding region. Therefore, AML1/EVI-1 fusion protein is a chimeric transcription molecule that consists of RHD of AML1 and two zinc-finger domains of EVI-1. In SKH1 cells, two major transcripts in sizes of 8.2 and 7.0 kb are transcribed from the *AML1/EVI-1* chimeric gene, and Western analysis shows that AML1/EVI-1 is a 180 kDa chimeric molecule. AML1/EVI-1 promotes pre-existing stem cell disorder into acute leukemia through multiple

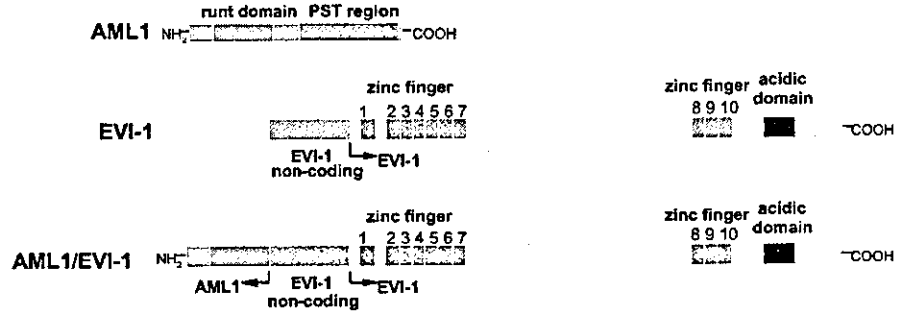

Figure 1 Schematic structure of wild-type AML1, EVI-1 and AML1/EVI-1. Wild-type AML1 possesses RHD at the N-terminus and PST region at the C-terminus. In AML1/EVI-1, N-terminal AML1 sequences are abruptly interrupted at the end of the RHD and followed by almost the entire coding region of EVI-1

mechanisms. I will describe the unique functions of each DNA-binding domain of AML1/EVI-1 fusion molecule in detail below.

Dominant-negative effects over AML1

AML1 transactivates Tww-tk-Luc reporter that contains PEBP2 sites derived from the TCRβ promoter (Tanaka *et al.*, 1995). However, AML1/EVI-1 does not have such transactivation ability, presumably because it does not contain the PST domain that is necessary for the reporter transactivation. Notably, AML1/EVI-1 reduces the reporter transactivation by AML1 in a dose-dependent manner. The gel shift assay shows that AML1/EVI-1 binds to the PEBP2 site more tightly than AML1 does. Therefore, AML1/EVI-1 could dominantly interfere with the AML1-induced transactivation by competing for the specific DNA binding. Deletion mutation analysis of AML1/EVI-1 shows that RHD is responsible for the dominant-negative effect. To exert transcription activation, AML1 needs to heterodimerize with PEBP2β through its RHD. AML1 mainly locates in the nucleus, while PEBP2β locates in the cytoplasm (Tanaka *et al.*, 1998). Immunofluorescence analysis shows that AML1/EVI-1 locates in the nucleus with or without RHD. AML1/EVI-1 seems to drag PEBP2β into the nucleus because co-expression of the full-length AML1/EVI-1 relocates PEBP2β from the cytoplasm to the nucleus. This effect is RHD-dependent because AML1/EVI-1 lacking RHD does not have such effect, which is consistent with the fact that AML1 associates with PEBP2β through RHD. Since PEBP2β more effectively associates with AML1/EVI-1 than AML1, PEBP2β more efficiently translocates into the nucleus by AML1/EVI-1 than by AML1. This differential effect between AML1/EVI-1 and AML1 presumably causes the dominant-negative effect of AML1/EVI-1 over AML1 and accounts for one of the mechanisms through which this chimeric protein contributes to leukemogenesis.

Recently, we found that AML1/EVI-1 interacts with C-terminal binding protein (CtBP), which is essential for repressing the AML1-induced transactivation (Izutsu *et al.*, 2002) (Figure 2). CtBP was originally identified as a protein that interacts with a C-terminal portion of adenovirus E1A protein. To date, two highly related proteins, termed CtBP1 and CtBP2, have been identified in both mice and humans. CtBP belongs to a member of co-repressor proteins that mediates repression by associating with several transcription factors, including Krüppel-like factor (BKLF), friend of GATA (FOG) and T-cell factor (TCF). Although it is not clear how CtBP mediates transcriptional repression, it is supposed that histone deacetylase (HDAC)-1 that interacts with CtBP is involved in the process. We previously determined that EVI-1 interacts with CtBP exclusively through one of the two potential CtBP-binding amino-acid sequences (N-terminal PFDLT and C-terminal PLDLS) that locate next to the second zinc-finger

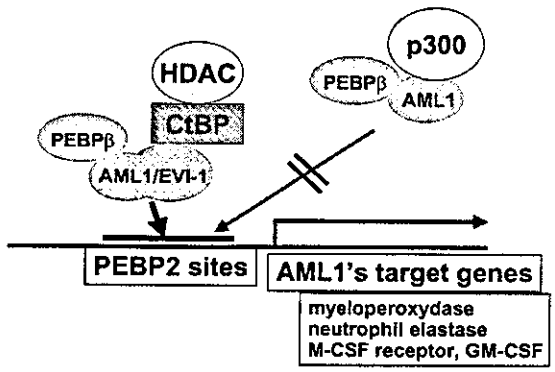

Figure 2 AML1/EVI-1 exerts dominant-negative effects over wild-type- AML1. AML1 becomes an active transcription factor by heterodimerizing with PEBP2β through RHD. PEBP2β increases the DNA-binding ability of AML1 and protects AML1 from the ubiquitin system-mediated degradation. As AML1/EVI-1 binds to PEBP2β more tightly than AML1, AML1/EVI-1 competes AML1 out from the PEBP2 sites. Moreover, AML1/EVI-1 associates with a co-repressor CtBP and thereby recruits HDAC. AML1/EVI-1 actively represses the transcription of potential AML1 target genes. CtBP, C-terminal binding protein; HDAC, histone deacetylase; PEBP2, polyomavirus enhancer binding protein 2

domain (Izutsu *et al.*, 2001). These two sequences are conserved in AML1/EVI-1 and one of them mediates interaction with CtBP. AML1/EVI-1 interferes with the AML1-induced transactivation, while AML1/EVI-1 with mutations in PLDLS does not. Therefore, AML1/EVI-1 requires CtBP as a co-repressor to dominantly inhibit the AML1-induced transactivation. An HDAC inhibitor trichostatin A relieves this dominant-negative effect of AML1/EVI-1. Therefore, it is conceivable that AML1/EVI-1 positively prevents the AML1-induced transcription by recruiting HDAC through CtBP.

AML1/EVI-1 blocks granulocytic differentiation of a murine myeloid cell line 32Dcl3 induced by G-CSF (Tanaka *et al.*, 1995). Parental 32Dcl3 cells acquire mature granulocytic phenotype characterized by cytoplasmic granules and segmented/circular nucleus when cultured with G-CSF for several days. A robust induction of MPO mRNA is also observed by Northern analysis. 32Dcl3 cells overexpressing AML1/EVI-1 maintain immature morphological phenotypes characterized by a large unsegmented nucleus even in the presence of G-CSF (Izutsu *et al.*, 2002). In parallel, there is poor induction of MPO mRNA in these cells. Thus, overexpression of AML1/EVI-1 blocks G-CSF-induced differentiation of 32Dcl3 cells into mature granulocytic phenotype. On the contrary, in 32Dcl3 cells overexpressing mutant AML1/EVI-1 that is unable to interact with CtBP, the morphological changes and induction of MPO mRNA are indistinguishable from those of parental 32Dcl3 cells. These results suggest that AML1/EVI-1 blocks G-CSF-induced granulocytic maturation by interacting with CtBP. Taken together, CtBP-mediated repression of gene transcription could be one of the mechanisms for AML1/EVI-1-mediated block in granulocyte differentiation.

Repression of transforming growth factor β (TGFβ)-mediated growth inhibition

Cell growth and differentiation are tightly regulated by delicate balance of growth factors and growth-inhibitory factors. TGFβ inhibits proliferation of a wide range of cell types including epithelial, endothelial and hematopoietic cells. Binding of TGFβ to the heteromeric serine/threonine kinase receptor complex leads to direct phosphorylation of intracellular signal transducers Smad2 and Smad3, followed by the formation of heteromeric complexes with a common signal transducer Smad4, and subsequent their translocation into the nucleus. Once in the nucleus, Smad complexes are thought to act as a transcriptional activator to inhibit cellular proliferation. We demonstrated that EVI-1 perturbs TGFβ signaling and inhibits proliferation of a wide variety of cells. EVI-1 represses transcriptional activation of TGFβ-responsive reporters such as the *plasminogen-activator inhibitor-1* (PAI-1) or *p15* promoter-Luciferase construct (Kurokawa *et al.*, 1998a). There is no effect of EVI-1 on the basal activity of these promoters, indicating that this inhibitory effect works specifically toward TGFβ-induced transcriptional activation. In growth inhibition experiment, EVI-1 antagonizes the anti-proliferative effects of TGFβ against TGFβ-sensitive Mv1Lu cells. Biochemically, most of retinoblastoma (Rb) proteins in the EVI-1-expressing cells remain in the hyperphosphorylated forms even in the presence of TGFβ, whereas the control cells treated with TGFβ accumulate hypophosphorylated Rb and show a decreased level of Rb protein. The first zinc-finger domain of EVI-1 mediates interaction with Smad3, and the small region next to the second zinc-finger domain (repression domain) is required to repress TGFβ signaling. The association between EVI-1 and CtBP is essential for the inhibition of *PAI-1* reporter construct. Trichostatin A treatment alleviates EVI-1-mediated repression of TGFβ-responsive reporters, suggesting that HDAC is involved in this repression. Consistent with this, Mv1Lu cells overexpressing EVI-1 with a mutated PLDLS show normal growth inhibition as parental Mv1Lu cells when treated with TGFβ.

Similar to EVI-1, AML1/EVI-1 inhibits transactivation of TGFβ-responsive promoters (Kurokawa *et al.*, 1998b). The constitutive expression of AML1/EVI-1 or EVI-1 in 32Dcl3 cells overcomes TGFβ-mediated inhibition of cell growth. From these data, AML1/EVI-1 and EVI-1 can potentially block growth inhibition of hematopoietic cells mediated by TGFβ. MOLM-1 is a human megakaryoblastoid cell line carrying the inv(3)(q21q26) and endogenously expressing a truncated form of EVI-1 proteins in which the C-terminal amino acids of wild-type EVI-1 are replaced by five amino acids (Ogawa *et al.*, 1996b). EVI-1 in MOLM-1 cells retain both the first zinc-finger and the repression domains that are required for repression of TGFβ signaling. Thus, MOLM-1 cells do not respond to TGFβ-mediated growth inhibition. However, it becomes TGFβ sensitive when treated with antisense oligonucleotides complementary to the sequence encoding the N-terminus of the first zinc-finger domain of EVI-1. Just like wild-type-EVI-1, AML1/EVI-1 physically interacts with Smad3 and inhibits the Smad3 activity (Figure 3). AML1/EVI-1 also associates with CtBP through the CtBP-binding consensus sequence PLDLS. As endogenous CtBP proteins are detected in SKH1 cells, it could be possible that TGFβ signaling is repressed in this leukemia cell line. While the interaction of CtBP and HDAC1 is clearly demonstrated, association between AML1/EVI-1 and HADC1 is not yet determined. However, it is speculated that AML1/EVI-1 recruits HDACs through CtBP to repress TGFβ-responsive transcription. Thus, in leukemia with the

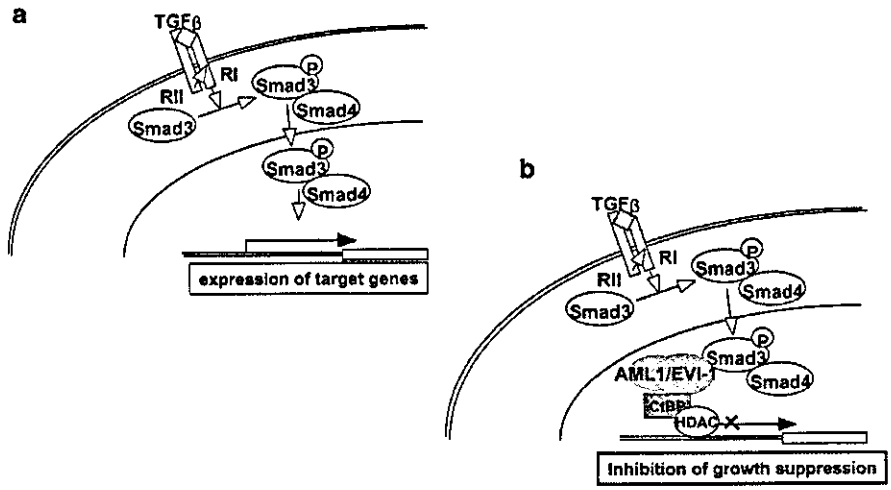

Figure 3 AML1/EVI-1 represses TGFβ-mediated growth inhibitory signal. (**a**) Upon TGFβ binding to the heteromeric serine/threonine kinase receptor complex, type II receptor activates type I receptor and thereby phosphorylates Smad3. Phosphorylated Smad3 forms a complex with Smad4 and translocates into the nucleus, where they work as a transcription factor to inhibit cellular growth. (**b**) AML1/EVI-1 binds to Smad3 through the first zinc-finger domain in EVI-1 and actively interferes with Smad3 transcriptional activity. Recruitment of HDAC by EVI-1 via CtBP is an underlying mechanism. TGFβ transforming growth factor β; R I, type I receptor; R II, type II receptor; CtBP, C-terminal binding protein; HDAC, histone deacetylase

t(3;21) translocation, AML1/EVI-1 may contribute to leukemogenesis by blocking the growth-inhibitory effect of TGFβ.

Inhibition of c-Jun N-terminal kinase

Mitogen-activated protein (MAP) kinase cascades are important signaling pathways that are involved in a wide variety of biological response mechanisms. In vertebrates, at least three pathways have been identified: ERK, c-Jun N-terminal kinase (JNK) and p38. Generally, ERK signaling is involved in controlling cell proliferation and differentiation, while JNK or p38 signaling plays an important role in triggering apoptosis in response to cellular stresses such as UV light, γ-radiation, osmotic shock, protein synthesis inhibitors, tumor necrosis factor-α (TNFα) and interleukin-1. EVI-1 acts as an inhibitor of JNK, either when overexpressed JNK is unstimulated or stimulated by UV light, anisomycin, sorbitol and TNFα (Kurokawa *et al.*, 2000). However, EVI-1 does not affect the kinase activities of overexpressed ERK and p38. Furthermore, Rat1 cells overexpressing EVI-1 show downregulated endogenous JNK activity. In MOLM-1 cells, the endogenous JNK activity is reduced. However, when treated with EVI-1 antisense oligonucleotide, the JNK activity is restored. The same results are obtained in the experiment using human endometrial carcinoma cell line HEC1B that expresses EVI-1 at a high level. Consistently, the presence of EVI-1 actually reduces phosphorylated substrates of c-Jun. JNK activation requires phosphorylation on two specific amino-acid residues Thr183 and Tyr185 by MMK4 or MMK7. Although EVI-1 physically interacts with JNK, it does not affect its phosphorylation status. However, the binding between JNK and c-Jun is significantly reduced by concomitant expression of EVI-1. This suggests that EVI-1 inhibits c-Jun phosphorylation by inhibiting the association between JNK and c-Jun (Figure 4). EVI-1 associates JNK through the first zinc-finger domain. This interaction is essential for inhibition of JNK activity. EVI-1 constitutively associates with JNK in HEC1B cells, and this interaction is increased by the treatment with UV light.

We also demonstrated that EVI-1 inhibits apoptotic cell death by interfering with JNK signaling pathway. 293 cells undergo apoptosis via JNK signaling induced by UV light. Overexpression of EVI-1 in 293 cells significantly represses apoptosis induction by UV light. On the other hand, HEC1B cells are resistant to apoptosis, presumably because they express endogenous EVI-1. However, HEC1B cells treated with EVI-1-specific antisense oligonucleotide easily undergo apoptosis. Stress activation of JNK promotes upregulation of FasL expression in T lymphocytes, which is one of the mechanisms potentially causing apoptosis. FasL expression induced by UV stimulation in Jurkat cells is prevented by EVI-1 depending on the first zinc-finger domain. EVI-1 actually blocks apoptosis induction by UV light, and represses activation of FasL reporter stimulated by UV light and anisomycin in Jurkat cells. Furthermore, EVI-1 inhibits apoptosis induction by TNFα in U937 cells by repressing JNK activity. In conclusion, EVI-1 blocks both the molecular and biological activities of JNK.

It has not been determined whether AML1/EVI-1 possesses the similar anti-apoptotic effect as EVI-1 does. However, because this function is dependent on the first zinc-finger domain in EVI-1, it is reasonable to speculate that AML1/EVI-1 also prevents apoptosis induction by inhibiting JNK.

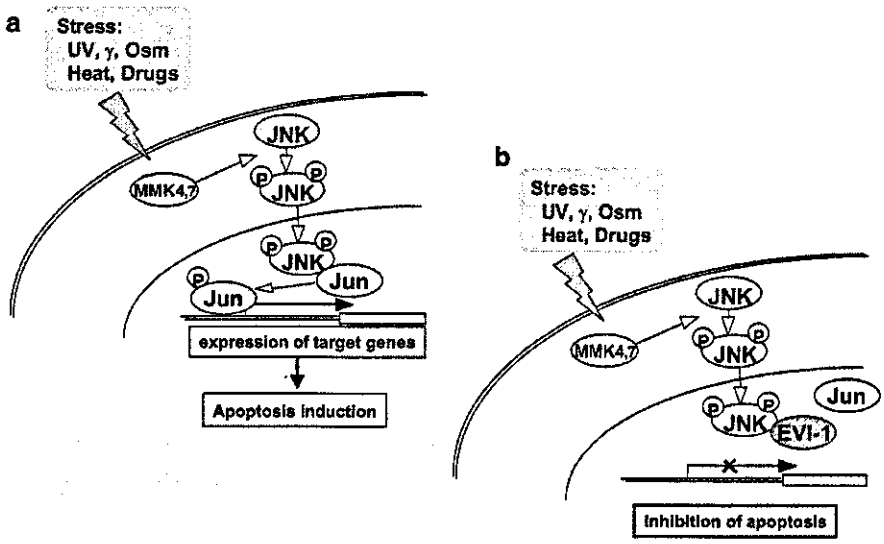

Figure 4 EVI-1 inhibits JNK activity. (a) Various kinds of stresses such as UV light, γ-radiation, osmotic shock, heat shock and protein synthesis inhibitor activate JNK by phosphorylation. Activated JNK translocates into the nucleus, phosphorylates transcription factors such as Jun and triggers apoptosis induction. (b) EVI-1 blocks association between JNK and its substrates and thereby represses JNK activity to induce apoptosis. JNK, c-Jun N-terminal kinase

Stimulation of proliferation

AP-1 (Fos/Jun heterodimer or Jun/Jun homodimer) is activated by growth stimuli, including growth factors, phorbol esters such as 12-*o*-tetradecanoylphorbol 13 - acetate (TPA) and various oncogene products, and stimulates transactivation through TPA responsive site (TRE). AP-1 functions as a positive or negative regulator in a variety of cellular differentiation and proliferation processes. EVI-1 raises AP-1 activity in NIH3T3 cells and embryonal carcinoma (EC) cell line P19 (Tanaka *et al.*, 1994). EVI-1-transfected P19 cells show differentiated phenotypes, characterized by flattened and enlarged morphology. These changes are indistinguishable from the morphological changes seen in P19 cells treated with retinoic acid or transfected with c-Jun. The stage-specific embryonic antigen SSEA-1, known as a stem cell marker in EC cells, is detected in parental P19 cells, but not in EVI-1-transfected P19 cells. In contrast to SSEA-1, the heat shock protein Hsp47, known as a differentiation marker, is detected in EVI-1-transfected P19 cells, but not in parental cells. In addition, expression of c-Jun and c-Fos is increased in EVI-1-tansfected cells. EVI-1 enhances the activity of the *c-Fos* promoter in NIH3T3 and P19 cells, depending on the second zinc-finger domain (Figure 5). It is shown that the second zinc-finger domain is essential for both the activation of AP-1 and transactivation of the *c-Fos* promoter.

In order to evaluate the transforming activity of AML1/EVI-1, the fusion cDNA is introduced into Rat1 fibroblasts (Kurokawa *et al.*, 1995). Rat1 cells expressing AML1/EVI-1 form macroscopic colonies in soft agar, while the mock-transfected cells produce tiny, barely macroscopic ones. This indicates that AML1/EVI-1 is a transforming gene. Introduction of AML1/EVI-1 into the Rat1 clones harboring BCR/ABL confers enhanced capacity for anchorage-independent growth. AML1/EVI-1 also stimulates AP-1 activity. As the second zinc-finger domain is required for both transformation of Rat1 cells and an increase in AP-1 activity, it is conceivable that AML1/EVI-1 transforms Rat1 cells by AP-1 activation. Notably, AML1/EVI-1-transformed colonies express c-Jun mRNA. Since AML1/EVI-1 exaggerates the transforming activity of BCR/ABL, AML1/EVI-1 could perform a critical role in leukemic progression of CML.

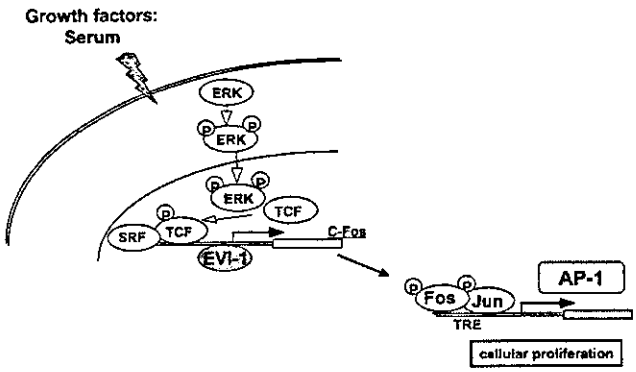

Figure 5 EVI-1 stimulates AP-1 activity. EVI-1 activates *c-Fos* transcription. Fos associates with Jun to form a heterodimer and stimulates TRE-mediated transcription. Increased AP-1 activity by EVI-1 promotes cellular proliferation. ERK, extracellular-regulated kinase; TCF, ternary complex factor; SRF, serum response factor; TRE, TPA responsive element; AP-1, activation protein 1

Conclusion

Similar to other AML1-related chimeras, AML1/EVI-1 exerts dominant-negative effects over AML1-induced transcription. However, target genes that may have a key role in leukemogenesis are still not elucidated. On the other hand, almost the entire coding region of EVI-1 is expressed under the AML1 promoter in leukemic cells carrying AML1/EVI-1 chimeric gene. Since ectopic expression of EVI-1 is thought to be involved in leukemic transformation of CML and MDS, EVI-1 portion in AML1/EVI-1 fusion molecule may also play a critical role in leukemogenesis. Notably, AML1/EVI-1 and EVI-1 share similar functions; the first zinc-finger and the repression domains exhibit anti-growth repression effect by blocking TGFβ signaling, the first zinc-finger domain exhibits anti-apoptotic effect by repressing JNK signaling, and the second zinc-finger domain exhibits proliferation stimulation effect by increasing AP-1 activity. All these effects are related to leukemic cell proliferation, directly or indirectly. The molecular mechanisms of leukemogenesis by AML1/EVI-1 are summarized in Figure 6. The multi-functions of AML1/EVI-1 fusion molecule are mostly demonstrated by *in vitro* experiments. Our next step is to generate AML1/EVI-1 leukemia model mice and demonstrate these functions *in vivo*. It is plausible that AML1/EVI-1 knock-in mice are embryonic lethal in the mid-gestation because of the dominant-negative effects of AML1/EVI-1 over normal AML1, as is the case with AML1/ETO knock-in mice. Thus, we are planning to make conditional AML1/EVI-1 knock-in mice to enable temporal and special expression control of the chimeric molecule.

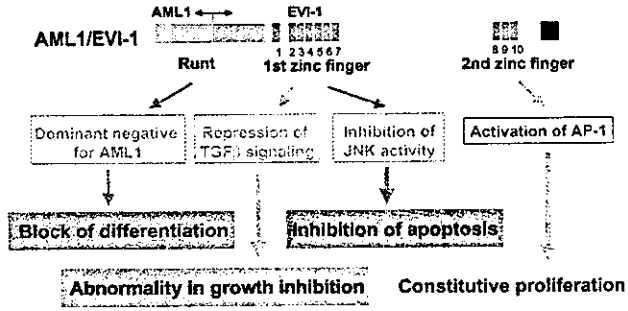

Figure 6 AML1/EVI-1 is a multi-functional oncoprotein. AML1/EVI-1 develops hematopoietic stem cell disorders to acute leukemia by at least four unique functions: dominant-negative effect over AML1, repression of TGFβ signaling, inhibition of JNK activity and activation of AP-1

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Functional analysis of a dominant-negative ΔETS TEL/ETV6 isoform

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Abstract

A transcriptional repressor TEL belongs to the ETS family transcription factors and acts as a tumor suppressor. We identified five alternatively spliced TEL isoforms generated possibly through exon skipping mechanisms, by using reverse transcriptase-polymerase chain reaction analysis. Among them, we examined molecular and biological functions of a ΔETS-TEL isoform (TEL-f). This isoform abrogated specific DNA-binding capacity to and trans-repressional ability through the ETS-binding site. Regardless, it showed dominant-negative effects over wild-type-TEL (TEL-a)-mediated transcriptional repression partly through sequestration of TEL-a from nucleus to cytoplasm. Moreover, TEL-f dominantly interfered with TEL-a-mediated erythroid differentiation in MEL cells and growth suppression in NIH3T3 cells. Interestingly, TEL isoforms without the entire (Δ exons 6 + 7-TEL) or a part (Δ exon 7-TEL) of ETS domain were expressed more frequently in myelodysplastic syndrome-derived leukemia than in myelodysplastic syndrome before transformation. This observation suggests that accumulation of the dominant-negative ΔETS-TEL molecules could be a related phenomenon to leukemic progression of myelodysplastic syndrome.
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Keywords: TEL; Isoform; ETS; Dominant-negative effects; Sequestration; Tumor suppressor; Erythroid differentiation; Myelodysplastic syndrome; Leukemic transformation

The *TEL* gene (also known as *ETV6*) that encodes a member of the E26 transformation-specific (ETS) family of transcription factors [1] was originally identified by virtue of its fusion with the *platelet derived growth factor receptor β* gene in a patient of chronic myelomonocytic leukemia (CMMoL) carrying a chromosomal abnormality t(5;12)(q33;p13) [1]. TEL shares with other ETS proteins the evolutionarily conserved ETS domain at the C-terminus that is responsible for DNA binding to the ETS-binding consensus site (EBS). TEL also shares with a subset of other ETS proteins a N-terminal weakly conserved domain referred to as helix–loop–helix (HLH) or pointed domain. TEL itself is known to act as a transcriptional repressor through association with multiple co-repressors such as mSin3A and SMRT/N-CoR [2], and histone deacetylase (HDAC)-3. *FLI-1* [3], *Id1* [4], and *stromelysin-1* [5] have been identified as target genes for TEL.

TEL is widely expressed throughout mouse embryonic development and in most human and mouse tissues. It is essential to mouse development since its inactivation by homologous recombination results in embryonic lethality at E10.5–11.5. The knockout embryos show defects in yolk sac angiogenesis and intra-embryonic apoptosis of mesenchymal and neural cells, while they present normal yolk sac hematopoiesis [6]. Analysis of chimeric mice with *TEL*(−/−) ES cells uncovered an essential role of TEL in establishing hematopoiesis of all lineages in neonatal bone marrow, although *TEL*(−/−) ES cells contributed to both primary and definitive fetal hematopoiesis [7]. As for hematopoietic lineage-specific roles, we reported that TEL stimulates erythroid differentiation in murine erythroleukemia MEL cells when induced by chemical compounds such as hexamethylene bisacetamide (HMBA) and dimethyl sulfoxide [8]. It is also suggested by the

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analysis using Ras-transformed NIH3T3 cells in vitro and in vivo that TEL exerts tumor suppressive functions in part through transcriptional repression of *stromelysin-1* gene [5].

The *TEL2* gene encodes a protein highly structurally related to TEL [9,10]. TEL2 has several alternatively spliced isoforms that may exhibit different functions depending on their structures. By reverse transcriptase-coupled polymerase chain reaction (RT-PCR) analysis, we first observed five TEL isoforms variously lacking its functional domains in human mononuclear cells of peripheral blood. It is plausible that expression of these isoforms affects the functions of wild-type-TEL. Because we demonstrated dominant-negative effects of a ΔHLH-TEL mutant over wild-type-TEL [8], we investigated functional alterations of a ΔETS-TEL isoform in this study. This isoform lost specific DNA-binding and dominantly interfered with wild-type-TEL-mediated transcriptional repression. Moreover, the ΔETS-TEL isoform also repressed wild-type-TEL-induced erythroid cell differentiation and fibroblast growth suppression. Notably, ΔETS-TEL isoforms were frequently expressed in myelodysplastic syndrome (MDS)-derived leukemia cases. These findings indicate that accelerated expression of the dominant-negative TEL isoforms could be connected with transformation of MDS.

Materials and methods

Pathologic samples and RNA extraction. After obtaining informed consent, mononuclear cells were isolated from bone marrow samples from 10 patients with various types of MDS (6 cases with refractory anemia (RA), 2 cases with refractory anemia with excess of blasts (RAEB), 2 cases with CMMoL), 7 cases with MDS-derived leukemia, and 14 healthy volunteers. Clinical diagnoses of MDS subtypes were performed according to the French–American–British classification. Total RNA was extracted from 0.5 to 10×10^6 of bone marrow mononuclear cells using Isogen (Nippon Gene).

Plasmid construction. pME18S-FLAG-TEL, pCXN2-FLAG-TEL, pCDNA3-FLAG-TEL, and pSRαMSVtk*neo*-FLAG-TEL were described previously [8,11]. To obtain the cDNA encoding TEL-f isoform, a C-terminal part of TEL sequences that contains the deletion of both exons 6 and 7 was amplified by PCR using a primer set of 5′ MDS-PCR12 primer (5′-CAGGAGAACAACCACCAGGAG-3′) and TEL-ES2 primer (5′-GGGAATTCGGCCACTCATGATTTCA TCTGGGGTTTTCATAAACCTGCTATTCTCCCAATGGGCAT GCG-3′) and full-length TEL cDNA as a template. 5′ MDS-PCR12 primer contains the sequence within exon 5. TEL-ES2 primer includes the antisense sequence corresponding to the 3′ end of exon 5 connected with the 5′ end of exon 8. FLAG-tagged N-terminal TEL cDNA and the resultant C-terminal PCR fragment were subcloned together into *Eco*RI site of expression vector pME18S, pCDNA3, pCXN2, and pSRαMSVtk*neo*. HA-tagged N-terminal TEL cDNA and the C-terminal PCR fragment were also inserted together into *Eco*RI site of pME18S and pCAGIPuro. The reporter plasmids $(EBS)_3$tkLuc and $(mEBS)_3$tkLuc were described previously [8].

Retroviral stock preparation. Both 10 μg of retroviral construct and 40 μg of ψ packaging plasmid were transfected to exponentially growing 1×10^6 Cos7 cells in 90 mm tissue culture plate using DEAE-dextran method. Forty-eight hours later, culture media were replaced with 5 ml DMEM containing 5% fetal calf serum (FCS). The supernatant media were harvested after 24 h incubation, filtered with a 0.45 μm pore-sized filter, and stored at −80 °C as retroviral stocks. Harvest of retroviral stocks was repeated until 5 days post-transfection.

Cell culture. MEL-B8 (a Friend virus-transformed mouse erythroleukemia cell line), Cos7, and NIH3T3 cells were grown in DMEM with 10% FCS. HeLa cells were cultured in MEM with 10% FCS. To induce erythroid differentiation in MEL cells, 5 mM HMBA (Sigma–Aldrich) was added to the culture. Erythroid differentiation was determined by counting the percentage of hemoglobin-producing cells following benzidine staining.

Isolation of stable transfectants. Stable MEL clones expressing either TEL-a or TEL-f were isolated as described previously [8]. Five $\times 10^5$ NIH3T3 cells were infected with mock or retroviruses carrying the FLAG-tagged TEL-a cDNA to establish stable transfectants. Infected cells were selected with 0.8 mg/ml G418 and cloned by limiting dilution. Survival clones were screened for expression of the isoform by Western analysis using anti-FLAG (Sigma–Aldrich) antibody. The mock and the TEL-a expressing clones were further transfected with the HA-tagged TEL-f cDNA cloned into pCAGIPuro plasmid using Transfast transfection reagent (Promega). Survival clones after selection with 0.5 μg/ml of puromycin were again screened for additional expression of TEL-f by Western analysis using anti-FLAG and anti-HA (Boehringer–Mannheim) antibodies.

RT-PCR analysis and sequencing. One microgram of total RNA was reverse-transcribed with 400 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen) and a random hexamer (Takara). One-fiftieth of the synthesized cDNA was directed to PCR analysis. The PCR condition was as follows: 94 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and final extension at 72 °C for 7 min. The primers used for amplification of full-length TEL and ETS domain were EX-10 (5′-ATGTCTGAGAC TCCTGCTCAGTG-3′) and A-ETS-3 (5′-TGGTTCCTTCAGCATT CATCT-3′) and ETS-3 (5′-GCACTCCGTGGATTTCAAAC-3′) and A-ETS-3, respectively. The products were electrophoresed on 2% agarose gels containing ethidium bromide. All PCR fragments were subcloned into pCR2.1-TOPO vector (Invitrogen) and subsequently cycle-sequenced in forward and reverse directions using M13 forward (−20) (5′-GTAAAACGACGGCCAG-3′) and reverse (5′-CAGG AAACAGCTATGAC-3′) primers and ABI BigDye terminators. DNA sequences were analyzed using ABI Prism 3100 Genetic Analyzer.

Cell fractionation. To fractionate cells, cell pellets of MEL clones were resuspended in hypotonic buffer (10 mM sodium phosphate, pH 7.0, 5 mM EDTA, 1 mM DTT, 1 mM Na_3VO_4, and 1 mM PMSF) and homogenized using Dounce homogenizer (Wheaton Science Products). Nuclear and cytoplasmic fractions were separated by centrifugation at 5000 rpm for 10 min. Equal proportions of the nuclear and cytoplasmic fractions were subjected to Western analysis.

Immunoprecipitation and Western analysis. Cos7 cells were transfected with expression plasmids using DEAE-dextran method and lysed. Immunoprecipitants with anti-FLAG M2 antibody obtained from lysates of the transfected Cos7 cells were eluted with FLAG oligopeptide. Western analysis using anti-FLAG or anti-HA antibody was performed as described previously [12]. The blots were visualized by ProtoBlot AP system (Promega).

Electrophoretic mobility shift assay. TEL-a and TEL-f cDNAs subcloned into pCDNA3 expression plasmid were in vitro translated using TNT Coupled Wheat Germ Extract System (Promega). The procedures for electrophoretic mobility shift assay (EMSA) and the oligonucleotides used in this assay were reported previously [8].

Luciferase assay. HeLa cells were transfected with 1 μg $(EBS)_3$ tkLuc or $(mEBS)_3$tkLuc reporter plasmid alone or along with 0.1–1.0 μg of expression plasmids using Tfx-20 (Promega). To keep the transfection efficiency as constant as possible among the samples to be compared, total amount of DNA in terms of weight was adjusted to be equal by adding the expression plasmid pME18S. Luciferase assays were performed as described previously [8]. A total of 20 ng of

pRL-CMV plasmid was co-transfected as an internal control for transfection efficiency and the data were normalized to the *Renilla* luciferase activity. All transfection experiments were performed at least four times and the representative data are presented.

Colony assay. For a soft agar assay, cells of each transfected derivative were trypsinized, suspended in DMEM containing 0.3% agar and 20% FCS at a density of 2×10^4 cells/3.5 cm dish, and plated onto a bottom layer containing 0.6% agar. Colonies >0.125 mm in diameter were enumerated after 14 days. The numbers of colonies are presented as a mean value in tetraplicate experiments.

Results

Various types of TEL isoforms are expressed in peripheral blood mononuclear cells of healthy individuals

Because another TEL family member TEL2 has various types of isoforms [10], we looked at whether TEL also shows isoforms especially in hematopoietic cells. To this end, we performed RT-PCR analysis that amplifies almost full-length TEL cDNA using mRNAs derived from peripheral blood mononuclear cells of four healthy individuals as templates and a primer set located within exons 1 and 8. Various PCR fragments of different sizes were amplified in all reactions as shown in Fig. 1A. To determine structures of these PCR fragments, we subcloned them into pCR2.1-TOPO vector for sequence analysis. The results are listed in Fig. 1B. In addition to wild-type-TEL (TEL-a), we detected various types of alternatively spliced TEL isoforms (TEL-b, c, d, e, and f) in which one, two or four exons were deleted. TEL-c, d, and f seemed to be C-terminally truncated proteins with out-of-frame stop codons. As judged from their structures, some of them could antagonistically work on TEL-a. Actually, a ΔHLH deletion mutant with the similar structure to TEL-e has been demonstrated to exert dominant-negative effects over TEL-a [8]. To investigate the possibility that TEL-f lacking the DNA-binding ETS

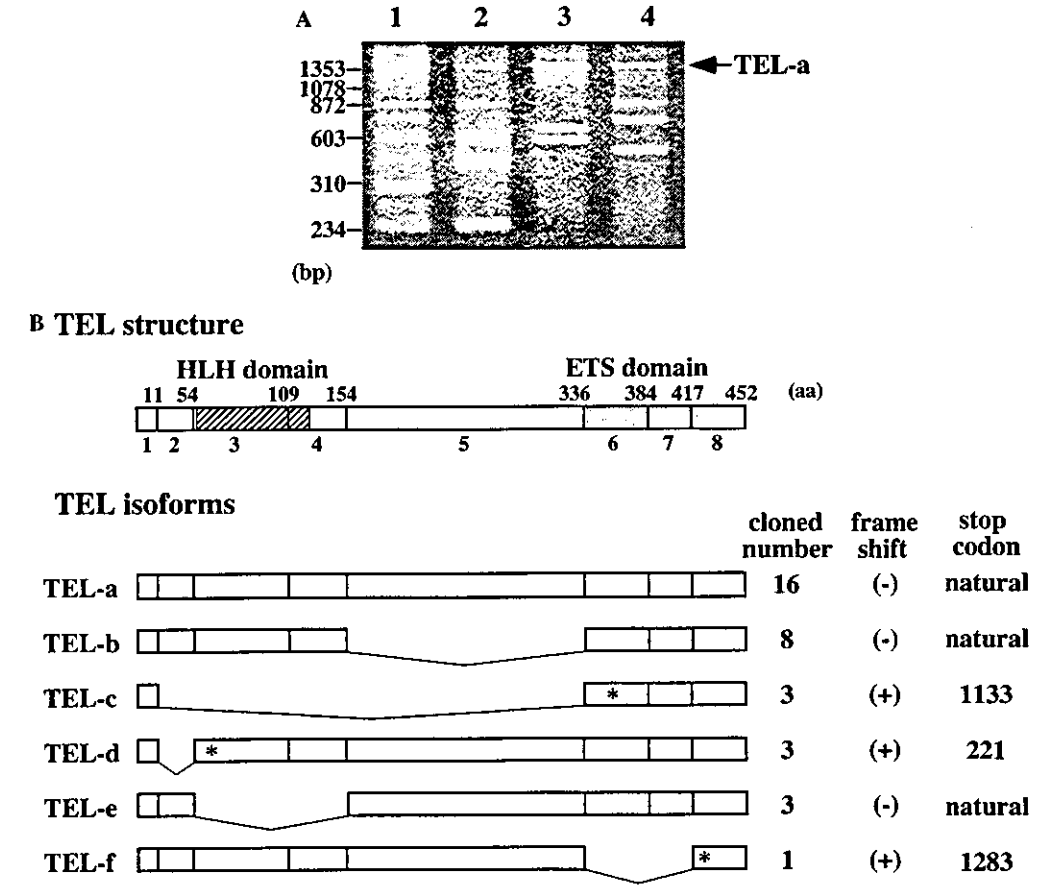

Fig. 1. Schematic description of six alternatively spliced TEL isoforms. (A) Ethidium bromide-staining of the RT-PCR products that were amplified using a primer set in exons 1 and 8 is presented. An arrow indicates full-length fragments derived from TEL-a. We cloned several alternative isoforms shown below from these products. (B) Structure of full-length TEL is shown in the upper panel. Exons 1–8 are presented by open boxes and exon numbers are indicated below the boxes. Amino acid numbers at the end of each exon are also revealed above the boxes. HLH and ETS domains are shown by striped and shaded boxes, respectively. Structures of expected protein isoforms (TEL-a, TEL-b, TEL-c, TEL-d, TEL-e, and TEL-f) encoded by six alternatively spliced TEL transcripts are shown in the lower panel. Positions of stop codons generated by frame-shift are indicated by asterisks. Numbers of obtained clones and positions of stop codons are presented on the right side of each isoform (numbers adopted by Golub et al. [1]). aa, amino acids.

domain is also a dominant-negative isoform, we constructed TEL-f expression plasmids and compared molecular and biological functions of TEL-a and TEL-f.

TEL-f lacks specific DNA-binding capacity

We first analyzed DNA-binding affinity of TEL-a and TEL-f by using EMSA with a radioactive EBS oligonucleotide probe and in vitro translated TEL-a and TEL-f proteins. As shown in Fig. 2, TEL-a proteins generated a specific DNA–protein complex (indicated with an asterisk 1) that was detected as a broad band possibly because of its weak binding to DNA. The complex was not seen in the mock control and was supershifted with anti-TEL antibody (indicated with an asterisk 2). This broad band represented a specific binding of TEL-a to the EBS probe since the binding was canceled by cold specific competitors but not by non-specific competitors. In contrast, TEL-f did not show the specific DNA–protein complex as TEL-a did. Therefore, we conclude that TEL-f lacks DNA-binding capacity and may lose trans-repressional abilities of TEL-a.

TEL-f is a negative regulator for transcriptional repression by TEL-a

In order to get more insights into functional alterations of TEL-f, we investigated transcriptional regulatory properties of TEL-a and TEL-f on $(EBS)_3$tkLuc reporter. We transfected $(EBS)_3$tkLuc or $(mEBS)_3$tkLuc (mutated EBS-containing $(EBS)_3$tkLuc) along with TEL-a or TEL-f expression plasmid into HeLa cells and evaluated luciferase activities. Co-transfection of $(EBS)_3$tkLuc with TEL-a expression plasmid resulted in a dose-dependent decrease in the luciferase activity (Fig. 3A, left panel). This repression appeared to be EBS-mediated since TEL-a did not repress the activity of $(mEBS)_3$tkLuc (Fig. 3A, right panel). In contrast, no repression was observed when TEL-f was expressed with $(EBS)_3$tkLuc reporter (Fig. 3B).

Although TEL-f showed neither EBS-binding nor trans-repression, it could be possible that it affects TEL-a-mediated transcriptional repression through heterodimerizing with TEL-a. Notably, co-expression of TEL-f abolished the transcriptional suppression by TEL-a in a dose-dependent manner (Fig. 3C). These data suggest that TEL-f has dominant-negative effects over the TEL-a-mediated transcriptional repression.

TEL-f associates with TEL-a

To determine whether TEL-f actually heterodimerizes with TEL-a in vivo, we co-expressed FLAG-tagged TEL-a with HA-tagged TEL-a or TEL-f and performed immunoprecipitation with anti-FLAG antibody followed by Western analysis with anti-HA antibody.

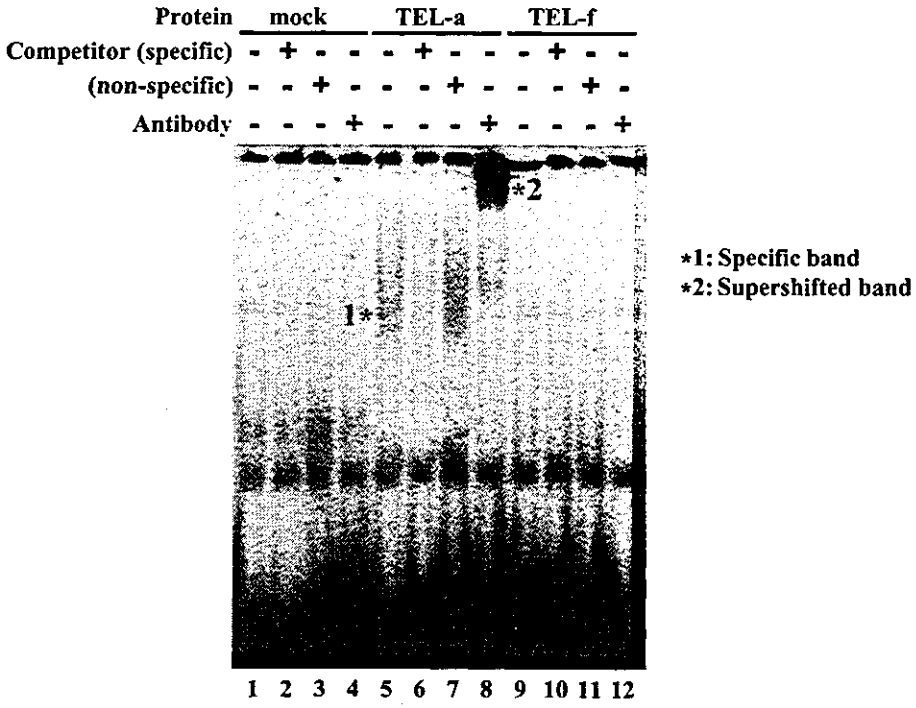

Fig. 2. TEL-f lacks specific DNA-binding capacity. EMSA was carried out using the [^{32}P]-labeled EBS probe and in vitro translated mock (lanes 1–4), TEL-a (lanes 5–8) or TEL-f (lanes 9–12) proteins. A 300-fold molar excess of specific competitors (lanes 2, 6, and 10) or non-specific competitors (lanes 3, 7, and 11) that contain mutations in the ETS-binding consensus sequence, or anti-TEL antibody (lanes 4, 8, and 12) was also added to the reactions. Asterisks 1 and 2 indicate specific DNA–protein complex and supershifted complex, respectively.

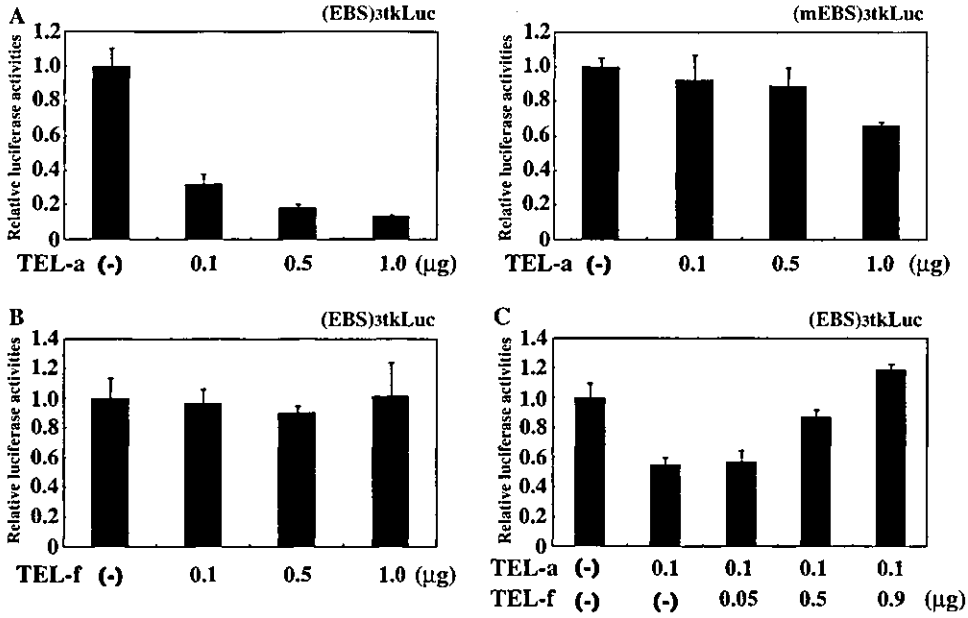

Fig. 3. TEL-f shows dominant-negative effects over TEL-a-mediated transcriptional repression. (A) HeLa cells were transfected with 1 μg $(EBS)_3$tkLuc (left panel) or $(mEBS)_3$tkLuc (right panel) reporter plasmid alone or along with 0.1, 0.5 or 1.0 μg TEL-a expression plasmid. Bars show relative luciferase activities to the level when a control plasmid pME18S was co-transfected with the corresponding reporter plasmid, and present average results of duplicate experiments. (B) HeLa cells were transfected with 1 μg of $(EBS)_3$tkLuc reporter plasmid alone or along with 0.1, 0.5 or 1.0 μg of TEL-f expression plasmid. The results are presented as relative luciferase activities. (C) HeLa cells were transfected with 1 μg $(EBS)_3$tkLuc reporter plasmid alone (lane 1) or along with 0.1 μg TEL-a expression plasmid (the other lanes). 0.05, 0.5, and 0.9 μg (lanes 3, 4, and 5) of TEL-f expression plasmid were also co-transfected. The results are presented as relative luciferase activities.

Expression of each protein was confirmed by Western analysis with anti-FLAG and anti-HA antibodies (Fig. 4B). To avoid or reduce contamination of heavy chains from the antibody used for immunoprecipitation, immunoprecipitants were eluted with FLAG oligopeptide. As expected, both HA-tagged TEL-a and TEL-f were co-immunoprecipitated with anti-FLAG antibody (Fig. 4A). This indicates that TEL-f associates with TEL-a in vivo and that heterodimerization with TEL-f without DNA-binding abilities attenuates TEL-a's transcriptional functions.

TEL-f is localized within cytoplasm and partly pulls out TEL-a from nucleus

Although many basic amino acids lie scattered in ETS domain, a classical stretch of basic amino acids that forms a nuclear localization signal (NLS) seems not to be formed. However, it is possible that subcellular localization of TEL-f could be changed [13]. To assess the relative amounts of TEL-a and TEL-f in the nuclear and cytoplasmic compartments, we performed Western analysis on MEL cells constitutively expressing FLAG-tagged TEL-a or TEL-f after separation of the nuclear and cytoplasmic fractions. As shown in Fig. 5A, TEL-f was predominantly present in the cytoplasmic fraction, while TEL-a was found in the nuclear fraction. Since TEL-f had a potential to heterodimerize with TEL-a, we speculated that TEL-f might affect subcellular localization of TEL-a. Surprisingly, both TEL-a and TEL-f were evenly detected in the nuclear and cytoplasmic fractions, when TEL-f was co-expressed with TEL-a in MEL cells (Fig. 5B). These data indicate that TEL-f might inhibit molecular properties of TEL-a at least partly through exporting it from nucleus to cytoplasm through heterodimerization.

TEL-f blocks erythroid differentiation in MEL cells

MEL cells are Friend virus-induced erythroleukemia cell line cells [14,15] and are blocked roughly at proerythroblast stage of differentiation. Treatment with HMBA causes them to reinitiate erythroid differentiation culminating in the synthesis of hemoglobin and other red cell-specific proteins, terminal cell divisions, and loss of tumorigenicity. We previously reported that TEL stimulates erythroid differentiation in MEL cells induced by chemical compounds, depending on HLH domain [8]. To evaluate the capacity of TEL-f to affect erythroid differentiation, we established MEL clones stably expressing TEL-a (Ta-108 and Ta-113) or TEL-f (Tf-1 and Tf-2) by introducing FLAG-tagged TEL-a or TEL-f expression plasmid (pCXN2-FLAG-TEL-a or pCXN2-FLAG-TEL-f) into MEL cells (Fig. 6A). After adding HMBA to the culture of MEL clones, the proportion of MEL cells undergoing

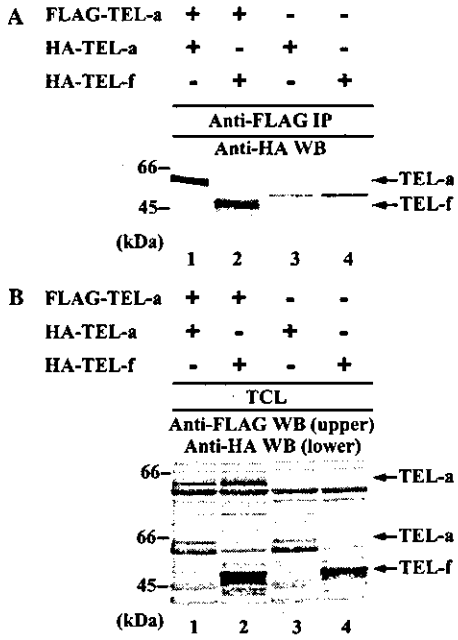

Fig. 4. TEL-f associates with TEL-a in vivo. (A) Cos7 cells were transfected with HA-tagged TEL-a or TEL-f expression plasmid alone (lane 3 or 4) or along with FLAG-tagged TEL-a expression plasmid (lane 1 or 2) using DEAE-dextran method. Immunoprecipitants with anti-FLAG antibody of the lysates were eluted with FLAG oligopeptide and analyzed by Western analysis with anti-HA antibody. Arrows indicate co-immunoprecipitated HA-tagged TEL-a or TEL-f proteins. (B) The same lysates were subjected to Western analysis with anti-FLAG or anti-HA antibody to confirm the expression of transfected cDNAs. Arrows indicate overexpressed FLAG-tagged TEL-a proteins, or HA-tagged TEL-a or TEL-f proteins. TCL, total cell lysates.

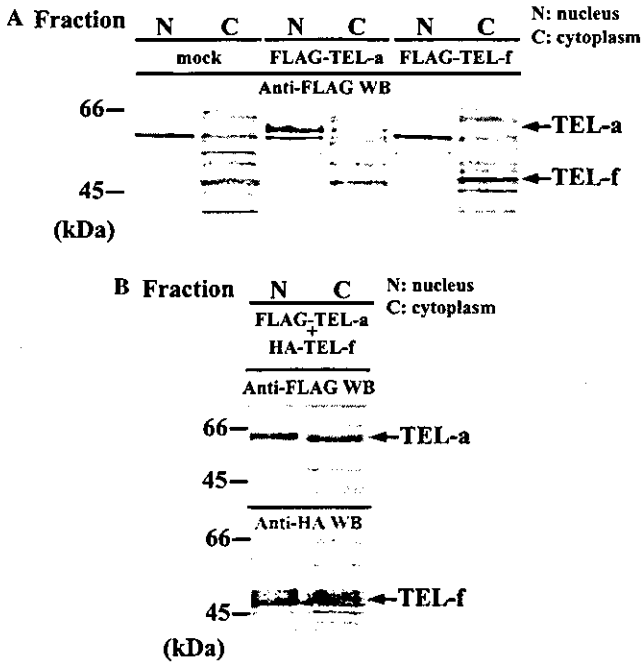

Fig. 5. Subcellular localization of TEL-a and TEL-f in MEL cells. (A) Lysates from MEL clones stably expressing either FLAG-tagged TEL-a or TEL-f were separated into the nuclear and cytoplasmic fractions and equal proportions of these fractions were analyzed by Western analysis with anti-FLAG antibody. Arrows indicate TEL-a in the nucleus and TEL-f in the cytoplasm. (B) A lysate from a MEL clone stably expressing both FLAG-tagged TEL-a and HA-tagged TEL-f was prepared as in A and analyzed by Western analysis with anti-FLAG and anti-HA antibodies. Arrows indicate TEL-a and TEL-f that were distributed almost equally into nucleus and cytoplasm when TEL-a and TEL-f were co-expressed.

erythroid differentiation was calculated by counting benzidine-positive cells. As reported before, mock clones (M-106 and M-107) became benzidine positive on day 3 and MEL clones stably expressing TEL-a revealed a higher proportion of benzidine positivity (Fig. 6B). Importantly, TEL-f expressing cells significantly reduced their abilities to become benzidine-positive compared to the mock clones. Thus, TEL-f blocks erythroid differentiation in this model system. It is conceivable that TEL-f exerts this effect by repressing the function of endogenous TEL-a proteins.

TEL-f stimulates anchorage-independent growth and overcomes growth-suppressive effects of TEL-a in NIH3T3 cells

It has also been demonstrated that expression of TEL-a in Ras-transformed NIH3T3 cells inhibits cell growth in liquid and soft agar cultures [5]. To test whether TEL-f modulates the growth of NIH3T3 cells, we first infected these cells with mock or recombinant retroviruses expressing FLAG-tagged TEL-a (pSRαMSVtk*neo*-FLAG-TEL). After isolating clones (M-2 or Ta-5) by limiting dilution, expression of TEL-a was confirmed by Western analysis (Fig. 7A). We then transfected HA-tagged TEL-f expression plasmid (pCAGIPuro-HA-TEL-f) into M-2 or Ta-5 and isolated clones (M-2-Tf-1 or Ta-5-Tf-1). Expression of TEL-f was also confirmed by Western analysis. As shown in Fig. 7B, expression of TEL-f stimulated colony formation in soft agar culture, while that of TEL-a inhibited it. Notably, concomitant expression of TEL-a and TEL-f prevented the abilities of TEL-a to repress anchorage-independent growth. All these data suggest that TEL-f not only shows the transforming activities but also interferes with the tumor suppressive function of TEL-a in the murine fibroblasts.

ΔETS-TEL isoforms are frequently detected in MDS-derived leukemia

Because TEL functions as a stimulator for erythroid differentiation and a tumor suppressor, it may be possible that dominant-negative TEL isoforms contribute to ineffective erythropoiesis or leukemic transformation in MDS. Thus, we studied expression

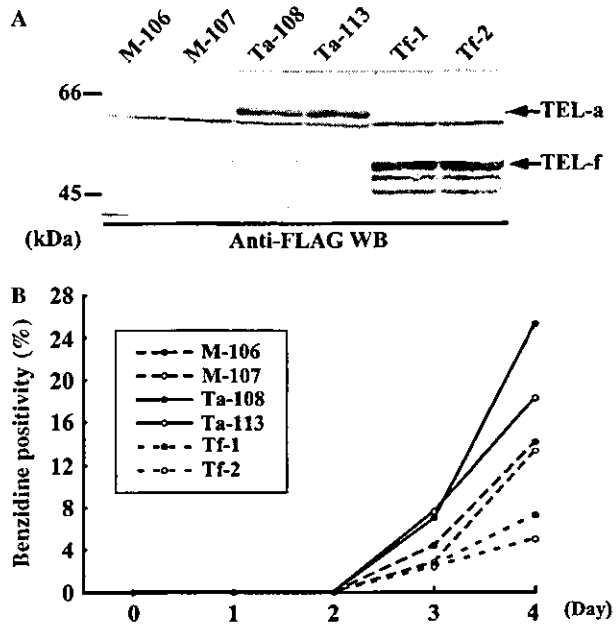

Fig. 6. TEL-f inhibits erythroid differentiation in MEL cells. (A) Expression of TEL-a or TEL-f in each MEL clone. These clones were obtained as described in Materials and methods. Arrows indicate overexpressed TEL-a and TEL-f proteins. (B) Time courses of erythroid differentiation after HMBA treatment in controls (M-106 and M107), or TEL-a (Ta-108 and Ta-113) or TEL-f (Tf-1 and Tf-2) expressing cells. Cell commitment to terminal differentiation was determined by benzidine staining and percent of benzidine-positive cells was calculated at different time points.

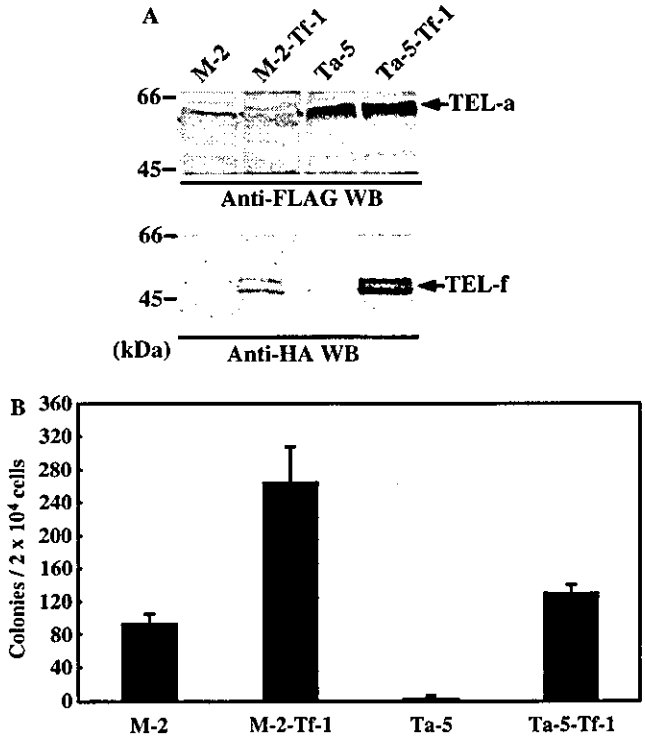

Fig. 7. TEL-f relieves TEL-a-mediated growth suppression in NIH3T3 cells. (A) Expression of TEL-a and/or TEL-f in each NIH3T3 clone. These clones were obtained as described in Materials and methods. Arrows indicate overexpressed TEL-a and TEL-f proteins. (B) Transformation assays were performed as described in Materials and methods. Bars show means and standard deviations of colony counts in four independent experiments that were normalized to ones with 2×10^4 NIH3T3 cells.

of ΔETS-TEL isoforms in bone marrow cells derived from MDS patients by RT-PCR analysis with a primer set that amplifies a sequence encoding ETS domain (Fig. 8B). A full-length ETS domain-encoding sequence of 522 bp was constantly amplified in all the samples examined, including 14 normal controls, 17 MDS samples, and three leukemia cell lines (U937, K562, and HL60) (Fig. 8A). In addition to this, a smaller faint fragment of 278 bp was observed in almost half of normal controls and MDS cases with subtypes of RA, RAEB, and CMMoL. Importantly, this smaller fragment of 278 bp was constantly amplified in all the cases with MDS-derived leukemia except one (case 7 with MDS-derived leukemia) and three leukemia cell lines. Sequencing analysis revealed that this smaller fragment has a sequence lacking both exons 6 and 7 (Fig. 8B). The faint band of 421 bp just below the full-length band in case 1 with MDS-derived leukemia turned out to be a fragment lacking only exon 7. Moreover, the smallest product of 203 bp observed in case 7 with MDS-derived leukemia deleted a 3′ part of exon 5 as well as both entire exons 6 and 7. Unfortunately, we did not have sufficient samples of these patients to determine whether there are present mutations within exons or splicing junctions that cause exon skipping.

Discussion

We demonstrate in this study that the *TEL* gene is alternatively spliced in normal hematopoietic cells, possibly leading to generation of distinct protein isoforms. Depending on their structures, some may be loss-of-function isoforms and the other may be dominant-negative ones. TEL-f, one of these isoforms that lacks the DNA-binding ETS domain, does not bind to DNA and antagonizes wild-type-TEL (TEL-a)-mediated trans-repressive effects on EBS. Possibly losing NLS in ETS domain, TEL-f resides in the cytoplasm but not in the nucleus. Notably, both TEL-a and TEL-f are almost evenly distributed into cytoplasm and nucleus when they are co-expressed. Since TEL-f heterodimerizes with TEL-a, it may dominantly interfere with TEL-a's transcriptional natures partly through exporting TEL-a from nucleus to cytoplasm. Moreover, even in the nucleus, heterodimerizing with TEL-f that does not bind to DNA may reduce TEL-a's transcriptional activities. TEL-f dominantly represses TEL-induced erythroid differentiation in MEL cells and growth suppression in NIH3T3 cells. These data collectively suggest that changes in expression pattern of TEL isoforms

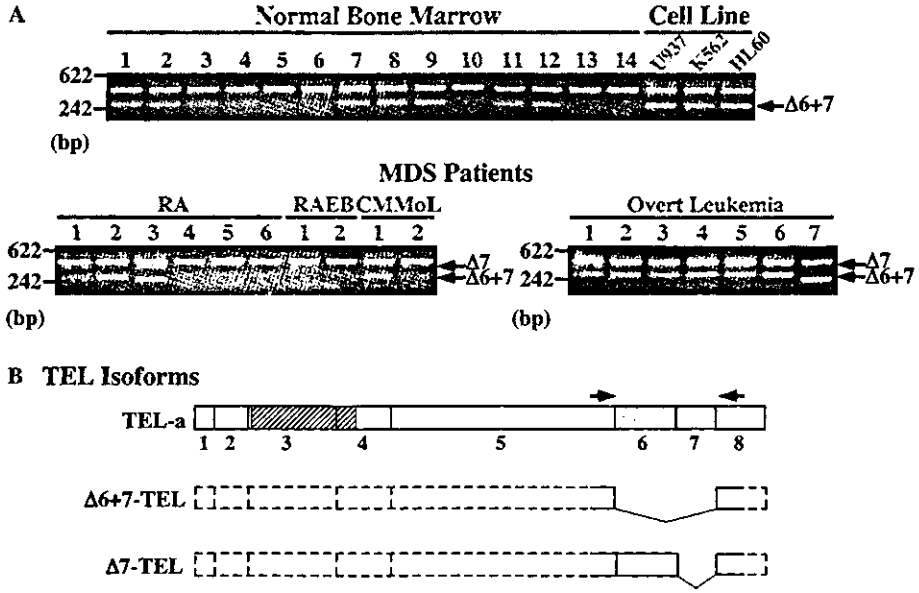

Fig. 8. RT-PCR analysis of TEL isoforms for ETS domain in MDS samples. (A) Expression of TEL isoforms for ETS domain in normal, MDS or MDS-derived leukemia bone marrow cells as well as three leukemia cell lines (U937, K562, and HL60). In the upper panel, results of PCR analysis on normal bone marrow and three leukemia cell lines using a primer set specific for ETS domain splice variants are shown. The main splice variant (Δ6 + 7-TEL) is indicated by an arrow on the right side. In the lower panel, results of PCR analysis on bone marrow from MDS with various subtypes and MDS-derived leukemia are shown. Two splice variants (Δ6 + 7-TEL and Δ7-TEL) are indicated by arrows on the right side. (B) Positions of the primer set used to amplify ETS domain are shown by arrows above the schematic structure of TEL-a. Structures of two possible isoforms (Δ6 + 7-TEL and Δ7-TEL) observed in the PCR analysis are presented with dotted lines below.

could cause its functional inactivation in both molecular and biological manners.

Alternative splicing is an important cellular mechanism that leads to temporal and tissue specific expression of unique mRNA products and is observed in up to 35–60% of human genes [16]. Also in a number of genes that encode transcriptional regulators, several splicing variants have been identified. TEL2 that belongs to the TEL subclass in the ETS family transcription factors also has several isoforms [10]. TEL2 isoforms either contain or lack N-terminal HLH domain and also vary at the C-terminus. By using RT-PCR method, we here first isolated five different alternatively spliced transcripts of TEL in hematopoietic cells that conceivably give different protein isoforms. Splicing patterns of TEL appear to be more complex than those of TEL2. TEL isoforms either possess or lose N-terminal HLH domain, internal domain between HLH and ETS domains or C-terminal ETS domain, while all TEL2 isoforms described contain both internal and ETS domains. Thus, the presence of isoforms lacking ETS domain is characteristic of TEL. However, we detected intrinsic expression of only TEL-a but not TEL-f in three leukemia cell lines (U937, K562, and HL60) by using immunoprecipitation followed by Western analysis (data not shown). In any case, it is plausible that a diversity of all these isoforms is generated through exon skipping mechanisms, regulation of which is not fully understood.

Frequently observed are functional differences in molecular and biological ways among isoforms in a variety of proteins. TEL2 isoforms are also endowed with different molecular properties depending on combination of their used exons. Among TEL2 isoforms, only the full-length TEL2 isoform TEL2-b has both DNA-binding and trans-repressional properties and the N-terminally truncated isoforms TEL2-c and TEL2-a show only DNA-binding affinity without transcriptional effects. Similarly, each TEL isoform seems to differ in molecular functions. We recently reported that a TEL deletion mutant lacking HLH domain possesses DNA-binding activities but loses trans-repressional abilities possibly through inabilities to bind to mSin3A. Because this mutant has almost the same structure as a TEL isoform TEL-e that results from the deletion of both exons 3 and 4, TEL-e conceivably loses trans-repressional abilities. We also have shown in this study that TEL-f resulting from the deletion of both exons 6 and 7 and lacking the entire of ETS domain loses both DNA-binding and trans-repression. Interestingly, Fenrick et al. [5] have described the similar loss of trans-repressional abilities in an artificial ΔETS deletion mutant lacking a part of ETS domain (a.a. 373–385). Moreover, these alternatively spliced isoforms without either HLH or ETS domain dominantly interfere with TEL-a-induced transcriptional repression. Therefore, relative expression levels of each splicing variant may determine TEL's overall transcriptional activities. There