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Oligomerization of the ABL Tyrosine Kinase by the Ets Protein TEL in Human Leukemia

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TEL is a member of the Ets family of transcription factors which are frequently rearranged in human leukemia. The mechanism of TEL-mediated transformation, however, is unknown. We report the cloning and characterization of a chromosomal translocation associated with acute myeloid leukemia which fuses TEL to the ABL tyrosine kinase. The TEL-ABL fusion confers growth factor-independent growth to the murine hematopoietic cell line Ba/F3 and transforms Rat-1 fibroblasts and primary murine bone marrow cells. TEL-ABL is constitutively tyrosine phosphorylated and localizes to the cytoskeleton. A TEL-ABL mutant containing an ABL kinase-inactivating mutation is not constitutively phosphorylated and is nontransforming but retains cytoskeletal localization. However, constitutive phosphorylation, cytoskeletal localization, and transformation are all dependent upon a highly conserved region of TEL termed the helix-loop-helix (HLH) domain. TEL-ABL formed HLH-dependent homo-oligomers in vitro, a process critical for tyrosine kinase activation. These experiments suggest that oligomerization of TEL-ABL mediated by the TEL HLH domain is required for tyrosine kinase activation, cytoskeletal localization, and transformation. These data also suggest that oligomerization of Ets proteins through the highly conserved HLH domain may represent a previously unrecognized phenomenon.

The aberrant expression of transcription factors is emerging as a central paradigm in the pathogenesis of human acute leukemias. The genes encoding transcription factors are frequently the targets of chromosomal translocations, resulting in the disruption of either the coding or regulatory sequences of the genes. For example, acute lymphoid leukemias are frequently associated with overexpression of transcription factors such as MYC or SCL/TAL-1 as a result of their juxtaposition with either immunoglobulin or T-cell receptor regulatory elements, respectively (6, 9).

Leukemias of the myeloid lineage are more frequently associated with the formation of chimeric transcription factors which result from in-frame fusion of the two genes disrupted by a chromosomal translocation. This mechanism of transformation is exemplified by rearrangement of either the α or β subunit of core binding factor (CBF). The t(8;21) chromosomal translocation results in fusion of AML1, the DNA-binding α subunit of CBF, to ETO, a zinc finger protein of unknown function (12, 30, 33). AML1-ETO transformation is thought to be mediated by transcriptional repression of genes normally activated by CBF (29). Similarly, rearrangement of the β subunit of CBF in inv(16)-associated leukemia is thought to interfere with normal CBF function (18, 23). A unifying theme in all of these examples is the expression of a transcription factor with aberrant transactivation properties.

We recently reported an unusual transcription factor chimera: the fusion of a TEL, a new member of the Ets family of transcription factors, to the platelet-derived growth factor β receptor (PDGFβR), a receptor tyrosine kinase (17). The TEL-PDGFβR fusion is invariably associated with chronic myelomonocytic leukemia with t(5;12) chromosomal translocation, but its mechanism of transformation has not been demonstrated. A unique feature of the TEL-PDGFβR fusion is that TEL does not contribute a DNA-binding domain to the fusion protein, suggesting that unlike other transcription factor chimeras, TEL-PDGFβR might transform through a mechanism independent of DNA binding or transcriptional regulation. Other Ets proteins have been shown to be rearranged in human malignancies, including ERG in myeloid leukemia (46) and FLI-1, ERG, and ETV1 in Ewing's sarcoma (10, 20, 48). Similarly, overexpression of Fli-1 and Spi-1 has been implicated in the pathogenesis of murine erythroleukemias (3, 31). In all of these examples, however, the Ets DNA-binding domain is aberrantly expressed.

To explore the mechanism by which TEL may be contributing to the pathogenesis of human leukemia, we have studied additional chromosomal translocations occurring near the *TEL* gene locus on 12p13. We report here the positional cloning and functional characterization of a t(9;12;14) translocation associated with acute myeloid leukemia which results in fusion of TEL to another tyrosine kinase, ABL. Like the TEL-PDGFβR fusion, the TEL DNA-binding domain is not incorporated into the fusion protein. We show that TEL-ABL is a tyrosine-phosphorylated cytoskeletal oncoprotein whose activity is dependent upon oligomerization mediated by an amino-terminal TEL domain. This region, highly conserved among a subset of Ets proteins, has been termed the helix-loop-helix (HLH) (17, 45) or pointed domain (21), but its function is unknown. The TEL-ABL fusion represents a new mechanism of ABL activation in human leukemia and suggests that a principal role of the TEL HLH domain may be to serve as an

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ABL oligomerization motif. The results of these experiments further suggest that the HLH domain may play a previously unrecognized role in the protein-protein interactions of Ets factors.

MATERIALS AND METHODS

Pulsed-field gel electrophoresis (PFGE) and Southern hybridization. Patient bone marrow and healthy volunteer peripheral blood samples were obtained with informed consent. The patient marrow sample was obtained from an 81-year-old man with acute undifferentiated myeloid leukemia with the following complex karyotype: 45, add(X)(p11), Y, −3, der(3) t(3;15)(q12;q15), t(4;7)(q21;q22), −5, t(9;12;14)(q34;p13;q22), del(12)(p11p13), der(15) del(15)(q11q15), t(3;15)(q12;q15), der(18) t(3;18)(q11;q1?2), +mar, as described previously (22). Erythrocytes were removed by dextran sedimentation followed by hypotonic lysis (7). Agarose plugs containing leukocytes were prepared and digested with restriction endonucleases as previously described (4). Electrophoresis was performed with a CHEF II pulsed-field apparatus (Bio-Rad), and the DNA was transferred to Hybond-N nylon membranes (Amersham). The *TEL* cDNA probe is a 1.4-kb *Eco*RI fragment containing the entire *TEL* coding sequence. The probe was labeled with ^{32}P by random priming, and Southern hybridization was performed as previously described (44).

Northern (RNA) hybridization and RNase protection. RNA was prepared either from patient bone marrow or control peripheral blood mononuclear cells or from HL-60 cells by using guanidinium acid-phenol-chloroform (RNAzol; Tel-Test). For Northern hybridization, 15 μg of total RNA was electrophoresed through a 1% agarose-formaldehyde gel and transferred to a Hybond-N nylon membrane (Amersham). After UV cross-linking, the membrane was hybridized to a ^{32}P-labeled 1.4-kb *TEL* cDNA probe as previously described (44). RNase protection was performed as previously described (2, 17). Briefly, 5 to 10 μg of total RNA was hybridized with approximately 50,000 cpm of probe overnight at 57°C. Digestion conditions were 10 mg of RNase A per ml and 10 U of RNase T_1 (Calbiochem) per ml for 1 h at 18°C. Protected fragments were purified by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation and then separated on 8 M urea sequencing gels. The numbering of nucleotides in *TEL* is based on the numbering of the sequence deposited in GenBank under accession number U11732 (17).

Anchored PCR and RT-PCR. The anchored PCR method utilized was adapted from the method of Frohman (13). Three micrograms of total RNA from patient bone marrow was reverse transcribed with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) and oligonucleotide primer Q_T (5′-TGA GCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTT-3′). An aliquot of the cDNA was used as a template for 30 cycles of PCR (1 cycle consists of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C) with *TEL* primer 541 (*TEL* nucleotides [nt] 541 to 560; 5′-CCTCCCACCATTGAACTGTT-3′) and primer Q_0 (5′-CCAGTGAGCAGAGTGACG-3′). A second round of amplification was then performed with nested *TEL* primer 701 (*TEL* nt 701 to 720; 5′-AGAACAACCACCAGGAGTCC-3′) and primer Q_1 (5′-GAGGACTCGA GCTCAAGC-3′). The resulting PCR product was gel purified, cloned into pBluescript KS+ and sequenced by using a Sequenase 2.0 kit (United States Biochemical). For *TEL-ABL* reverse transcription-PCR (RT-PCR), cDNA prepared from patient bone marrow RNA or control peripheral blood cells was amplified with *TEL* primer 541 and *ABL* primer G (GenBank accession number M14752, nt 626 to 649; 5′-GTGATTATAGCCTAAGACCCGGAG-3′) in 35 cycles of PCR. The PCR product was cloned and sequenced as described above.

Cell transformation studies. The retroviral expression vector pSRα/MSV/TK/neo was used in all experiments and has been previously described (32). A full-length *TEL-ABL* cDNA clone was reconstructed by splicing together a wild-type *TEL* clone, a wild-type c-*ABL* clone, and an RT-PCR-generated fragment spanning the *TEL-ABL* junction. The *TEL-ABL* ΔEX mutant was created by digestion with *Eco*47III and *Xmn*I followed by recircularization of the plasmid. The *TEL-ABL* ΔEB mutant was created by digestion with *Eco*47III and *Bst*EII, Klenow polymerase fill-in, and recircularization of the plasmid. The *BCR-ABL* (p185) and c-*ABL* constructs have been previously described (32). Retrovirus was produced by transfecting the various constructs into the retrovirus-producing cell line 293T or Bosc 23, as previously described (36). Retrovirus-containing supernatant was harvested 48 h after transfection. Viral titers were assessed by infecting Rat-1 cells with each retrovirus and harvesting the cells 48 h later, at which time Western blot (immunoblot) analysis was performed with an anti-ABL antibody. The amount of ABL fusion protein detected was compared by eye to the amount of endogenous c-ABL as a positive control, and the viral stocks were diluted such that approximately equivalent levels of ABL fusion protein were detected per cell. We have found that this method of determining viral titer is superior to methods based upon neomycin resistance.

For the fibroblast transformation studies, 5×10^4 Rat-1 cells were plated in soft agar 48 h after retroviral infection, and the formation of colonies was assessed after 3 weeks in culture, as previously described (24). For the Ba/F3 assay, Ba/F3 cells were infected with retrovirus and maintained in interleukin 3 (IL-3)-containing medium (RPMI 1640 supplemented with 10% fetal bovine serum and 2 U of IL-3 [Genzyme] per ml) for 48 h, at which point the cells were washed twice in RPMI 1640 and then maintained in IL-3-free medium in 96-well plates at a concentration of 10^4 cells per well. The ability to grow in the absence of IL-3 was assessed by light microscopy after 2 weeks of culture. The murine bone marrow transformation studies were performed as previously described (25). Briefly, fresh bone marrow from the femurs and tibias of BALB/c mice was infected with retroviruses for 3 h, and 5×10^6 cells were plated in 6-cm-diameter dishes in RPMI 1640 supplemented with 5% fetal bovine serum. Outgrowth of nonadherent hematopoietic cells was assessed after 3 weeks of culture and scored positive if the cell count exceeded 10^6 cells per ml.

Immunoblotting. Rat-1 cells were lysed in 2% sodium dodecyl sulfate (SDS)-containing buffer 48 h after infection, as previously described (1, 14). Total-cell lysates were subjected to electrophoresis through SDS-containing polyacrylamide gels. The proteins were transferred to nitrocellulose membranes with a semidry transfer apparatus (Bio-Rad). The membranes were then probed with either a monoclonal anti-ABL antibody 21-24 (Oncogene Sciences) or with the antiphosphotyrosine antibody 4G10 (UBI). Bands were visualized by using an ECL chemiluminescence kit (Amersham).

Immunofluorescence. Rat-1 cells were subjected to indirect immunofluorescence antibody staining 48 h following retroviral infection with ABL constructs. Cells grown on glass coverslips were fixed in 2% paraformaldehyde and permeabilized with 0.1% Triton X-100. After the cells were blocked in 5% normal goat serum and 0.2% bovine serum albumin for 1 h, mouse anti-ABL monoclonal antibody 21-24 (Oncogene Sciences) was added for 1 h. After the cells were washed, secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Vector Laboratories, Burlingame, Calif.) was added for 1 h. After the cells were washed in phosphate-buffered saline–0.05% Tween 20, the coverslips were mounted on glass slides. For actin colocalization experiments, cells were incubated for 20 min with 1 μg of rhodamine-conjugated phalloidin (Sigma) per ml following permeabilization. The cells were visualized with an Olympus BH-2 fluorescence microscope and photographed with Kodak Gold II ASA 400 print film.

In vitro translation. *TEL-ABL* and *TEL-ABL* ΔEX were subcloned into pBluescript KS+ (Stratagene), and the full-length *TEL* cDNA was cloned into pCDNA3 (Invitrogen). TEL-ABL ΔC and TEL-ABL ΔEXΔC constructs were created by deleting sequence downstream of a *Bgl*I site, thereby deleting the C-terminal 670 ABL amino acids. In vitro transcription-translation was performed by using a TNT rabbit reticulocyte lysate kit (Promega) according to the manufacturer's specifications. Proteins were labeled by [^{35}S]methionine incorporation. Ten microliters of the programmed lysate diluted to 300 μl with lysis buffer was immunoprecipitated with anti-ABL monoclonal antibody 21-24. Immunoprecipitates were resolved on SDS-polyacrylamide gels, and the signal was visualized by fluorography with Amplify (Amersham) according to the manufacturer's instructions.

RESULTS

PFGE demonstrates rearrangement of the *TEL* gene. Bone marrow cells of an 81-year-old man with acute undifferentiated myeloid leukemia and a complex karyotype including t(9;12;14)(q34;p13;q22) were analyzed for rearrangement of the *TEL* gene. DNA prepared from patient bone marrow was examined by PFGE Southern blotting with a *TEL* cDNA probe. In patient DNA, this probe detects two novel bands, whereas the 150-kb wild-type band is missing (Fig. 1A). This result suggested that the cDNA probe spanned the translocation breakpoint on one copy of chromosome 12 (resulting in two novel bands) and that the *TEL* gene was deleted from the other copy of chromosome 12. This finding supports our previous fluorescence in situ hybridization observation that the 1,390-kb yeast artificial chromosome 964c10 containing the *TEL* gene spans this patient's t(9;12;14) translocation breakpoint and is deleted from the other copy of chromosome 12 (patient 4 in reference 22).

A chimeric *TEL* transcript is expressed. The pattern of *TEL* expression was assessed by subjecting patient bone marrow RNA to Northern blot analysis by using a *TEL* cDNA probe. Transcripts 7 and 4 kb long were seen in control HL-60 RNA (Fig. 1B). In contrast, a single >7-kb transcript was expressed in patient cells; none of the wild-type *TEL* transcripts was detected. This supports the hypothesis that a chimeric *TEL* transcript is formed as a result of the translocation, accompanied by deletion of the other *TEL* allele.

A series of riboprobes scanning the *TEL* coding sequence was used to localize the *TEL* breakpoint by RNase protection. As shown in Fig. 1C, probe BX (*TEL* nt 194 to 574), which

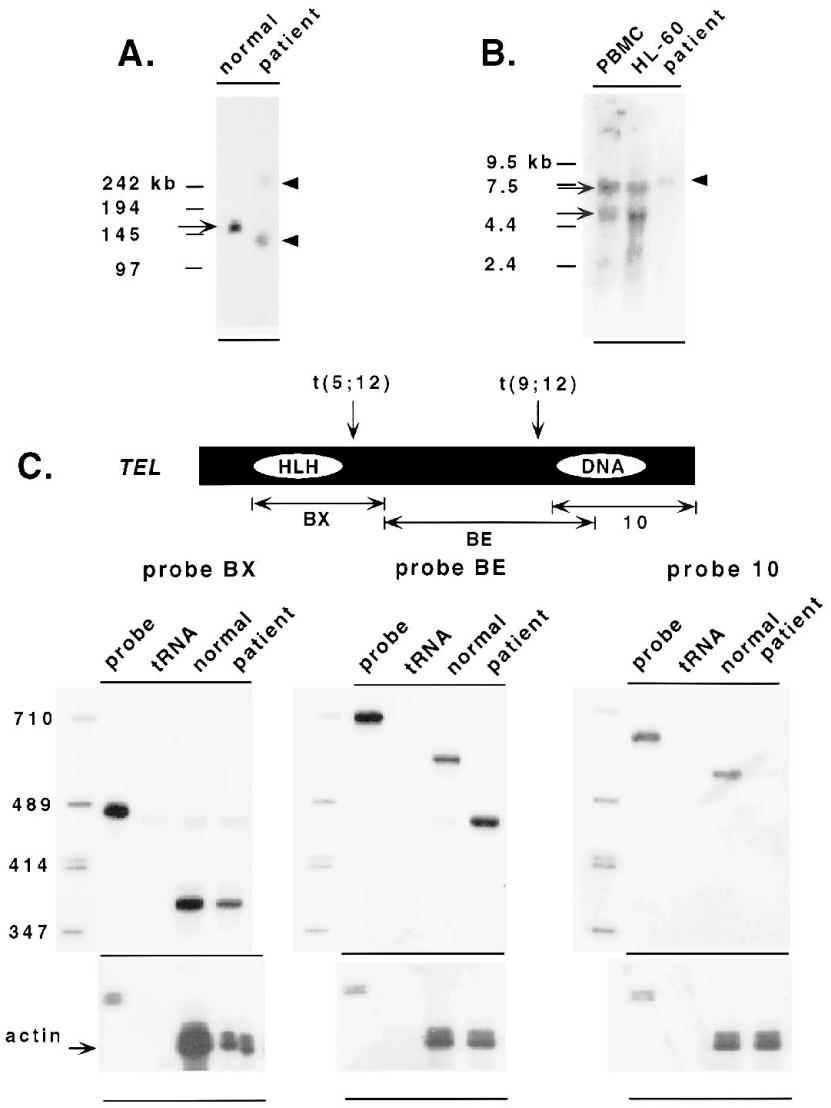

FIG. 1. Mapping the t(9;12) translocation breakpoint. (A) PFGE Southern blotting. Control (normal) or patient bone marrow DNA embedded in agarose plugs was digested with *Sfi*I and probed with a full-length *TEL* cDNA probe. This probe identifies a 150-kb fragment in control cells (arrow). In patient cells, however, this band is missing, and two novel bands are seen (arrowheads). (B) Northern blot analysis. RNA derived from control peripheral blood mononuclear cells (PBMC), the myeloid leukemia cell line HL-60, or patient bone marrow was probed with a full-length *TEL* cDNA probe. Whereas 7- and 4.5-kb transcripts are seen in PBMC and HL-60 (arrows), only a single >7-kb transcript is seen in patient RNA. (C) RNase protection. Riboprobes spanning the *TEL* coding sequence were used to localize the translocation breakpoint within the *TEL* message. The locations of the probes are shown at the top of the figure, illustrating the locations of the HLH domain and the DNA-binding domain. A γ-actin probe was hybridized simultaneously as a measure of RNA integrity. The probe lanes contain undigested probe, and in the tRNA lanes tRNA was used to assess nonspecific protection of the probes. Probe BX is fully protected (380 nt) by both control (normal) and patient RNA, indicating that the t(9;12) breakpoint does not occur within this probe. Probe BE, however, gives a fully protected 568-nt band in control RNA but is only partially protected (460 nt) by patient RNA. A small amount of the 460-nt fragment is also seen in control RNA, corresponding to a minor splice variant occurring at *TEL* nt 1033. Probe 10 is fully protected by control RNA and is not protected at all by patient RNA, indicating that *TEL* sequences 3′ of *TEL* nt 1033 are not expressed.

detects the t(5;12) breakpoint in chronic myelomonocytic leukemia, was fully protected by patient and control RNAs, indicating that a breakpoint did not occur within this region of *TEL*. Probe BE (*TEL* nt 574 to 1142), when tested in HL-60 cells, yields a predominant 568-nt fully protected fragment and a minor 460-nt fragment corresponding to a naturally occurring splice variant at *TEL* nt 1033 (1a). In contrast, patient RNA protected only the 460-nt fragment. These findings suggested that a translocation breakpoint occurred at *TEL* nt 1033.

Probe 10 (*TEL* nt 1037 to 1580) yields a fully protected 543-nt fragment in control RNA, whereas this probe is not protected at all by patient RNA. The internal γ-actin probe shows that equivalent amounts of RNA were present in each sample. Probe 10 was particularly informative, because it established the likely transcriptional orientation of the chimeric *TEL* message. The intense signals seen with 5′ probes BX and BE compared with the lack of a signal with 3′ probe 10 suggested that a chimeric *TEL* transcript was driven by the *TEL* promoter, with fusion occurring downstream of *TEL* nt 1033. Probe 10 also demonstrated the absence of significant levels of a reciprocal *TEL* fusion transcript which would fully protect the probe and confirmed the absence of expression of wild-type *TEL* as suspected from the PFGE and Northern blot analyses.

Anchored PCR identifies a *TEL-ABL* fusion. The results of Northern blot analysis and RNase protection suggested that

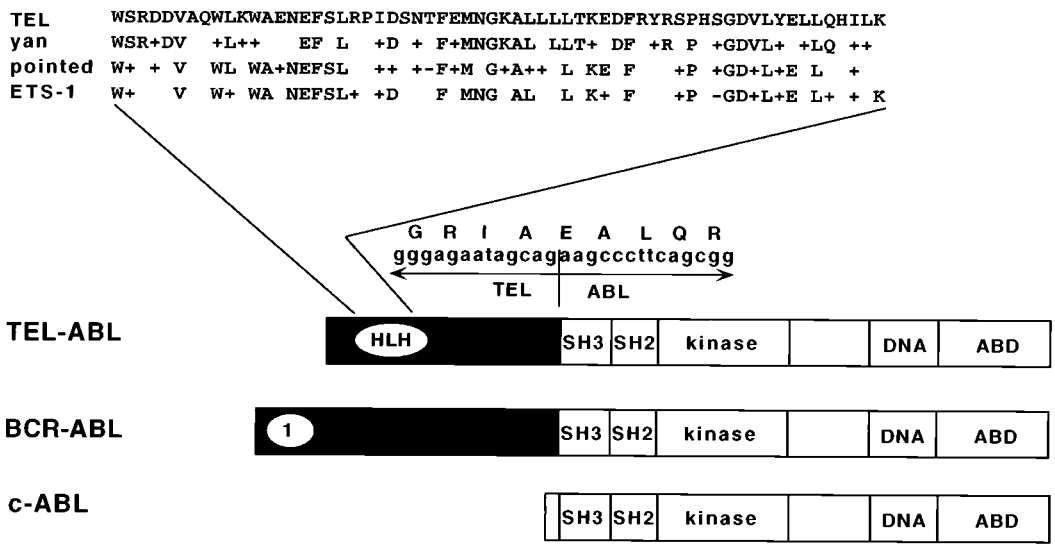

FIG. 2. Schematic diagram of the TEL-ABL chimera. The *TEL-ABL* chimera results from fusion of the amino portion of *TEL* to exon 2 of *ABL*. The identical *ABL* sequences are incorporated into the *TEL-ABL* chimera as in *BCR-ABL*. The TEL-ABL chimera fuses TEL amino acids 1 to 336 to ABL amino acids 27 to 1131 (numbering based on that of sequences deposited in GenBank under accession numbers U11732 and M14752). Several of the mapped ABL domains are shown, including the carboxy-terminal DNA-binding domain and actin-binding domain (ABD). BCR domain 1, located at the BCR amino terminus, encodes a coiled-coil oligomerization motif. The nucleotide and amino acid sequences surrounding the *TEL-ABL* breakpoint are shown. Shown at the top of the figure is the amino acid homology between the TEL HLH domain and other members of the Ets family, including *Drosophila* yan and pointed and human ETS-1. Plus signs indicate similarity; dashes are used to maximize alignment.

TEL was fused to another gene at *TEL* nt 1033. An anchored PCR approach using *TEL*-specific primers was therefore utilized to obtain sequence from the fusion partner. We and others have used this method to cross translocation breakpoints in cases where limited patient RNA was available (8, 16, 17). The resultant PCR product was cloned and sequenced. Sequence analysis showed that this sequence was identical to the wild-type *TEL* sequence until *TEL* nt 1033, where the sequences diverged further. A search of the GenBank database demonstrated that the divergent sequence was identical to intron 1b of the *ABL* gene which maps to 9q34, one of the cytogenetic breakpoints in the t(9;12;14) chromosomal translocation.

The oligo(dT)-containing primer used in anchored PCR is intended to anneal to the mRNA poly(A) tail. Promiscuous annealing to stretches of A-rich sequence, however, can lead to the PCR selection of short, aberrantly spliced transcripts (15a). Suspecting that the anchored PCR had selected for a rare splice variant or pre-mRNA of a *TEL-ABL* fusion, RNA-based PCR was performed with *TEL* sense primer 541 and *ABL* antisense primer G. A 697-bp PCR product was generated from patient RNA but not from control RNA (not shown). Sequence analysis of this fragment revealed wild-type *TEL* sequence until nt 1033, followed by in-frame fusion to exon 2 of *ABL*. RNase protection using the chimeric cDNA as a probe showed that this was indeed the predominant *TEL-ABL* splice form expressed in the leukemic cells (not shown). *BCR-ABL* chimeras similarly result from fusion of *BCR* sequences to exon 2 of *ABL*, as shown in Fig. 2. The result of the TEL-ABL chimera is fusion of the amino portion of TEL, including the HLH domain, to ABL. Notably, the TEL DNA-binding domain is not incorporated into the TEL-ABL fusion, and the reciprocal ABL-TEL fusion transcript which would contain the TEL DNA-binding domain is not detectable by Northern blot analysis or RNase protection (not shown).

TEL-ABL transforms fibroblasts and hematopoietic cells. In order to test the transforming potential of the TEL-ABL chimera, the full-length *TEL-ABL* fusion cDNA was reconstructed. The portion of the fusion surrounding the breakpoint was generated by RT-PCR from patient material and was therefore sequenced in its entirety to confirm wild-type sequence. Additional mutations which conceivably could have arisen in the patient within *TEL* or *ABL* sequences distant from the breakpoint cannot be excluded. The full-length cDNA was cloned into the retroviral expression vector pSRα/MSV/TK-neo (32). *BCR-ABL* (p185) cloned into the same vector served as a positive control for transformation, and c-*ABL* served as a negative control. The ability of TEL-ABL to induce anchorage-independent growth of Rat-1 fibroblasts was then assessed. TEL-ABL and BCR-ABL each induced colony formation, whereas c-ABL was nontransforming in this assay (Table 1).

To investigate the ability of TEL-ABL to transform hematopoietic cells, retrovirus harboring *TEL-ABL* or *BCR-ABL* or the vector alone was used to infect the IL-3-dependent hematopoietic cell line Ba/F3. Forty-eight hours following infection, the cells were deprived of IL-3 and plated in 96-well trays at a concentration of 10^4 cells per well. After a period of 14 days,

TABLE 1. Transforming potential of TEL-ABL

Construct	Transforming potential in cells[a]	
	Rat-1	Ba/F3
Control (mock infected)	0	0/96
Vector only	ND	0/96
c-*ABL*	1	ND
TEL-ABL	54	96/96
BCR-ABL	63	96/96

[a] The average number of colonies from two plates of Rat-1 cells grown in soft agar and number of IL-3-independent cultures generated from Ba/F3 cells are shown. ND, not done.

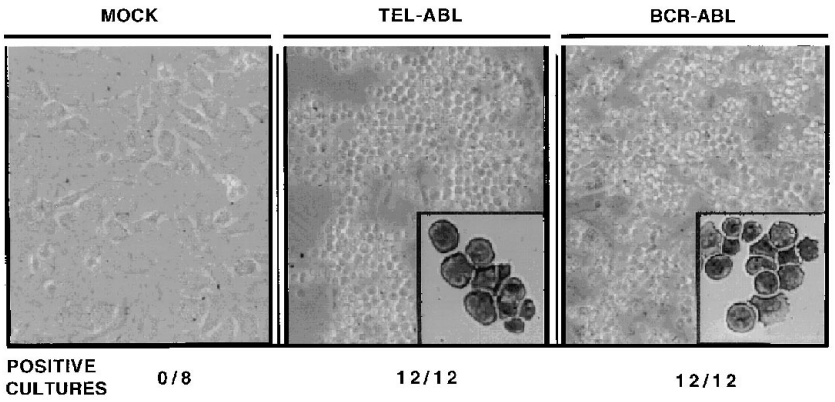

FIG. 3. Transformation of mouse bone marrow by TEL-ABL. Normal BALB/c bone marrow was either mock infected or infected with retroviruses harboring cDNA encoding TEL-ABL or BCR-ABL. After 3 weeks of culture, only adherent stromal cells were seen in mock-infected cultures, whereas nonadherent hematopoietic outgrowths were seen in *TEL-ABL* and *BCR-ABL* virus-infected cultures. The insets show higher magnification of the transformed hematopoietic cells.

cultures growing in the absence of IL-3 were scored. Ba/F3 cells infected with *TEL-ABL* or *BCR-ABL* retrovirus grew in the absence of IL-3, whereas cells infected with virus containing the control construct did not (Table 1). This result indicates that TEL-ABL is capable of replacing the mitogenic or antiapoptotic signal normally supplied by the cytokine IL-3.

The analysis of TEL-ABL transformation was next extended to control bone marrow progenitors in order to determine whether TEL-ABL was capable of transforming nonestablished cell lines. The ability of TEL-ABL to transform primary murine bone marrow cells was assessed by infecting freshly harvested normal BALB/c bone marrow cells with the *TEL-ABL* retrovirus. *BCR-ABL* cloned into the same expression vector served as a positive control for transformation. Cultures of 2×10^7 cells were assessed for outgrowth of nonadherent hematopoietic cells after 3 weeks of culture in medium containing 5% fetal bovine serum without supplemental hematopoietic growth factors. Hematopoietic cell outgrowths were seen in 12 of 12 cultures infected with *TEL-ABL* retrovirus, 12 of 12 cultures infected with *BCR-ABL* retrovirus, and 0 of 8 mock-infected cultures, in which only bone marrow stromal elements were seen (Fig. 3). Fluorescence-activated cell sorting (FACS) analysis of both TEL-ABL- and BCR-ABL-transformed hematopoietic cells demonstrated expression of the lymphoid-specific marker B220 and absence of expression of the myeloid-specific marker Mac-1 (not shown). These findings are consistent with a lymphoid origin of the TEL-ABL- and BCR-ABL-transformed cells observed in this system. It should be noted, however, that this culture system has been optimized for lymphoid growth and is not intended for use in the determination of lineage specificity of transforming proteins.

TEL-ABL is constitutively phosphorylated. The experiments described above suggest that the biological activity of TEL-ABL is remarkably similar to that of BCR-ABL, despite the fusion partners for the ABL kinase being completely different. One well-established function of the BCR portion of the BCR-ABL chimera is the constitutive activation of the ABL kinase, an event critical for transformation (24, 37). To assess the tyrosine phosphorylation status of TEL-ABL, total-cell lysates were prepared from Rat-1 cells infected with *TEL-ABL* or c-*ABL* retrovirus or mock infected, and the lysates were analyzed by Western blotting. Blotting with an anti-ABL monoclonal antibody demonstrated the expression of an approximately 180-kDa protein in *TEL-ABL* retrovirus-infected cells (Fig. 4A). The endogenous 150-kDa c-ABL protein was not highly expressed in Rat-1 cells but was readily visible in cells overexpressing c-ABL. Blotting with an antiphosphotyrosine antibody revealed a predominant 180-kDa phosphoprotein in TEL-ABL-expressing cells, whereas c-ABL was not phosphorylated (Fig. 4B). A 62-kDa phosphoprotein was seen in *TEL-ABL* retrovirus-infected cells, as has been previously reported for *BCR-ABL* retrovirus (1, 11, 51, 52). These results suggest that the 180-kDa TEL-ABL protein is highly expressed, and like BCR-ABL, is constitutively phosphorylated. The data also suggest that the 62-kDa phosphoprotein may be a substrate for the TEL-ABL tyrosine kinase.

TEL-ABL is localized to the cytoskeleton. The subcellular localization of TEL-ABL was determined by indirect immuno-

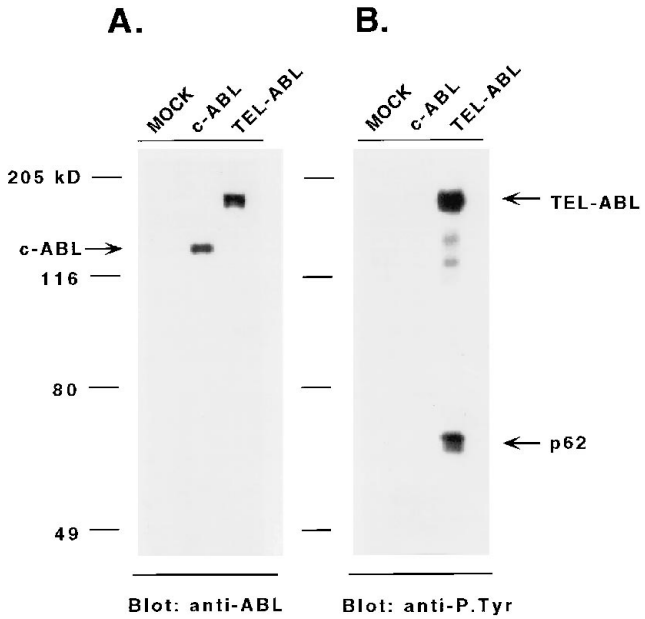

FIG. 4. TEL-ABL immunoblotting. (A) Total-cell lysates of Rat-1 fibroblasts either mock infected or infected with c-*ABL* or *TEL-ABL* retrovirus were analyzed by Western blotting with monoclonal anti-ABL antibody 21-24. Mock-infected cells express little of the 150-kDa c-ABL protein, but this band is readily visible in cells overexpressing c-ABL. The predicted 180-kDa band is seen in TEL-ABL-expressing cells. (B) The blot shown in panel A was stripped and reprobed with an antiphosphotyrosine antibody (anti-P.Tyr). c-ABL, while expressed in c-*ABL*-infected cells, was not tyrosine phosphorylated. TEL-ABL, however, was constitutively phosphorylated. In addition, a prominent 62-kDa phosphoprotein was seen in TEL-ABL-expressing cells.

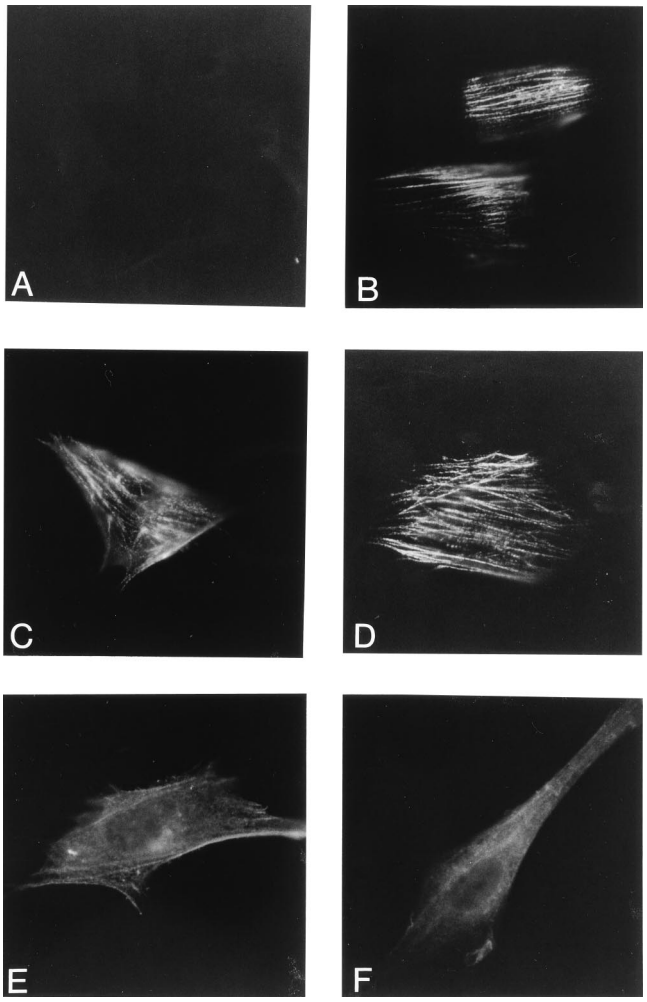

FIG. 5. Immunofluorescence localization of TEL-ABL. Rat-1 cells infected with various retroviral constructs were stained with anti-ABL antibody 21-24 and visualized with FITC-conjugated anti-mouse immunoglobulin G. (A) Vector only. The low levels of endogenous c-ABL are not detected by this method. (B) TEL-ABL. TEL-ABL is seen predominantly in linear cytoplasmic arrays resembling stress fibers. (C) BCR-ABL. A cytoskeletal association of BCR-ABL similar to that of TEL-ABL is seen. (D) TEL-ABL K293H. This kinase-inactive, nontransforming TEL-ABL mutant retains cytoskeletal association. (E) TEL-ABL ΔEX. Deletion of the TEL HLH domain causes TEL-ABL ΔEX to be expressed more diffusely in the cytoplasm. Some suggestion of cytoskeletal association remains. (F) TEL-ABL ΔEB. Like TEL-ABL ΔEX, this mutant lacking the TEL HLH domain is found more diffusely in the cytoplasm.

fluorescence staining of Rat-1 cells expressing TEL-ABL. It has been previously shown that fusion of BCR to ABL results in the relocalization of ABL from the nucleus to the cytoplasm. It was therefore important to determine what effect fusion of TEL to ABL would have on the predominantly nuclear localization of c-ABL. Fibroblasts infected with vector alone showed no appreciable staining (Fig. 5A), reflective of the fact that c-ABL is not highly expressed in Rat-1 cells. Cells expressing TEL-ABL, however, showed prominent staining in linear cytoplasmic arrays resembling stress fibers (Fig. 5B). Dual color counterstaining with rhodamine-conjugated phalloidin, which has a high affinity for actin filaments, demonstrated that TEL-ABL indeed colocalized with cytoskeletal actin filaments (not shown). BCR-ABL showed similar cytoskeletal localization (Fig. 5C), although this was less prominent than with TEL-ABL. A similar cytoskeletal pattern was seen in Ba/F3 cells expressing TEL-ABL (not shown).

TEL-ABL transformation is kinase dependent. Because there are several potential mechanisms by which a transcription factor-tyrosine kinase chimera might be transforming, we tested the dependence of TEL-ABL transformation on ABL tyrosine kinase activity. Substitution of arginine for lysine at ABL amino acid 219 (K219R; ABL numbering based on the numbering in the sequence deposited in GenBank under accession number M14752) ablates a critical ATP binding site within the SH1 domain and has been previously shown to inhibit ABL kinase activity (37). TEL-ABL harboring the K219R mutation (TEL-ABL K219R) could be highly expressed but, as expected, was not tyrosine phosphorylated (not shown). TEL-ABL K219R was then tested for its ability to confer IL-3 independence to Ba/F3 cells. Whereas TEL-ABL was transforming (96 of 96 cultures positive), TEL-ABL K219R was not (0 of 96 cultures positive). While TEL-ABL K219R was nontransforming, it retained its ability to associate with the cytoskeleton (Fig. 5D). These experiments demonstrate that TEL-ABL transformation is kinase dependent, but phosphorylation of TEL-ABL is not required for cytoskeletal association.

The TEL HLH domain is required for TEL-ABL transformation. We next addressed the dependence of TEL-ABL transformation on the TEL portion of the chimera. In particular, we tested whether the TEL HLH domain was critical for TEL-ABL transforming capability. Two TEL-ABL deletion mutants were constructed: TEL-ABL ΔEB, with TEL amino acids 39 to 182 deleted, and TEL-ABL ΔEX, with TEL amino acids 39 to 115, including the entire HLH domain, deleted. While TEL-ABL ΔEB and TEL-ABL ΔEX could be highly expressed in Rat-1 cells (Fig. 6A), only the full-length TEL-ABL protein was tyrosine phosphorylated (Fig. 6B). Immunofluorescence staining of Rat-1 cells expressing the TEL-ABL constructs demonstrated that whereas the full-length TEL-ABL protein was associated with the cytoskeleton (Fig. 5B), TEL-ABL ΔEX (Fig. 5E) and TEL-ABL ΔEB (Fig. 5F) were localized more diffusely in the cytoplasm. Finally, the transforming potentials of the TEL-ABL deletion mutants were tested in Rat-1 fibroblasts, Ba/F3 cells, and primary murine bone marrow cells. As shown in Table 2, neither *TEL-ABL ΔEB* nor *TEL-ABL ΔEX* was transforming in any of the assays. These data show that the TEL HLH domain is essential for TEL-ABL phosphorylation, for cytoskeletal association, and for transformation.

TEL encodes an oligomerization domain. We speculated that in the case of the TEL-PDGFβR fusion, the role of the TEL portion of the chimera might be to contribute an oligomerization motif, because dimerization of PDGFβR has been shown to be essential for its tyrosine kinase activity (17, 19). Similarly, BCR has been shown to contain an oligomerization motif which is required for ABL activation (27). To determine whether TEL-ABL self-associated, TEL-ABL was expressed in an in vitro transcription-translation system. TEL-ABL was cotranslated with a C-terminal-truncated form of TEL-ABL (TEL-ABL ΔC), and the proteins were immunoprecipitated with a C-terminal antibody which recognizes full-length TEL-ABL but not TEL-ABL ΔC. As shown in Fig. 7, TEL-ABL ΔC coimmunoprecipitated with full-length TEL-ABL, indicating that TEL-ABL self-associates in vitro.

If TEL-ABL oligomerization is mediated through the TEL HLH domain, then TEL-ABL ΔEX, the nontransforming form of TEL-ABL which lacks the TEL HLH domain, should not oligomerize. Indeed, TEL-ABL ΔEXΔC, a C-terminal truncation of TEL-ABL ΔEX, did not coimmunoprecipitate with

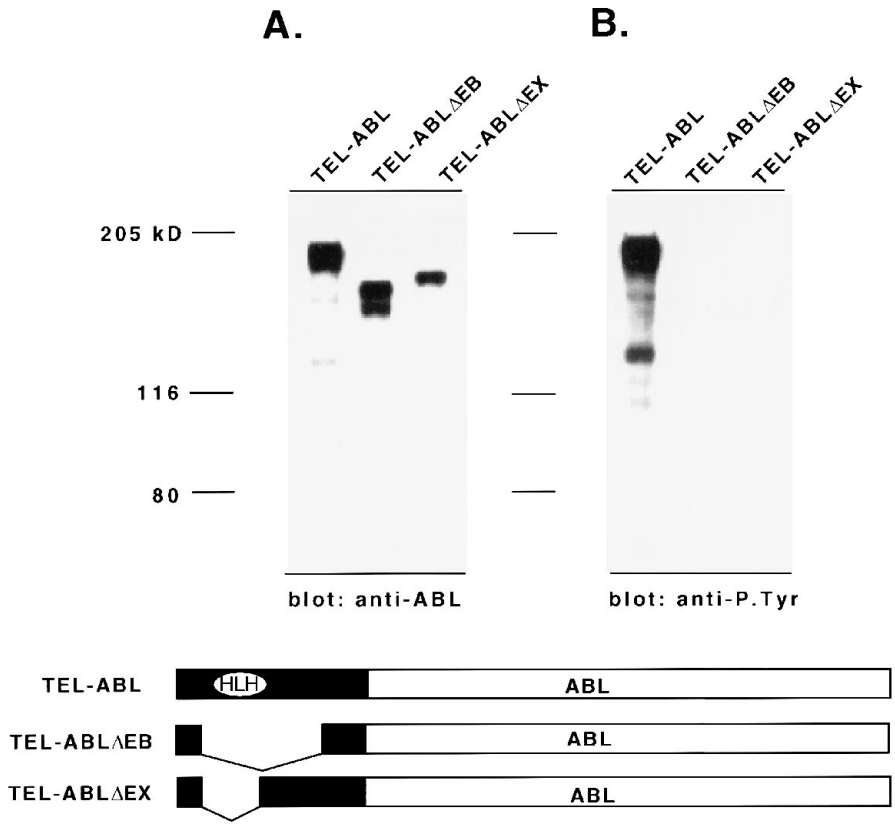

FIG. 6. TEL-ABL deletion mutant immunoblotting. The structures of the TEL-ABL deletion mutants are shown at the bottom of the figure. In TEL-ABL ΔEX, 76 amino acids (TEL amino acids 39 to 115), including the HLH domain, were deleted. In TEL-ABL ΔEB, TEL amino acids 39 to 182 were deleted. (A) Western blotting of Rat-1 total cell lysates with an anti-ABL antibody. The full-length TEL-ABL and TEL-ABL deletion mutants were all expressed. (B) The blot in panel A was stripped and reprobed with an antiphosphotyrosine antibody (anti-P.Tyr). The full-length TEL-ABL protein was tyrosine phosphorylated, but the HLH deletion mutants were not.

TEL-ABL ΔEX (Fig. 7). These experiments demonstrate that TEL-ABL oligomerization is dependent upon the TEL HLH domain.

The finding that the other (nontranslocated) *TEL* allele was deleted in the patient's leukemic cells led us to consider whether the wild-type TEL protein was also capable of interacting with TEL-ABL. Following cotranslation of wild-type TEL and TEL-ABL in vitro, TEL could be immunoprecipitated with an anti-ABL antibody, whereas TEL translated alone could not be immunoprecipitated with this antibody (Fig. 7). These experiments suggest that TEL encodes a protein-protein interaction motif which facilitates TEL-ABL oligomerization and is permissive to the formation of heterodimers between TEL-ABL and the normal TEL protein.

TABLE 2. Transformation by TEL-ABL deletion mutants

Construct	Transforming potential in cells[a]		
	Rat-1	Ba/F3	Marrow
TEL-ABL	83	96	12/12
TEL-ABL ΔEB	1	0	0/4
TEL-ABL ΔEX	0	1	0/4

[a] The average number of colonies from two plates of Rat-1 cells grown in soft agar, the number of IL-3-independent cultures generated from 96 Ba/F3 cultures, and the number of hematopoietic cultures established from control marrow cultures are shown.

Similar studies indicated that the wild-type TEL protein also self-associates in vitro (26).

DISCUSSION

The *TEL* gene was initially discovered because of its fusion in chronic myelomonocytic leukemia to the gene encoding the receptor tyrosine kinase PDGFβR (17). Our attempt to further elucidate the role of TEL in hematologic malignancy has now led us unexpectedly to another well-characterized tyrosine kinase, ABL. ABL has been extensively studied over the last decade because of its fusion to BCR as a result of the t(9;22) chromosomal translocation associated with chronic myeloid leukemia and acute lymphoblastic leukemia (42, 47). A number of the downstream targets of BCR-ABL have been identified, but its precise mechanism of transformation remains poorly understood. The identification of TEL, the only non-BCR fusion partner for ABL in human leukemia reported to date, may therefore shed some light on the critical events surrounding ABL-mediated transformation. Furthermore, the analysis of TEL-ABL transformation provides a powerful means of understanding the role of the TEL protein in neoplasia, despite its normal function being entirely unknown.

We report here a single case of TEL-ABL fusion in human leukemia, although an additional case has recently been reported in a child with acute lymphoblastic leukemia (35). While that case was not functionally characterized, only the amino-terminal 154 amino acids of TEL were present in the

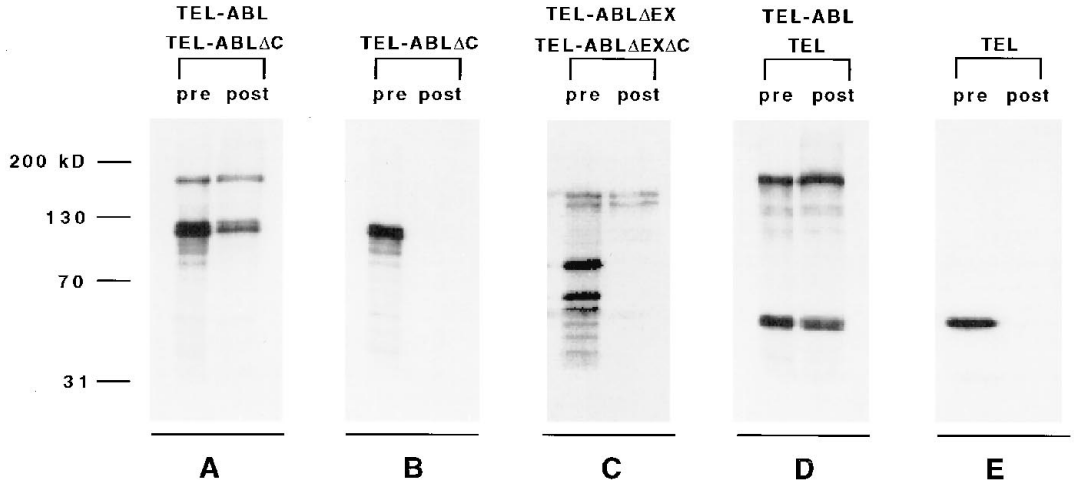

FIG. 7. In vitro dimerization of TEL-ABL. TEL-ABL or the HLH deletion mutant TEL-ABL ΔEX was cotranslated with C-terminal truncation mutant TEL-ABL ΔC or TEL-ABL ΔEXΔC or with wild-type TEL. [^{35}S]methionine labeled proteins were visualized on denaturing polyacrylamide gels. Pre refers to in vitro-translated proteins prior to immunoprecipitation with C-terminal monoclonal anti-ABL antibody 21-24. Post refers to the proteins recovered following anti-ABL immunoprecipitation. (A) TEL-ABL plus TEL-ABL ΔC. The 110-kDa truncated TEL-ABL ΔC protein coimmunoprecipitates with the full-length 180-kDa TEL-ABL protein, indicating that TEL-ABL oligomers form in vitro. (B) TEL-ABL ΔC alone. The 110-kDa truncated TEL-ABL is translated (pre) but is not recognized by the C-terminal anti-ABL antibody (post). This shows that the immunoprecipitation of TEL-ABL ΔC seen in panel A was not due to nonspecific binding of antibody to TEL-ABL ΔC. (C) TEL-ABL ΔEX plus TEL-ABL ΔEXΔC. TEL-ABL ΔEX is seen as a doublet at 165 and 170 kDa because of internal initiation of translation downstream of the start codon. TEL-ABL ΔEXΔC, the C-terminal truncation of TEL-ABL ΔEX, is seen as an approximately 100-kDa protein accompanied by several smaller proteins resulting from internal initiation of translation. TEL-ABL ΔEXΔC does not coimmunoprecipitate with TEL-ABL ΔEX, indicating that TEL-ABL mutants lacking the TEL HLH domain do not oligomerize. (D) TEL-ABL plus wild-type TEL. Coimmunoprecipitation of the 50-kDa wild-type TEL protein with the 180-kDa full-length TEL-ABL protein shows that TEL-ABL forms heterodimers with the normal TEL protein. (E) TEL alone. Translation of the normal TEL protein alone shows that TEL itself is not recognized by the anti-ABL antibody.

TEL-ABL fusion compared with the 336 TEL amino acids which we report here. These data suggest that the TEL-ABL fusion can be associated with both myeloid and lymphoid leukemias and that the TEL sequences upstream of amino acid 154 are likely to be sufficient for ABL activation.

We investigated the transforming potential of TEL-ABL in three cell culture systems: assays of fibroblast anchorage dependence, hematopoietic cell line growth factor dependence, and normal murine bone marrow cultures. TEL-ABL was transforming in all three assays, indicating that TEL-ABL is a dominant transforming oncoprotein. The testing of TEL-ABL transformation in multiple systems is critical, since it has been documented that the requirements for ABL-mediated transformation differ in different cell types (14, 15, 38). Although TEL-ABL and BCR-ABL appear to have indistinguishable biological activity, there are extensive differences between TEL and BCR. The shared structural features which make TEL and BCR capable of activating ABL in human leukemia remain to be elucidated.

We hypothesized that the TEL portion of the TEL-ABL chimera would be critical for the biological activity of TEL-ABL. To test this hypothesis, the deletion mutants TEL-ABL ΔEX and TEL-ABL ΔEB were tested for transforming potential. Neither mutant exhibited transforming activity in these assays, and both were found to have markedly decreased tyrosine phosphorylation than the full-length TEL-ABL. In addition, immunofluorescence studies demonstrated that TEL-ABL was strikingly restricted to linear cytoplasmic arrays resembling actin stress fibers, whereas the TEL-ABL mutants lacking the TEL HLH domain were distributed more diffusely in the cytoplasm. The association of BCR-ABL (28) and c-ABL (49) with actin has previously been demonstrated to occur through the ABL carboxy terminus. Association of BCR-ABL with actin filaments, however, is also strengthened by the first 63 amino acids of BCR, which are required for BCR homotetramerization (27). The functional significance of TEL-ABL cytoskeletal localization remains unclear, but recent evidence suggests that BCR-ABL phosphorylates cytoskeletal proteins such as paxillin, which is thought to play a role in signal transduction (43). The inability of TEL-ABL ΔEX to transform may be due to its inability to autophosphorylate, to associate with the cytoskeleton, or both. The kinase-inactive mutant TEL-ABL K219R, however, is not phosphorylated and is nontransforming yet retains its cytoskeletal localization. This suggests that TEL-ABL transformation is absolutely kinase dependent, but cytoskeletal association is not.

In TEL-ABL ΔEX, 76 amino acids from the TEL portion of the TEL-ABL chimera, including the TEL HLH domain, are deleted. The TEL HLH domain is highly conserved among a subset of Ets proteins including ETS-1, ETS-2, FLI-1, ERG, GABPα, and the *Drosophila* proteins yan and pointed. While this non-DNA binding portion of Ets proteins has been reported to bear weak homology to the basic HLH proteins MYC, MYO-D, and ID (45), protein-protein interactions through this domain have not been previously reported, and the true structure of the domain has yet to be determined. This domain has alternately been termed the 5′ Ets domain (39), domain B (50), and the pointed domain (21). To explore the possibility that TEL-ABL oligomerization was mediated through the TEL HLH domain, an in vitro transcription-translation approach was utilized. TEL-ABL was shown to form oligomers in vitro, whereas TEL-ABL ΔEX did not. We speculate that this failure to self-associate is responsible for its lack of transforming activity. It is also possible that the failure of TEL-ABL ΔEX to transform is due in part to its diminished cytoskeletal association. Further experiments are needed to address this question and to establish whether TEL-ABL forms simple homodimers or whether higher-order complexes are also formed, as is the case with BCR (27).

One intriguing finding with the patient studied was that in

addition to one *TEL* allele being rearranged, the other allele was deleted, resulting in loss of wild-type *TEL* function in the leukemic cells. We and others have observed similar loss of the residual *TEL* allele in other leukemias with translocations disrupting the *TEL* gene (16, 41). One hypothesis to explain this finding is that TEL loss of function alone is leukemogenic. That is, TEL might have tumor suppressor activity. Arguing against this is the observation that TEL-ABL transforms cells in a dominant, kinase-dependent fashion, suggesting that ABL activation is a critical component of TEL-ABL transformation. We favor the hypothesis that interaction of TEL-ABL with the normal TEL protein might abrogate the activation of TEL-ABL by preventing the formation of TEL-ABL oligomers. In support of this, wild-type TEL physically interacted with TEL-ABL in vitro. Deletion of the residual *TEL* allele in leukemic cells would circumvent this phenomenon, possibly resulting in the potentiation of TEL-ABL activity. A better understanding of the functional significance of *TEL* deletion in TEL-ABL-induced leukemia will require further in vivo analysis of TEL-ABL activity and the characterization of additional patients with TEL-ABL-positive leukemia.

The experiments described in this report establish a role for TEL, a putative transcription factor, in the activation of the tyrosine kinases PDGFβR and ABL through chromosomal translocation. It is possible that the principal function of the TEL portion of the TEL-ABL chimera is to facilitate oligomerization, a phenomenon which has been shown to be critical for a number of neoplasia-associated tyrosine kinases (5, 40). In addition, the observation that the TEL HLH domain encodes an oligomerization motif has implications for the Ets family of transcription factors. Given the high degree of homology between the TEL HLH domain and that of other Ets proteins, it seems unlikely that this self-association is restricted to TEL. Interestingly, a splice form of the *Drosophila* gene *pointed* which lacks the HLH domain (pointed^{P1}) is a constitutive transcriptional activator, whereas pointed^{P2}, which includes this domain, is regulated by the Ras pathway (34). Whether this difference in transcriptional activation is mediated through oligomerization remains to be determined. The ability of the TEL HLH domain to mediate self-association suggests that Ets factor protein-protein interactions through this highly conserved domain may represent a previously unrecognized phenomenon.

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