ENL, the Gene Fused With HRX in t(11;19) Leukemias, Encodes a Nuclear Protein With Transcriptional Activation Potential in Lymphoid and Myeloid Cells

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HUMAN LEUKEMIASES are frequently associated with specific chromosomal translocations that result in the oncogenic conversion of cellular proto-oncogenes located at the translocation breakpoints.1,2 In acute leukemias, these proto-oncogenes often encode transcription factors, suggesting that aberrant gene regulation plays an important role in leukemogenesis.1,2 Recently, a gene designated HRX (also called MLL, ALL-1, or HTRX17) has been cloned and shown to be disrupted by recurring 11q23 translocations. The central portions of the predicted Hrx and trithorax proteins contain sequences similar to metal-binding zinc fingers that may mediate interactions with DNA or heterologous proteins. An area of Hrx near its amino terminus that is not conserved with Drosophila trithorax contains so-called“AT hook” motifs that may mediate DNA interactions based on the role of similar motifs in HMG-I(Y) proteins.7

Translocations involving HRX are seen in the majority of cases of infant acute lymphoblastic leukemia (ALL) and treatment-induced secondary acute myelogenous leukemia (AML).8-10 These translocations cluster in a restricted 8-kb region of the HRX gene and fuse nearly identical N-terminal portions of Hrx, containing the AT hook motifs (but lacking the zinc fingers), to a variety of heterologous proteins in different 11q23 translocations. Fusion partners Enl, FeYAF-4, AF-9, AF-6, and AFX1 have been cloned and characterized for the t(11;19), t(4;11), t(9;11), t(6;11), and t(X;11), respectively.3,5,11-14 Enl, Fel, and AF-9 are all positively charged, serine- and proline-rich proteins containing nuclear targeting sequences, suggesting that they may be involved in transcriptional regulation. Partial sequence analysis of AFX1 indicates that it is also rich in serines and prolines, and AF-6 contains short stretches rich in prolines, charged amino acids, serines, or glutamines. Furthermore, AF-9 and Enl share significant amino acid similarity and are greater than 80% identical in their N-terminal and C-terminal regions.5 Based on their structural features, it has been proposed that the fusion proteins created by t(11;19), t(4;11), t(9;11), and t(X;11) translocations may function as chimeric transcription factors.

In the present study, we have analyzed the transcriptional properties of Enl. We show that Enl is a nuclear protein that can activate transcription from minimal promoters in both lymphoid and myeloid cells, as well as in yeast. Deletion mutagenesis demonstrated that the minimal portion of Enl required for activation of transcription was localized to its C-terminal 90 amino acids. This region is highly conserved between Enl and the t(9;11) fusion partner AF-9 and is retained in all Hrx-Enl and Hrx-AF9 fusion proteins. Thus, the leukemogenic contribution and transcriptional activation potential of Enl colocalize to its highly conserved carboxy terminus, suggesting that Hrx-Enl chimeric proteins mediate alterations in the transcription program of t(11;19)-bearing cells.

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MATERIALS AND METHODS

Plasmid constructions. Plasmids coding for GAL4 fusion proteins were derived from pSG424, which expresses the GAL4 DNA binding domain (amino acids 1-147) from the SV40 early promoter.15 GAL4-ENL fusion constructs (Fig 1) were made by inserting various fragments of the ENL cDNA into pSG424 downstream and in frame with GAL4 (1-147) using standard cloning techniques. The resulting plasmids expressed GAL4 fused with all of Enl (GE13), amino acids 1-559) or various portions of Enl (Fig 1). Reporter constructs contained two tandem copies of the GAL4 consensus binding site (CAT)6 driven by the herpes simplex virus thymidine kinase promoter (G4tkCAT),17 the adenovirus Elb promoter (G4ElbCAT),18 or the liver/bone/kidney alkaline phosphatase promoter (G4APCAT).19 The reference plasmid used in all experiments was pSVtk-Luc, which contains the SV40 origin of replication and expresses the luciferase gene under control of the HSV thymidine kinase promoter.

Mammalian cell transfections and transactivation assays. Hu-

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man lymphoid (REH, Jurkat) and myeloid (U937) cell lines were transfected as previously described. Briefly, 7.5 × 10⁶ cells were washed in serum-free RPMI medium; resuspended in 2.0 mL RPMI containing 0.75 μg each of reporter and expression plasmid, 0.50 μg of pSVtk-Luc, and 10 μg diethyl aminoethyl (DEAE)-dextran; and electroporated at 300 V, 960 μF. After 24 hours of incubation in 5 mL of RPMI containing 10% fetal calf serum, cells were harvested and CAT and luciferase assays were performed as described.

Transcriptional activation in yeast. Fragments of ENL cDNA were cloned into pAS₂ in-frame with the GALA DNA binding domain. Yeast strain Y190 was transformed to tryptophan and histidine, but including 50 mmom 3-aminotriazole (Sigma, St Louis, MO) and incubated at 30°C for 3 to 5 days. His⁺ colonies were screened for β-galactose activity using a filter lift assay. Yeast harboring pSE1111 (GALA-SNF1) and pSE1112 (GALA-SNF4) were used as positive controls for activation studies (provided by S. Elledge).24

Western blotting. COS-7 cells were transfected with 10 μg of each GAL4-ENL fusion plasmid per 60-mm plate as previously described.23 Cell extracts were prepared after 48 hours, electrophoresed on 10% polyacrylamide gels, and transferred to nitrocellulose by standard techniques. Blots were probed with a rabbit anti-GAL4 antiserum (kindly provided by Dr J. Lipsick, Stanford University, Stanford, CA) and visualized using diaminobenzidine as the substrate for horseradish peroxidase (HRP)-conjugated goat antirabbit IgG.

Immunohistochemistry. COS-7 cells were transfected with constructs that expressed full-length Enl (pBEX-ENLT) or Hrx-Enl (pBEX-HRX-ENLT) containing a nine-amino acid hemagglutinin (HA) epitope at their carboxy-termini. After 48 hours, cells were fixed in 3.7% formaldehyde and probed with a mouse anti-HA antibody (provided by Dr T. Wang, Stanford University, Stanford, CA). Cells expressing epitope-tagged Enl or Hrx-Enl were detected using HRP-conjugated goat anti-mouse IgG.

RESULTS

Transcriptional activation by GAL4-Enl in hematopoietic cells. The ability of Enl to activate transcription of a reporter gene was first tested by transient transfection experiments in human lymphoid cells. The activator plasmid GE₁⁻⁵⁵⁹, which expressed a fusion protein containing full-length Enl, was cotransfected into the Jurkat T-cell lymphoblast cell line along with a reporter gene construct containing two tandem copies of the GAL4 DNA binding site upstream of the HSV thymidine kinase promoter driving expression of the CAT gene (G4tkCAT). As a control for background expression of CAT, pSG424, which expresses amino acids 1-147 of GAL4 and does not transactivate transcription, was cotransfected with pG4tkCAT. GE₁⁻⁵⁵⁹ consistently transactivated the CAT gene, resulting in CAT activity approximately 10-fold higher than background (Figs 1 and 2). In similar experiments, GE₁⁻³⁵⁹ was cotransfected into Jurkat cells with a reporter gene construct containing the GAL4 DNA binding sites upstream of the CAT gene driven by the adenovirus E1b promoter (G4E1bCAT, Fig 1) or the liver/bone/kidney alkaline phosphatase promoter (G4APCAT, data not shown). Mean CAT conversion using these reporters was less than 2%, although both reporters could be transactivated by GAL4-VP16 fusions used as controls (data not shown).

To define the transactivation domain of En1, GAL4 fusion constructs were designed to express various C-terminal or N-terminal portions of Enl (Fig 1). Transcriptional activity was then determined by cotransfection of each plasmid along with G4tkCAT or G4E1bCAT into Jurkat cells (Figs 1 and 2). All constructs that contained the C-terminal 90 amino acids of Enl-activated transcription of G4tkCAT (GE₁⁻⁵⁵⁹ 24% of maximal conversion, GE₁⁻³⁵⁹ 36%, GE₀⁻⁵⁵⁹ 100%, and GE⁻⁵⁵⁹ 42%). Constructs lacking the C-terminus (GE₁⁻⁴⁰⁴, GE₁⁻²¹₂, and GE₁⁻¹⁴⁷) showed little or no activation of the reporter above background levels (8% to 13% conversion). Transfections performed with GE₀⁻⁵⁵⁹ demonstrated that the C-terminal 90 amino acids of Enl were sufficient for activation of the reporter gene. However, none of the GAL4-Enl fusions was able to activate transcription of the CAT gene from the E1b promoter (G4E1bCAT, Fig 1).

The transcriptional transactivation potential of Enl was then analyzed in precursor B cells (REH cell line) and in myeloid cells (U937 cell line) by cotransfection experiments with the GAL4-Enl fusion constructs and the G4tkCAT reporter gene (Fig 3). Although cell-type differences in relative activities were seen, all constructs expressed the C-terminal

![Fig 1. Transactivation in Jurkat cells. Quantitation of transactivation, expressed as percent CAT conversion, for pSG424 and GAL4-ENL fusions in Jurkat cells is shown for the G4E1bCAT and G4tkCAT reporters. Transfections were performed in duplicate and values represent the averages of two to four transfections, with standard deviation bars shown. The most potent activator, GE₁⁻⁵⁵⁹ was normalized to 100% conversion. Schematic diagrams of the constructs are shown on the left: (□) ENL sequences.](https://www.bloodjournal.org/doi/figure/10.1182/blood-1996-07-0505)

![Fig 2. Representative TLC plate of CAT assays performed on Jurkat cell extracts. Cells (3 × 10⁶) were transfected with 300 ng of each activator DNA and 300 ng of G4tkCAT reporter DNA. Lane pSG424 demonstrates the basal level of CAT activity in the absence of transactivation.](https://www.bloodjournal.org/doi/figure/10.1182/blood-1996-07-0506)
90 amino acids of Enl activated transcription of G4kCAT. In REH and U937 cells, amino acids 470-559 of Enl were sufficient for transactivation. Again, no activation of GE1bCAT was observed (data not shown). Thus, the ability of Enl to activate transcription in lymphoid and myeloid cells did not extend to all promoters tested, but rather was confined to the HSV tk promoter, suggesting promoter-specific restrictions of its transactivation properties.

To ensure that lack of activation by GE1-147, GE1-221, and GE1-147 was not due to differences in levels of protein expression, Western blot analysis of extracts from transiently transfected COS7 cells was performed (Fig 4). Using antibodies specific for GAL4, similar levels of protein expression were observed for all constructs (data not shown for GE1-470-559).

**Transactivation by ENL in yeast.** The portion of Enl that was shown to contain transcriptional activation potential in lymphoid and myeloid cells is also highly conserved in a heterologous mammalian protein [AF-9, the t(9;11) fusion partner for Hrx] and in a recently reported yeast protein named Anc1. To test the transcriptional properties of Enl in yeast, the carboxy-terminal 155 amino acids were fused in-frame with GAL4 in the pAS2 vector. The resulting GAL4-Enl470-559 protein was assessed for its ability to activate two different GAL4 reporter genes, the *Escherichia coli* lacZ gene under control of the GAL1 promoter and UASc and the HIS3 gene driven by its minimal promoter and the GAL1 UASG. Yeast expressing GAL4-Enl470-559 grew on medium containing the inhibitor 3-aminotriazole that required activation of the HIS3 gene (Table I). However, they did not activate expression of the β-galactosidase gene as assessed by a filter blot assay (Table 1). The C-terminal 90 amino acids of Enl was also able to transactivate expression of HIS3 (Table 1). Therefore, the carboxy-terminal portion of Enl that demonstrated promoter-selective activation in human lymphoid and myeloid cells showed similar properties in yeast.

**Nuclear localization of Enl.** It has previously been shown that Enl contains a nuclear targeting sequence, suggesting that Enl is a nuclear protein. Our observations that Enl can activate transcription from minimal promoters further supported this hypothesis. To test this directly, a plasmid was constructed to express full-length Enl containing a nine-amino-acid hemagglutinin epitope tag at the C-terminal end (pBEX-ENLT). COS7 cells were transfected with pBEX-ENLT, incubated for 48 hours, and stained with an antiepipitope antibody. As predicted, Enl was exclusively seen in the nucleus, with only background staining in the cytoplasm (Fig 5A). Negative controls performed with no primary antibody, or transfections performed with only vector DNA, showed minimal diffuse background staining (data not shown). Localization of the Hrx-Enl fusion protein that is created by the t(11;19) was similarly analyzed by the construction of an epitope-tagged fusion (pBEX-HRX-ENLT). Immunohistochemistry of COS7 cells transiently transfected with pBEX-HRX-ENLT demonstrated the nuclear localization of Hrx-Enl (Fig 5B).

**DISCUSSION**

Molecular analyses have demonstrated that nearly all HRX breakpoints seen in de novo and secondary leukemias with
11q23 rearrangements cluster in a restricted 8-kb region of HRX. The majority of HRX translocations thus fuse nearly identical N-terminal portions of Hrx, containing the AT hooks, to a variety of partners. Recently, five of these partner genes have been cloned, and are designated AF-4/FEL, AF-9, ENL, AF-6, and AFX1. Sequence analysis has shown that Fel, AF-9, and Enl contain nuclear targeting signals, and that all five proteins are unusually rich in serines and prolines, features that are associated with transcriptional activators. It has been hypothesized that Fel, AF-9, AFX1, and Enl may represent a class of transcriptional transactivators and that the Hrx-Fel, Hrx-AF-9, Hrx-AFX1, and Hrx-Enl fusions function as chimeric oncogenic transcription factors. In addition to the features described above, AF-9 and Enl share two regions of high similarity (Fig 6). These proteins are 82% identical and 92% similar at their N-termini and 82% identical and 91% similar at their C-termini. Recently, a yeast protein (Anc1) that shares significant similarity with Enl and AF-9 has been identified based on its inability to complement mutations in the actin gene. Similar to Enl, Anc1 is a nuclear protein, but its function remains unknown.

In the present study, we have constructed GAL4-ENL fusion genes to directly test whether Enl could influence transcription from minimal promoters. Using transient transfection experiments, we have shown that Enl has a limited but measurable potential to activate transcription of synthetic reporter genes in T-lineage and B-lineage lymphoid cells, as well as in myeloid cells. GAL4-Enl fusion proteins were able to transactivate the HSV thymidine kinase promoter but not two other minimal promoters in these cell lines. It should be noted that the thymidine kinase promoter used in these experiments contains the TATA box as well as upstream sequences, including two Sp1 binding sites and a CAAT element, whereas the Elb and alkaline phosphatase promoters contain only the TATA elements. A similar promoter-restricted activity was observed in yeast, in which Enl was able to transactivate only the HIS3 gene. Thus, our data suggest that the transcriptional activation potential of Enl is limited to a subset of promoters, unlike the properties of many transcriptional activators that have been characterized. These observations suggest that Enl may interact with a conserved component of the transcriptional machinery that is not essential for all promoters or may not be accommodated by the minimal TATA element. Such factors that are components of, or interact with, the basal transcription complex have been described. Alternatively, Enl may be a relatively weak activator that potentiates the transcription of promoters with a low threshold for activation, such as the HSV tk promoter, but does not activate the minimal (TATA box only) promoters of Elb and alkaline phosphatase. Under our transfection conditions, Gal4-Enl is at least 10-fold less...

25. Welch MD, Drubin DG: A nuclear protein with sequence similarity to proteins implicated in human acute leukemias is important for cellular morphogenesis and actin cytoskeletal function in Saccharomyces cerevisiae. Mol Biol Cell (in press)


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