The Human L(3)MBT Polycomb Group Protein Is a Transcriptional Repressor and Interacts Physically and Functionally with TEL (ETV6)*

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H-L(3)MBT, the human homolog of the Drosophila lethal(3) malignant brain tumor protein, is a member of the polycomb group (PcG) of proteins, which function as transcriptional regulators in large protein complexes. Homozygous mutations in the l(3)mbt gene cause brain tumors in Drosophila, identifying l(3)mbt as a tumor suppressor gene. The h-l(3)mbt gene maps to chromosome 20q12, within a common deleted region associated with myeloid hematopoietic malignancies. H-L(3)MBT contains three repeats of 100 residues called MBT repeats, whose function is unknown, and a C-terminal α-helical structure, the SPM (SCM, PH, MBT domain, which is structurally similar to the SAM (sterile alpha motif) protein-protein interaction domain, found in several ETS transcription factors, including TEL (translocation Ets leukemia). We report that H-L(3)MBT is a transcriptional repressor and that its activity is largely dependent on the presence of a region containing the three MBT repeats. H-L(3)MBT acts as a histone deacetylase-independent transcriptional repressor, based on its lack of sensitivity to trichostatin A. We found that H-L(3)MBT binds in vitro to TEL, and we have mapped the region of interaction to their respective SPM/SAM domains. We show that the ability of TEL to repress TEL-responsive promoters is enhanced by the presence of H-L(3)MBT, an effect dependent on the H-L(3)MBT and the TEL interacting domains. These experiments suggest that histone deacetylase-independent transcriptional repression by TEL depends on the recruitment of PcG proteins. We speculate that the interaction of TEL with H-L(3)MBT can direct a PcG complex to genes repressed by TEL, stabilizing their repressed state.

The human lethal(3) malignant brain tumor (h-l(3)mbt) gene, a recently cloned human homolog of the Drosophila l(3)mbt gene, encodes a member of the polycomb group (PcG) of proteins (1). Drosophila l(3)mbt functions as a tumor suppressor gene that, in the homozygous mutated state, leads to malignant transformation of adult optic neuroblasts and ganglion mother cells in the larval brain (2, 3). Because these mutant alleles have never been characterized, the biochemical role of the Drosophila L(3)MBT protein in suppressing tumorigenesis is not known.

The h-l(3)mbt gene is located on chromosome 20q12 (1), within the common deleted region identified in patients with deletions of the long arm of chromosome 20. 20q abnormalities are found in hematological disorders including the myeloproliferative disorders, especially polycythemia vera where it is identified in ~10% of the patients (4), the myelodysplastic syndromes, and in a fraction of acute myeloid leukemias cases (5–8).

H-L(3)MBT is a member of the PcG of chromatin-associated proteins originally defined by their ability to maintain long term repression of the homeotic genes that govern axial patterning during Drosophila embryogenesis (9–12). More than 15 PcG genes have been characterized to date, and subfamilies can be identified based on the presence of common protein domains. The human L(3)MBT protein contains three MBT repeats, making it a member of a subfamily that includes at least three other genes: h-l(3)mbt-like (13), SCMH1 (14), and SCLM2 (15). PcG proteins function in large multiprotein complexes to maintain long term repression of gene expression thereby permitting stable and heritable transmission of gene activity. This function is crucial to maintaining differentiated identity of cells over subsequent generations. The mechanism by which the PcG proteins maintain repression is unknown; however, recent evidence indicates that an important component of this repression occurs via chromatin remodeling. Another set of proteins, the trithorax group, is thought to antagonize PcG-mediated repression and maintain active gene expression. The trithorax group complex includes proteins with diverse activities including histone acetyltransferase (16) and ATP-dependent nucleosome remodeling (SWI/SNF) activities (17).

The h-l(3)mbt gene gives rise to three major mRNA species that encode protein isoforms that differ at their C-terminal region and contain respectively, 772, 752, and 738 amino acids. H-L(3)MBT protein contains three MBT repeats, which are 95–105-amino acid motifs of unknown function. Although analysis of the MBT motif has not shown homology with any catalytic domains or sequence-specific DNA binding domains (13), these repeats are highly conserved between the Drosophila and human homologs, suggesting that they have an important function.

The SPM domain present at the H-L(3)MBT C terminus is an α-helical structure of ~60 amino acids. The SPM domains in the Drosophila Sex Comb on Midleg (SCM) and polyhomeotic...
(PH) proteins have been found to mediate protein-protein interactions (18). The predicted α-helical secondary structure and conservation of hydrophobic residues prompt comparison of the SPM domain with the helix-loop-helix type motifs used for homotypic and heterotypic protein interactions in other transcriptional regulators. The SPM domain belongs to the extended family of SAM (sterile alpha motif) domains (also known as HLH or pointed domains) which are found in several regulatory proteins including kinases, adaptor proteins, and transcription factors (19, 20). These more distantly related proteins include several members of the ETS family of transcription factors (for review, see Ref. 21), such as TEL. The tel (translocation Ets leukemia) gene was identified and located at the chromosomal breakpoints of several leukemia-associated translocations (22–25). The fusion proteins derived from these translocations contain the N-terminal portion of TEL, including the SAM domain, fused to a variety of tyrosine kinase domains, such as the platelet-derived growth factor receptor (PDGFR) β, ABL, Janus kinase (JAK) 2, and NTRK3, or to transcription factors such as AML1 (22, 26, 27). Dimerization of the TEL-SAM domain results in the constitutive activation of the SPM domain with the helix-loop-helix type motifs used in transcriptional repression by TEL-AML1 (30–32). Even though the PcG-SPM and the TEL-SAM domains share very little sequence identity, the polyclonoe PH domain has been recently reported to form a helical polymer structure similar to the one formed by TEL-SAM, with both domains displaying closely related structural architecture (33). This homology prompted us to screen several SAM-containing ETS proteins for their ability to interact with H-L(3)MBT. We report that H-L(3)MBT is an HDAC-independent transcriptional repressor, and we have characterized its in vivo and in vitro physical association with TEL; this interaction requires their respective SPM and SAM domains. Using both the stemolysin-1 (matrix metalloprotease-3) promoter, which is physiologically regulated by TEL, and an artificial TEL-regulatable promoter, we show that H-L(3)MBT is targeted to these promoters through its interaction with TEL, enhancing the repressive effect of TEL on transcription. We believe that this is the first report of a functional interaction between a PcG protein and a mammalian DNA sequence-specific binding transcription factor. Given the pivotal role that ETS factors (and TEL in particular) play in human malignancies, the functional interaction between H-L(3)MBT and TEL provides a clue as to the potential role of PcG proteins such as L(3)MBT in cancer.

MATERIALS AND METHODS

Plasmid Construction—To generate a full-length h-L(3)mbt cDNA clone, total RNA from placenta (Research Genetics) was reverse transcribed using a GeneAmp RNA PCR Core kit (Roche Diagnostics). cDNA products were PCR-amplified with the Expand High Fidelity PCR System (Roche Diagnostics) in 5% dimethyl sulfoxide, using forward (5′-AGGCTGCATGACAGGATGAGCTGTTAAT-3′) and reverse (5′-TGAGAGGGGACCCCAAGGAGCTGTTAAT-3′) to generate a P1/P2 fragment, and P3 (5′-TGAGGGGGTGTCCCGGTCCTCA-3′), which is complementary to the end of P2) and P4 (5′-TCC- AAGCGGCTTCGGCCAGT-3′) to generate a P3/P4 fragment. Equimolar amounts of P1/P2 and P3/P4 were mixed and PCR amplified using P1 and P4. The resulting P1/P4 fragment encodes an in-frame H-L(3)MBT polypeptide lacking 390 amino acids corresponding to the MBT repeats. The GAL4-DDB-H-L(3)MBT construct was cloned by fragment cloning the h-L(3)mbt cDNA in-frame into pFA vector (Stratagene). For creation of the MBT mutant version of this GAL4 fusion construct, SOEing was applied using the P2 and P3 primers mentioned above, with P1 (5′-AGGCTGCATCTACCTGGAAGAG-3′) and P4 (5′-GCGG- TACGAGGCTTGGTCAAGCT-3′) amplifying with 5′-TCCTTCTGGCGCT- GAGGCCCAAGGGGAGGCGAC-3′ was used for P2, and 5′-GCGT- CACCGGCAAAGA-3′ was used for P3. For the mutant the pFA-h-l(3)mbt DNA was digested with BamHI and StuI, and the resulting fragment lacking SPM domain sequences was subcloned into the BamHI and StuI sites of pFA.

Cell Culture, Transient Transfection, and Luciferase Assay—NIH3T3, U2OS, and 293 cells were cultured in Iscove’s modified Dulbecco’s medium with 10% fetal bovine serum, 2 mm glutamine, and antibiotics and were trypsinized and replated to the needed density the day before transfection. NIH3T3 cells were transfected with Lipofectamine Plus reagent (Invitrogen); 293 cells were transfected using a calcium phosphate DNA precipitation technique (Promega, Promega, Madison, WI). Unless otherwise specified, 1 μg of expression construct (or empty vector), 2 μg of the reporter construct, and 0.2 μg of the cytomegalovirus-driven GFP expression plasmid (pEGFP-C3) control plasmid (Stratagene) were used for each transfection; the empty pcDNA3 expression vector was added, as needed, to keep the amount of transfected DNA the same for each sample. For the luciferase assays, cells were collected 48 h after transfection and lysed; luciferase activity was assessed using Luciferase Assay Substrate (Promega) on a LUMAT LB9501 (Berthold) luminometer. Luciferase activity was normalized according to the percent GFP-positive cells (as assessed by flow cytometry), to the total protein concentration. To rule out general effects of the transfected proteins on the cytomegalovirus promoter the mean fluorescence of the GFP peaks was determined for the samples and found to be comparable.

Immunoprecipitation Assays—After collection, cells were washed with PBS once, lysed by brief sonication in NET-N lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris pH 8, 1 mM EDTA) in the presence of proteinase inhibitors (10 μg/mL leupeptin, 10 μg/mL aprotinin, 0.5 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (10 mM NaF, 20 mM β-glycerophosphate), and then clarified by centrifugation. The protein concentration was adjusted to 1 mg/mL, and the lysates were incubated with 2 μg of specific antibodies for 1 h at 4°C. Protein-G agarose beads (Santa Cruz) were added to the extracts and mixed for at least 1 h at 4°C. The immune complexes were washed four times with NET-N lysis buffer, once with high salt buffer (1 mM NaCl, 20 mM Tris-HCl, pH 8), and once with low salt buffer (10 mM MgCl2, 50 mM HEPEs-KOH, pH 7.5); they were released by adding SDS sample buffer and boiled for 4 min. Samples were subjected to SDS-PAGE with 7.5% gel, transferred to a polyvinylidene difluoride membrane, and Western blotted with the following antibodies: FLAG-horse radish peroxidase-conjugated (M2, Sigma), HA mouse monoclonal antibody (12CA5, Roche Molecular Biochemicals), GAL4-DDB (RR51C, Santa Cruz), anti-TEL (gift of P. Marynen), and anti-H-L(3)MBT (MBT1) generated in our laboratory (see below). In each experiment, protein concentrations were determined by the Bradford method and used to assess the correct expression of the cotransfected expression plasmids.

Nuclear extracts were prepared in 0.4 mM NaCl, according to the protocol of Dignam and co-workers (35), and the immunoprecipitates from nuclear extracts were washed with a buffer containing 0.4 mM NaCl, 0.4 mM PIPES, 0.6% of GST Fusion Proteins in SPM Domain Assays—The GST fusion protein plasmids were transformed into the Escherichia coli BL21 strain (Novagen, Inc., Madison, WI). After overnight culture, protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 2–4 h. The bacterial pellets were then lysed in 1 ml of PBS-T buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Tris, 1.5 mM KH2PO4, 0.5 mM phenylmethylsulfonyl fluoride) and sonicated. 5 μg of GST-TEL fusion protein (or GST) was incubated with 20 μg of 5′0′ slurry glutathione-Sepharose 4B beads (Amersham Biosciences) in a total volume of 1 ml of PBS-T for 45 min at 4°C. The beads were washed three times with 1 ml PBS-T and then incubated...
three MBT repeats were cotransfected with the full-length H-L(3)MBT proteins lacking either the SPM domain or the residues in the SPM domain, epitope-tagged versions of mutant homodimerizes; to determine whether the self-binding ability analyzed by SDS-PAGE with 7.5% gel and autoradiography. The TNT-beads by boiling in SDS-gel loading buffer for 4 min. Proteins were transcribed and translated 35S-labeled protein in 1 ml of NET-N buffer for 2–4 h at 4 °C. The beads were then washed with 1 ml of NET-N six times. The bound proteins were released from the beads by boiling in SDS-gel loading buffer for 4 min. Proteins were analyzed by SDS-PAGE with 7.5% gel and autoradiography. The TNT-coupled reticulocyte lysate system (Promega) was used to generate in vitro translated proteins, following the procedures specified by the manufacturer.

Preparation of Anti-H-L(3)MBT Antisera and Coupling to Protein A Gel—Anti-H-L(3)MBT antisera were generated by immunizing rabbits with either a 174-amino acid fragment encoding the second half of the MBT repeats (MBT1) or a protein fragment containing the three MBT repeats (MBT2). Each fragment was bacterially expressed as a GST fusion protein using pGEX-4T expression plasmid (Amersham Biosciences) and purified using glutathione-Sepharose 4B beads. The antisera were affinity purified using N-hydroxysuccinimide-activated Sepharose columns (Amersham Biosciences) coupled to GST alone (first passage for negative selection) and then GST-MBT-coupled beads (second passage for positive selection). The affinity-purified MBT2 antibody (suitable for immunoprecipitation) was then coupled to recombinant protein A gel, using the Immunopure rProtein A IgG Plus Orientation kit from Pierce.

![Diagram](Image)

**Fig. 1. H-L(3)MBT homodimerizes through the SPM domain.** a, schematic representation of type I H-L(3)MBT (772 amino acids) protein and the H-L(3)MBT-ΔMBT and H-L(3)MBT-ΔSPM deletion mutants. b and c, H-L(3)MBT and its mutant expression vectors were coexpressed in 293T cells (as indicated). Protein-protein interactions were detected by communoprecipitation (IP) from cell lysates using an anti-HA monoclonal antibody (12CA5) and Western blotting (WB) with FLAG-horseradish peroxidase-conjugated monoclonal antibody (M2). Input protein lysates are shown in the left parts of the panels. The right parts of the panels show the communoprecipitated proteins.

with 2 μl of in vitro transcribed and translated [35S]-labeled protein in 1 ml of NET-N buffer for 2–4 h at 4 °C. The beads were then washed with 1 ml of NET-N six times. The bound proteins were released from the beads by boiling in SDS-gel loading buffer for 4 min. Proteins were analyzed by SDS-PAGE with 7.5% gel and autoradiography. The TNT-coupled reticulocyte lysate system (Promega) was used to generate in vitro translated proteins, following the procedures specified by the manufacturer.

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**RESULTS**

The SPM Domain in H-L(3)MBT Mediates Homotypic Interactions—PeG proteins form large multiprotein complexes, thus we examined the dimerization properties of H-L(3)MBT. First, we determined whether H-L(3)MBT protein could homodimerize using FLAG or HA epitope-tagged H-L(3)MBT cDNAs (shown in Fig. 1a), which we transfected into 293T cells. As shown in Fig. 1b (the 6th lane) the H-L(3)MBT protein clearly homodimerizes; to determine whether the self-binding ability resides in the SPM domain, epitope-tagged versions of mutant H-L(3)MBT proteins lacking either the SPM domain or the three MBT repeats were cotransfected with the full-length HA-H-L(3)MBT. Removal of the SPM domain is sufficient to abrogate homodimerization of H-L(3)MBT (Fig. 1b, 10th lane), whereas removal of the three MBT repeats had no effect on homodimerization (Fig. 1c, 4th lane). The protein membrane was stripped and probed for HA antibody to confirm the presence of equal amount of the HA-tagged proteins (not shown). We conclude that H-L(3)MBT self-associates in vivo and that self-association requires the SPM domain.

**H-L(3)MBT Has General Transcriptional Repressor Activity—**PeG proteins maintain a chromatin-repressed state, but unlike classic transcription factors they do not recognize specific DNA target sequences. Thus far, the only PeG protein shown to have sequence-specific DNA binding activity is pleiohomeotic, the Drosophila homolog of mammalian YY1 (36). Although PeG proteins act on chromatin, their repressive function can also be detected in transcriptional assays using transiently transfected reporter gene plasmids (which are believed to form less organized chromatin-like structures) (37). To evaluate the transcriptional regulatory activity associated with H-L(3)MBT, we fused it to the DNA-binding domain (DBD, amino acids 1–147) of GAL4. The GAL4-H-L(3)MBT construct was tested using a luciferase reporter plasmid that contains four copies of the GAL4 DNA recognition sequence positioned immediately upstream of a thymidine kinase (tk80) minimal promoter (diagrammed in Fig. 2a, upper panel). In-
increasing amounts of GAL4-H-L(3)MBT were cotransfected into 293 cells together with a constant amount of luciferase reporter plasmid; a GAL4-TEL fusion protein was included as a positive control for repression. The GAL4-H-L(3)MBT repressed transcription in a dose-dependent manner (Fig. 2a), and the magnitude of this trans-repression effect was similar to that observed with TEL. (The correct expression of the GAL4 fused protein was verified with an anti-GAL4 DBD antibody on the protein lysates (Western blot not shown).) Cotransfection of full-length H-L(3)MBT without the GAL4 moiety (pcDNA3-H-L(3)MBT) did not repress the promoter activity, excluding a general effect on transcription (Fig. 2a). Thus, H-L(3)MBT can function as a transcriptional repressor. Notably, repression of the reporter by GAL4-H-L(3)MBT was not influenced by the addition of a range of concentrations of trichostatin A, whereas repression by GAL4-TEL was partially reversed by it (Fig. 2b). This suggests that transcriptional repression by H-L(3)MBT does not depend on HDAC activity.

Repression by H-L(3)MBT Requires the MBT Repeats—To determine the regions of H-L(3)MBT involved in transcriptional repression, we generated constructs fusing the GAL4-DBD to the N terminus of H-L(3)MBT mutants in which either the three MBT repeats, the SPM domain, or the zinc finger region was deleted. These mutants (diagrammed in Fig. 3a) were assayed for expression by Western blot analysis (Fig. 3c) and were shown to localize in the cell nucleus by immunofluorescence staining (data not shown). Mutants lacking either the zinc finger or the SPM domain retained the ability to repress the GAL4-reporter gene (Fig. 3b). In contrast, the mutant missing the three MBT repeats had a minimal capacity to repress the GAL4-TK-LUC reporter (30% repression compared with ~90% repression for the wild type protein). Thus, the trans-repressive ability of H-L(3)MBT requires the presence of the MBT repeats for full activity, whereas the zinc finger and SPM domains appear to be dispensable for this function. Repression by H-L(3)MBT-Δ-SPM suggests that self-dimerization is not necessary for trans-repression; however, a role for protein dimerization cannot be completely excluded because the GAL4-DBD itself can direct the oligomerization of GAL4 and GAL4-derived proteins.

H-L(3)MBT and TEL Physically Interact through Their SPM/SAM Domains—The SPM domain contained in several PcG proteins shares structural homology with the SAM domain found in a subset of members of the ETS family of transcriptional factors (e.g. TEL) (33, 38, 39). To determine whether H-L(3)MBT and TEL interact in vivo we performed immunoprecipitation assays, using a rabbit polyclonal antibody (MBT2) to precipitate H-L(3)MBT followed by Western blotting for TEL. To avoid interference from the heavy chain of the antibody used to pull down H-L(3)MBT, it was necessary to cross-link covalently both the H-L(3)MBT antibody and the normal rabbit polyclonal IgG to protein A-agarose matrix. Using affinity-purified anti-H-L(3)MBT rabbit polyclonal antibody (MBT2 Ab) or normal rabbit IgG covalently coupled to protein A-agarose matrix was used to immunoprecipitate H-L(3)MBT from 1 mg of U2OS cell nuclear extract. The immunoprecipitated protein complexes were run on SDS-PAGE (7.5% gel) and subjected to Western blot (WB) analysis. Inputs in the first lanes of both panels for both MBT2 Antiserum and for MBT2 Antiserum plus mouse IgG as a control.
of H-L(3)MBT are not required for its interaction with TEL. In contrast, deletion of the SAM domain of TEL or the SPM domain of H-L(3)MBT completely abrogates heterodimerization (lanes 3, 7, and 9). Thus, these domains are involved in the physical association of TEL and H-L(3)MBT.

Recently, TEL2 (or TELB), a new member of the ETS family of transcription factors which is very similar to TEL, was cloned (39, 40). TEL2 is predicted to be structurally similar to TEL and has been shown to form homodimers as well as heterodimers with TEL (41). We tested whether H-L(3)MBT could also interact with TEL2 and found that HA-TEL2 coimmunoprecipitates with FLAG-H-L(3)MBT when the two proteins are coexpressed (Fig. 5b).

To further study the interaction between TEL and H-L(3)MBT, we used GST-based in vitro protein binding assays. [35S]-Radiolabeled H-L(3)MBT (and deletion-mutant proteins) were produced by in vitro transcription/translation (IVT) and tested for their ability to bind to a bacterially expressed GST-TEL fusion protein. The self-association of TEL with GST-TEL was used as a positive control. These studies determined that GST-TEL specifically binds H-L(3)MBT but not H-L(3)MBT-Δ-SPM (Fig. 5c). The interaction of TEL with H-L(3)MBT-Δ-SPM and with H-L(3)MBT-Δ-C2HC appears to be much less efficient than its interaction with the intact H-L(3)MBT. This is shown in Table I, where the interactions between GST-TEL and the IVT proteins are assigned a +, +/−, or − based on the relative amounts of input IVT protein detected after the pulldown.

TEL and FLI-1 are known to bind to each other via their SAM domains, but they do not interact with ETS-1 (42). We investigated whether in vitro translated H-L(3)MBT can bind to either of these SAM domain-containing ETS proteins. As shown in Fig. 5d, neither GST-FLI-1 nor GST-ETS-1 binds H-L(3)MBT in this assay. The association of in vitro translated TEL with GST-FLI-1 served as the positive control.

Cooperation of H-L(3)MBT and TEL in the Repression of Transcriptional Targets—To assess the functional impact of the physical interaction between H-L(3)MBT and TEL on transcriptional regulation, we generated a reporter gene construct whose luciferase expression is regulated by a minimal tk80 promoter element, and three concatamerized ETS binding sites (43). This artificial promoter/ enhancer sequence has been shown to drive high levels of luciferase activity in all of the cell lines tested (compared with the control tk80Luc reporter plasmid), probably reflecting the binding of endogenous transactivating ETS proteins (44). Transfection of the pcDNA3-H-L(3)MBT construct had no effect on the activity of the 3xEB8tk80Luc reporter plasmid, whereas pcDNA3-TEL somewhat repressed its activity. However, the combination of H-L(3)MBT and TEL significantly repressed luciferase expression (Fig. 6a), indicating that TEL binding its consensus binding site can recruit H-L(3)MBT to a promoter or enhancer regulatory region. Although previous studies have shown that the Drosophila PcG proteins can interact with the basic transcriptional apparatus (e.g., transcription factor IID) (45), we did not find any appreciable effect of H-L(3)MBT on the activity of either an SV40 promoter or a herpes simplex virus TK promot-

### Table I

| IVT 35S-Protein | Binding to GST-TEL |
|-----------------|-------------------|
| TEL             | +                 |
| H-L(3)MBT       | +                 |
| H-L(3)MBT-Δ-SPM | +/−               |
| H-L(3)MBT-Δ-C2HC| +/−               |
| H-L(3)MBT-Δ-SPM | −                 |

Fig. 5. H-L(3)MBT interacts with TEL and TEL2 but not with ETS1 or FLI-1. This interaction requires the SAM/SPM domain(s). a, the H-L(3)MBT and the TEL constructs were expressed in 293T cells in the indicated combinations (A–E). Protein lysates were subjected to immunoprecipitation (IP) using either a mouse anti-HA monoclonal antibody or mouse normal IgG as a control (for nonspecific pulldown). The anti-FLAG-horseradish peroxidase-conjugated antibody (M2) was used for Western blot analysis. Western blot of input proteins is shown on the right side of the panel (lanes 1–15) for H-L(3)MBT and on the top right panel for TEL. b, FLAG-H-L(3)MBT and HA-TEL2 cDNAs were expressed alone or in combination. Protein lysates were subjected to immunoprecipitation using a mouse anti-HA monoclonal antibody. Anti-FLAG-horseradish peroxidase conjugated antibody (M2) was used for Western blot analysis. Western blot of input protein is shown in the right three lanes of the panel. c, results of GST-TEL pulldown assays. The in vitro translated proteins are indicated on the top of each group of lanes. I, input; G, GST pulldown control; T, GST-TEL pulldown. d, results of GST-ETS-1 (lanes 1 and 5) and GST-FLI-1 (lanes 2 and 6) pulldown assays. In vitro translated TEL and H-L(3)MBT were used for lanes 1–4 and 5–8, respectively. [35S]Methionine-radiolabeled in vitro translated proteins were incubated with 5 μg of bacterially produced GST or GST fusion proteins immobilized on GSH-Sepharose. After incubation and washing, the bound proteins were separated by SDS-PAGE (7.5% gel), fixed, and stained with Coomassie to show the presence and purity of the purified GST fusion proteins. Input represents 5% of the total protein used in the pulldown assay.
er-driven reporter construct (Fig. 6a).

It was shown recently that TEL can repress transcription of stromelysin-1 (matrix metalloproteinase-3) gene by direct binding to its promoter (46). To study the functional relevance of the H-L(3)MBT-TEL interaction on a natural target of TEL, we cotransfected pcDNA3-h-l(3)mbt, pcDNA3-TEL, and pcDNA3-TEL/H9004-SAM alone, or in combination, with a rat stromelysin-1 promoter-regulated luciferase reporter gene plasmid (shown schematically in Fig. 6b). This promoter contains 754 bp of 5'-flanking sequence and at least two TEL binding sites (TBS), (shown at the top). Cells were collected 48 h after transfection; luciferase activity was normalized to a percentage of GFP-positive cells as assessed by flow cytometry and total protein concentration. The -fold repression was calculated as the -fold decrease in luciferase activity compared with the empty vector. Values shown are the average ± S.E. of two experiments performed in triplicate.

MBT repeats (the region required for repression by the GAL4-H-L(3)MBT fusion protein (see Fig. 3b)).

DISCUSSION

Koga et al. (1) and our group (6) have previously isolated the h-l(3)mbt gene, which we showed is located within the commonly deleted region of 20q-seen in patients with myeloid hematologic malignancies. We now report that H-L(3)MBT is a transcriptional repressor and has the ability to direct repression of specific promoters by binding to the DNA sequence-specific transcription factor TEL. We show that two protein motifs in H-L(3)MBT, the MBT repeats and the SPM domain, which are conserved in other PcG family members, are required for its repression and protein binding activities.

The Trans-repressing Ability of H-L(3)MBT Requires Mainly the Presence of the MBT Repeats—Removal of the MBT repeats from H-L(3)MBT eliminated much of its trans-repressing ability, demonstrating the role for these repeats in establishing repression, likely through protein-protein contacts. Future studies will determine whether all three MBT repeats are required for H-L(3)MBT function; however, the known MBT-containing PcG proteins possess two, three, or four MBT repeats, suggesting that the functional unit may require a minimum of two repeats. Little is known about the biological
relevance of the MBT repeats; however, these repeats appear to be indispensable to the function of the SCM protein in *Drosophila* (47). Three hypomorphic SCM alleles, which are mutated in the MBT repeats, interact genetically with PcG mutations more strongly than SCM null alleles, and the strongest interactions produce partial syntenic lethality (47). These SCM mutant proteins can still associate with polytene chromosomes, suggesting that although the MBT repeats have a critical function in the PcG complex, they may not be essential for protein localization.

**The SPM Domain of H-L(3)MBT Mediates Homotypic and Heterotypic Interactions—**We have demonstrated homodimerization of H-L(3)MBT and have determined that the SPM domain of H-L(3)MBT mediates both homotypic and heterotypic interactions. Structural studies of the TEL-SAM domain have shown this to be an oligomerization motif (41), yet other ETS proteins that contain a SAM domain, such as FLI-1, ERG, ETS1, ETS2, or GABPβ, lack the ability to form polymers. ETS1 is monomeric even at high concentrations (48), and in our *in vitro* experiments, we were unable to coimmunoprecipitate H-L(3)MBT with either ETS-1 or FLI-1. Rather, we found that TEL and TEL2 interact with H-L(3)MBT through their α-helical SAM/SPM domains. Further testing would define whether these proteins are unique among ETS proteins in their ability to interact with H-L(3)MBT. Kim et al. (41) defined a subset of ETS-SAM domains which maintain hydrophobic residues at the interacting surface, and their model predicts that TEL2, and the *Drosophila* YAN protein, would in fact oligomerize similarly to TEL.

In addition to binding H-L(3)MBT, the TEL-SAM domain is involved in several other interactions, such as the binding of TEL to TEL2 (39) or to FLI-1 (42). TEL is conjugated to SUMO-1 after interacting with UBC9, and this modification requires the SAM domain and largely involves a specific lysine residue (Lys-99) in the TEL-SAM domain (49, 50). Finally, the TEL-SAM domain is required to target TEL to specific subnuclear structures called TEL bodies (50).

**Functional Interaction of H-L(3)MBT and TEL—**We have demonstrated that H-L(3)MBT potentiates the repression established by TEL on the stromelysin-1 promoter and that H-L(3)MBT increases the efficiency of silencing of an artificial 3×ETSBS enhancer by TEL. Augmentation of TEL-mediated repression requires the direct physical association of H-L(3)MBT and TEL through their SPM and SAM domains, respectively. The available literature suggests that the role of the SAM domain in TEL-mediated repression may be highly context-dependent. For instance, TEL-mediated repression still occurs when the SAM domain is replaced by a leucine zipper dimerization motif (44), suggesting that dimerization but not corepressor binding to the SAM domain is essential. Yet TEL point mutants that fail to dimerize can retain their ability to bind mSin3A and maintain partial trans-repressor activity (51). An explanation for these apparently conflicting results could be that the SAM domain constitutes an interface for numerous protein contacts, including the H-L(3)MBT polycomb protein, which provide TEL with several mechanisms of repression. Transcriptional repression by TEL has been shown to involve at least two distinct regions and mechanisms of action. Repression by the central region of TEL involves the recruitment of a complex that includes N-CoR, SMRT, and mSin3A (which is also contacted by the SAM domain (51)); these corepressors recruit HDAC activity to DNA-binding proteins to actively repress transcription (52). The SAM domain of TEL has been shown to repress gene transcription through a different mechanism, which appears to be HDAC-independent (53).

We have shown that H-L(3)MBT functions as an HDAC-independent repressor and interacts with the TEL-SAM domain, which supports a model in which TEL recruits components of two separate repressor complexes through different regions of the protein. In an analogous manner, the retinoblastoma protein (pRB) represses cyclin E expression via an HDAC-dependent mechanism but utilizes PcG complexes for the HDAC-independent, long term silencing of cyclin A and cdc2 expression, which leads to arrest in G1 (54). In fact, this G1 block is maintained even if p16 is depleted and pRB becomes hyperphosphorylated, which suggests that PcG complexes help mediate irreversible repression by pRB. Short term inhibition of transcription might therefore be achieved through the recruitment of corepressors and HDACs to the promoter. Later, long term silencing might be established by an H-L(3)MBT-containing PcG protein complex that can maintain repression and would make HDACs available to other target genes in the cell. Additional work aimed at identifying other components of this complex is under way, which will help address the respective contributions of HDAC-dependent and -independent transcriptional repression to the regulation of TEL target genes.

**PcG Protein Recruitment to DNA through Canonical DNA-binding Factors—**With the exception of YY1 (36), none of the PcG proteins has been shown to bind DNA in a sequence-specific fashion (55). Notwithstanding, PcG complexes are found in stable association with chromatin on polytene chromosome in *Drosophila* (18) and with core promoter regions in chromatin immunoprecipitation assays (45). Although formation of a multiprotein complex could generate a DNA binding activity, more likely, PcG complex formation at DNA target sites is dependent on a mosaic of interactions of different PcG proteins with multiple DNA-binding proteins that act as recruiters (55). The physical and functional interaction of H-L(3)MBT with TEL presents some analogy to the interaction of PcG proteins in the *Drosophila* polycomb repressive complex 1 (PRC1) with the Zeste protein (56). Zeste is a sequence-specific DNA-binding factor, which has consensus binding sites in the promoter and regulatory regions of several homeotic genes (57); it has been proposed that the interaction of Zeste with PRC1 aids in the targeting of PcG proteins to repressed gene loci. TEL is a sequence-specific DNA-binding protein that is directly involved in establishing the repression of specific target genes. By binding both H-L(3)MBT and specific promoter sequences, TEL may bridge the PcG complex with specific regulatory elements.

**Significance of H-L(3)MBT/TEL Interaction—**Silencing by PcG proteins seems to depend on the state of transcriptional activity of the target gene because PcG complexes generally require a silenced gene as template (58). When the target is transcriptionally active, silencing is not established (59); whereas once established, the repressed state persists throughout embryonic development. Thus, the PcG complex has been proposed to function as a memory system that can stabilize previous regulatory events by “locking” them in. However, this model may be too simplistic, given that the vertebrate EED-EZH2 complex possesses HDAC activity (60), which could be involved in initiating gene repression.

We found that substituting a thymidine kinase minimal promoter with the much stronger SV40 promoter in the 3×EB-StkLuc construct led to a loss of repression by TEL (and, as a consequence, by TEL plus H-L(3)MBT) (data not shown). This is consistent with the principles of gene silencing observed in *Drosophila*, where high doses of trans-activators can antagonize PcG-mediated silencing (61).

The molecular mechanisms through which PcG proteins achieve and maintain repression are largely unknown. Purification of two different PcG protein complexes has identified...


proteins that could interact functionally with PcG proteins to repress gene expression, such as HDAC2, which is found in the EED-EZH2 complex (60) and the corepressor molecules SMRT and Sin3A, which are found in the Drosophila PRC1 (56). Although repression mediated through the EED-EZH2 complex is relieved by trichostatin A (a potent HDAC inhibitor), the PRC1 complex blocks chromatin remodeling by the trithorax group-related SWI/SNF complex in vitro through a mechanism that appear to be HDAC-independent (62). Trichostatin A is unable to relieve the repression brought about by H-L(3)MBT; this suggests that the mechanism of repression of H-L(3)MBT may be more similar to that of the PRC1 complex.



**PcG Genes in Hematopoiesis**—Several lines of evidence support a critical role for PcG proteins in regulating the early and late stages of hematopoiesis. Mice lacking bmi-1 display a progressive marrow hypoplasia similar to aplastic anemia and have a hypoplastic spleen and thymus (63). On the contrary, eed−/− mice have a propensity to develop both myeloproliferative and lymphoproliferative diseases later in life (64). Mice lacking mel-18 have a severe combined immunodeficiency, and mel-18−/− lymphocyte precursors respond poorly to interleukin-7 stimulation (65). PcG proteins regulate Hox gene expression, and there are numerous examples where dysregulated Hox expression profoundly perturbs hematopoiesis or regulates the commitment and differentiation processes. The ectopic stem cell compartment may signify that their physical and hematopoiesis in the murine bone marrow (67). The contemporary regulation of the commitment and differentiation processes.

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