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Enhancer-Binding Activity of the *tal-1* Oncoprotein in Association with the E47/E12 Helix-Loop-Helix Proteins

HAI-LING HSU, JIIN-TSUEY CHENG, QI CHEN,† AND RICHARD BAER*

Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9048

Received 24 January 1991/Accepted 18 March 1991

Almost 30% of patients with T-cell acute lymphoblastic leukemia (T-ALL) bear structural alterations of *tal-1*, a presumptive proto-oncogene that encodes sequences homologous to the helix-loop-helix (HLH) DNA-binding and dimerization domain. Analysis of the *tal-1* gene product reveals that its HLH domain mediates protein-protein interactions with either of the ubiquitously expressed HLH proteins E47 and E12. The resultant *tal-1*/E47 and *tal-1*/E12 heterodimers specifically recognize the E-box DNA sequence motif found in eucaryotic transcriptional enhancers. Hence, the *tal-1* protein shares biochemical properties with other tissue-specific HLH proteins that control cell type determination during myogenesis (e.g., MyoD1) and neurogenesis (e.g., achaete-scute). The data suggest that HLH heterodimers involving *tal-1* may function in vivo as transcriptional regulatory factors that influence cell type determination during hematopoietic development.

Gross rearrangements of the *tal-1* gene (also called *TCL5* or *SCL*) arise in patients with T-cell acute lymphoblastic leukemia (T-ALL) by either of two distinct mechanisms. Approximately 25% of T-ALL patients exhibit a precise 90-kb deletion (designated tal^d) of upstream sequence from one allele of the *tal-1* locus (12); the site specificity of tal^d deletion is apparently mediated by aberrant activity of the immunoglobulin recombinase (2, 12). An additional 3% of T-ALL patients harbor the translocation (1;14)(p34;q11), in which *tal-1* is transposed from its normal location on chromosome 1 into the T-cell receptor α/δ-chain gene on chromosome 14 (4, 5, 15, 17, 18, 23). Hence, alteration of the *tal-1* gene, by either tal^d recombination or t(1;14)(p34;q11) rearrangement, represents the most common genetic lesion known to be associated with human T-ALL (12).

Although its function is not understood, the *tal-1* gene product is homologous to a number of proteins that are involved in the control of cell growth and differentiation (4, 17). The region of homology is restricted to an approximately 60-amino-acid domain that has the potential to form two amphipathic alpha helices separated by an intervening loop (35). Most helix-loop-helix (HLH) proteins also contain clusters of basic amino acids at the amino-terminal flank of the HLH domain that potentially mediate sequence-specific DNA recognition. Indeed, several HLH proteins are proposed to function as transcriptional regulatory factors based on their ability to bind in vitro to the E-box nucleotide sequence motif (CANNTG) found in eucaryotic transcriptional enhancers (3, 6, 7, 9, 11, 14, 19, 25, 28, 29, 31, 35, 36). The enhancer-binding HLH proteins include E47 and E12, two distinct but related polypeptides encoded by the E2A gene (35). The E47 (or E12) protein binds the E box as a homodimer that is apparently stabilized by protein-protein interactions involving the amphipathic helices of their HLH domains. Interestingly, the E2A gene products (E47 or E12) can form heterologous complexes, presumably heterodimers, with other HLH proteins (e.g., MyoD1), and these heterodimers also bind the E-box sequence with high affinity (36).

Murre et al. (36) suggested that the enhancer-binding HLH proteins can be divided into at least two classes: those that are expressed in a broad spectrum of tissues and cell types (class A) and those expressed in a lineage-restricted fashion (class B). They also proposed that the ubiquitous class A proteins, such as E47 and E12, might influence tissue-specific gene expression by heterologous interaction with lineage-specific class B proteins. In support of this argument, they demonstrated the formation of enhancer-binding heterodimers involving the E2A gene products (E47 or E12) and MyoD1, a muscle-specific HLH protein that governs the myogenic conversion of multipotential mesodermal cells (20). MyoD1 shares close sequence homology with three distinct muscle-specific HLH proteins that are also capable of inducing myogenesis (9, 10, 21, 34, 39, 41). Notably, each member of the myogenic family of HLH proteins has now been shown to form enhancer-binding heterodimers with gene products of the E2A locus (9, 11, 19, 36). Moreover, the *Drosophila* homolog of E47/E12 (daughterless) forms enhancer-binding heterodimers with HLH proteins encoded by achaete-scute, a gene complex that controls neural development in *Drosophila* cells (13, 16, 36, 42). Considering the role of the myogenic and achaete-scute proteins in myogenesis and neurogenesis, respectively, it seems feasible that lineage-specific HLH proteins exist that control other major developmental pathways and that these proteins may also function by heterodimer formation with ubiquitously expressed HLH proteins such as E47 and E12. In view of the association between *tal-1* gene rearrangement and T-ALL formation, the *tal-1* gene product represents a worthy candidate for a lineage-specific HLH protein involved in hematopoietic development. In this report we demonstrate that *tal-1* interacts with the E47/E12 proteins in vitro and that the resultant protein complexes recognize the E-box motif in a sequence-specific fashion.

MATERIALS AND METHODS

Bacterial expression of fusion proteins. Bacterial expression of glutathione *S*-transferase (GST) proteins was based

* Corresponding author.
† Present address: Department of Zoology, University of Texas at Austin, Austin, TX 78712.

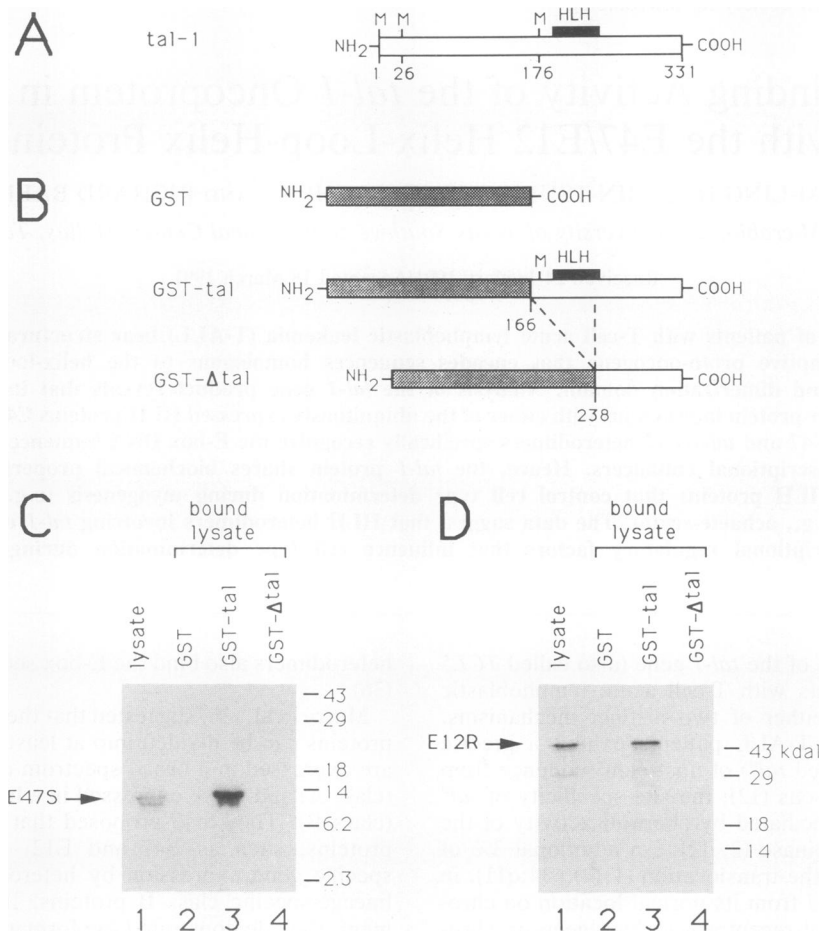

FIG. 1. Direct interaction between *tal-1* and the HLH proteins E47 and E12. (A) Schematic diagram of the *tal-1* gene product. Sequences corresponding to the HLH domain are indicated, and the positions of the three potential methionine initiation codons are indicated (1). (B) Schematic diagrams illustrating the composition of the GST proteins. GST is the 26-kDa wild-type GST encoded by expression vector pGEX-2T. GST-*tal* is a fusion protein composed of the GST sequence and the carboxy-terminal 156 amino acids of *tal-1*. GST-Δ*tal* is a fusion protein composed of GST and the carboxy-terminal 94 residues of *tal-1*. Sequences are numbered according to their positions in the wild-type *tal-1* protein. (C) Binding of E47S by the *tal-1* HLH domain. Each GST protein was incubated with [^{35}S]methionine-labeled E47S and subsequently exposed to glutathione-agarose beads. After extensive washing, the beads were treated with 50 μl of 15 mM glutathione, and the eluant was fractionated by electrophoresis on a 15% SDS-polyacrylamide gel. An aliquot of untreated reticulocyte lysate containing radiolabeled E47S was fractionated in lane 1. The other lanes represent radiolabeled E47S retained in the binding assays with wt-GST (lane 2), GST-*tal* (lane 3), and GST-Δ*tal* (lane 4). The bifurcation of the E47S band in lane 1 is due to overloading of the gel with reticulocyte lysate; electrophoresis of smaller aliquots of lysate results in a single E47S band. (D) Binding of E12R by the *tal-1* HLH domain. Each GST protein was incubated with [^{35}S]methionine-labeled E12R. Subsequent treatments were the same as those described for E47S.

on the pGEX vector system developed by Smith and Johnson (40). Plasmid pGEX-2T was used for expression of wild-type GST protein. Plasmid *tal*cZ1-0.95/pGEX-2T, used for expression of the GST-*tal* fusion protein, was generated by inserting a 0.95-kb *tal-1* cDNA fragment into the *Bam*HI site of pGEX-2T. Plasmid Δ*tal*c1-1/pGEX-1 contained a 1.7-kb *tal-1* cDNA fragment in the *Eco*RI site of pGEX-1 and was used for expression of the GST-Δ*tal* fusion protein. The GST proteins were expressed in plasmid-transformed JM101 cells and were purified to homogeneity by affinity chromatography on glutathione-agarose essentially as described by Smith and Johnson (40).

In vitro transcription-translation reactions. Linearized plasmid DNAs were used as templates for in vitro RNA transcription. Plasmids encoding E47S and E12R have been described by Murre et al. (35). Plasmid pV2C11Aα, prepared by Davis et al. (20), contains the MyoD1 reading frame within a 1.8-kb *Eco*RI fragment. Plasmid *tal*/T7pGEM contains a 0.95-kb *tal-1* cDNA fragment in the *Bam*HI site of pGEM4 (Promega); in this cDNA, in vitro translation initiates at an in-frame AUG codon corresponding to residue 176 of the *tal-1* gene product (Fig. 1A). RNA was synthesized from linearized plasmid DNA templates (2 μg) in 100-μl reactions (2 h at 37°C), using T3 or T7 RNA polymerase in the presence of RNasin (Promega). In vitro translation of RNA in rabbit reticulocyte lysates was conducted in either the presence or absence of [^{35}S]methionine (New England Nuclear) as instructed by the manufacturer (Promega).

Protein-protein binding assay. For each binding reaction, 20 μl of purified GST protein (0.25 μg/μl in buffer C) was mixed with 20 μl of a reticulocyte lysate containing [^{35}S]methionine-labeled E47S or E12L. After a short incubation (37°C, 30 min), the reaction mixture was diluted by the

addition of 760 μl of buffer C, 100 μl of 10% Triton X-100, and 100 μl of glutathione-agarose beads (7 mg/ml in buffer C; Sigma). After shaking for 2 h at 4°C, the beads were pelleted by centrifugation (5 s at 12,000 × *g*), and the supernatant was removed. The beads were subjected to three successive washes (shaking at 4°C for 10 min) in 1 ml of buffer C. Finally, the beads were resuspended in 50 μl of 15 mM glutathione (in buffer C), and the eluant was fractionated by electrophoresis on a sodium dodecyl sulfate (SDS)-polyacrylamide gel. Buffer C has been described elsewhere (40).

Preparation of antisera. Anti-*tal* antisera were produced by immunizing rabbits with the GST-Δ*tal* fusion protein (Fig. 1B) (26). Anti-*lyl* antisera were produced by immunizing rabbits with GST-Δ*lyl*, a fusion protein containing the carboxy-terminal 43 residues of the *lyl-1* protein (33). The specificities of the antisera were confirmed by immunoprecipitation of in vitro-translated *tal-1* and *lyl-1* polypeptides (data not shown) (26).

EMSA. Reading 5′ to 3′, sequences of the upper strand of each complementary pair of oligonucleotides are as follows: μE2, CCTGCAGCAGCTGGCAGGAA; μE5, GAACCAG AACACCTGCAGCA; and mutant μE2, CCTGCAGCCG CTTGCAGGAA. To prepare radiolabeled DNA, each oligonucleotide (20 μl at 10 μM) was mixed with an equivalent amount of its complementary oligonucleotide, heated at 88°C for 2 min, and annealed by slow cooling to room temperature. The oligonucleotides were then labeled by reaction with T4 polynucleotide kinase (New England BioLabs) in the presence of [γ-^{32}P]ATP (6,000 Ci/mmol; New England Nuclear); unincorporated label was removed by chromatography on Bio-Gel P6 (Bio-Rad). The electrophoretic mobility shift assays (EMSAs) were conducted as described by Davis et al. (19) except that *Escherichia coli* DNA (1 μg per EMSA reaction) was used as the nonspecific competitor.

RESULTS AND DISCUSSION

To determine whether *tal-1* interacts with E47 or E12, we developed a simple binding assay using *tal-1* fusion proteins produced in bacteria. *tal-1* cDNA sequences were inserted into a GST expression vector to generate fused GST-*tal* open reading frames. Wild-type GST and GST fusion proteins were then produced in *E. coli* and isolated to homogeneity by affinity chromatography on glutathione-agarose (40). The three GST polypeptides used in this study are illustrated in Fig. 1B: wt-GST, the wild-type GST; GST-*tal*, a fusion protein containing the carboxy-terminal 156 residues of *tal-1*, including the HLH domain and its associated basic region; and GST-Δ*tal*, a fusion protein containing the carboxy-terminal 94 residues of *tal-1* but not including the HLH domain. Each of the three GST proteins was mixed with a [^{35}S]methionine-labeled E47 polypeptide produced by in vitro translation in reticulocyte lysates; the polypeptide (designated E47S) consists of the carboxy-terminal 90 residues of E47, including the entire E47 HLH domain (35). After a short incubation, the GST proteins were absorbed to glutathione-agarose beads, and the beads were extensively washed so that retention of radiolabeled E47S on the beads would represent a measure of its specific interaction with the GST protein. The absorbed proteins were then eluted from the beads by competition with free glutathione, fractionated by denaturing polyacrylamide gel electrophoresis, and detected by autoradiography (Fig. 1C). In vitro translation of E47S RNA generates a radiolabeled polypeptide with the expected molecular weight for the 90-residue E47S protein (Fig. 1C, lane 1). As illustrated in lane 3, radiolabeled E47S

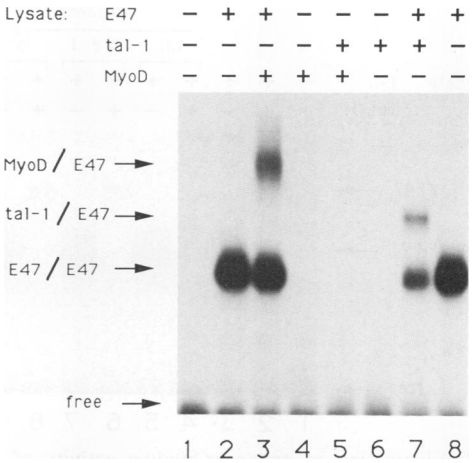

FIG. 2. DNA binding by heterodimers of the *tal-1* and E47 HLH proteins. ^{32}P-labeled μE2 oligonucleotide was incubated with the indicated combinations of in vitro-translated *tal-1*, MyoD1, and E47S polypeptides. After electrophoresis on a native 5% polyacrylamide gel, the radiolabeled oligonucleotides were detected by autoradiography. As illustrated, free μE2 oligonucleotides migrated rapidly toward the anode. Complexed oligonucleotides with slower electrophoretic mobilities are denoted with arrows, and the presumptive protein composition of each complex is indicated.

was readily absorbed to glutathione-agarose when incubated with the GST-*tal* fusion protein. Absorption of E47S was clearly mediated by interaction with the *tal* moiety of GST-*tal* since E47S absorption did not occur in the presence of wild-type GST alone (lane 2). Moreover, GST-Δ*tal*, which differs from GST-*tal* only in that it lacks the *tal-1* HLH domain (Fig. 1B), did not promote absorption of E47S (Fig. 1C, lane 4). Therefore, the specific interaction between *tal-1* and E47S is mediated by the HLH domain of *tal-1*.

The same assay was used to evaluate protein-protein interaction between *tal-1* and E12R, a polypeptide consisting of the carboxy-terminal 440 residues of E12 (35). In vitro translation of E12R RNA generates a major radiolabeled polypeptide of the expected size, as well as a number of minor products of lower molecular weights (Fig. 1D, lane 1). The intact E12R polypeptide and a subset of the smaller radiolabeled products were specifically absorbed to glutathione-agarose when incubated with the GST-*tal* fusion protein (lane 3) but not when incubated with GST (lane 2) or GST-Δ*tal* (lane 4). Hence, specific interaction between *tal-1* and E12 is also dependent on the HLH domain of *tal-1*.

The DNA-binding properties of *tal-1* were analyzed by an EMSA using as probes oligonucleotides representing each of two well-characterized E-box sequences from the murine immunoglobulin heavy-chain gene enhancer (μE2 and μE5) (22, 32). For these studies, a polypeptide consisting of the 156 carboxy-terminal residues of *tal-1* was produced by in vitro translation in reticulocyte lysates of in vitro-transcribed *tal-1* RNA. The MyoD1 protein (318 amino acid residues) and truncated polypeptides representing E12 (E12R; the carboxy-terminal 440 residues) and E47 (E47S; the carboxy-terminal 90 residues) were also generated by in vitro translation; each of these proteins, including the truncated *tal-1*, E12, and E47 polypeptides, contains its entire HLH domain along with the associated basic region. Figure 2 illustrates an EMSA in which a radiolabeled oligonucleotide representing the μE2 sequence was incubated with various combinations of in vitro-translated HLH polypep-

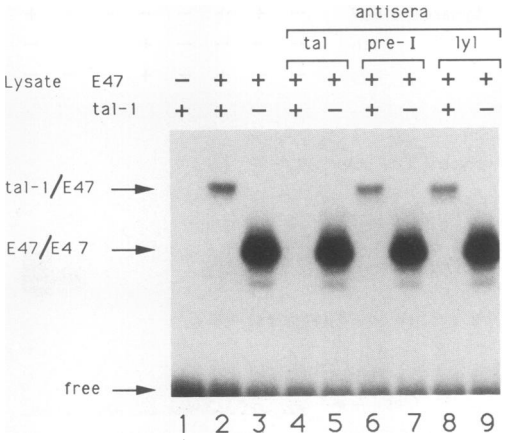

FIG. 3. Elimination of enhancer-binding activity of the *tal-1*/E47S heterodimer by anti-*tal* antibodies. ^{32}P-labeled μE5 oligonucleotide was incubated with the indicated combinations of in vitro-translated *tal-1* and E47S polypeptides. Protein-oligonucleotide complexes involving the E47S/E47S and *tal-1*/E47S dimers are designated with arrows. The indicated reactions were supplemented with 1 μl of rabbit anti-*tal* antiserum (lanes 4 and 5), preimmune serum (pre-I) from the same animal (lanes 6 and 7), or rabbit anti-*lyl* antiserum (lanes 8 and 9).

tides and fractionated by native polyacrylamide gel electrophoresis. The MyoD1, E12R, and E47S polypeptides generated protein-DNA complexes similar to those previously described (36). Hence, E47S bound specifically to the μE2 oligonucleotide (Fig. 2, lane 2), presumably as a homodimer. Although MyoD1 did not by itself bind μE2 (lane 4), in the presence of E47S a distinct complex was formed (lane 3) with an electrophoretic mobility consistent with that of the E47S/MyoD1 heterodimer (36). The behavior of the *tal-1* polypeptide in this assay was analogous to that of MyoD1. Thus, protein-DNA complex formation was not observed when the μE2 oligonucleotide was incubated with *tal-1* alone (lane 6) or with *tal-1* in the presence of MyoD1 (lane 5). In contrast, a novel protein-DNA complex was generated when μE2 was exposed to the *tal-1* and E47S polypeptides simultaneously (lane 7). Since this species is formed only when both *tal-1* and E47S are present, it likely represents a heterologous complex, presumably a heterodimer, involving both polypeptides (lane 7). Results of additional EMSAs indicate that *tal-1* also forms a heterologous DNA-binding complex with the E12R polypeptide (data not shown).

To confirm that the putative *tal-1*/E47 protein-DNA complex does indeed involve *tal-1*, we attempted to block its formation with *tal*-specific antibodies. For this purpose, antisera that recognize the carboxy-terminal domain of *tal-1* were prepared by immunizing rabbits with the GST fusion protein GST-Δ*tal* (Fig. 1B). As illustrated in Fig. 3, the *tal-1*/E47S protein-DNA complex was eliminated by incubation with anti-*tal* antisera (lane 4) but not with preimmune sera from the same animal (lane 6) or with irrelevant antisera (e.g., anti-*lyl*; lane 8). As expected, incubation with anti-*tal* antisera had no effect on formation of the E47S/E47S complex (lane 5).

The specificity of DNA binding by *tal-1* was also evaluated by EMSA using unlabeled DNA as the competitor. In these experiments, an excess of unlabeled μE2 oligonucleotide readily competed with radiolabeled μE2 for binding to the *tal-1*/E47S heterodimer; in contrast, a mutant μE2 oligonucleotide with base substitutions in the E-box motif (CCGCTT in place of CAGCTG) did not compete (data not shown). Similar results were obtained for the *tal-1*/E12R heterodimer. Hence, the *tal-1*/E47S and *tal-1*/E12R complexes bind DNA in a sequence-specific manner that is dependent on nucleotide residues within the E-box motif.

The experiments described above demonstrate that *tal-1* interacts directly with the ubiquitous HLH proteins E47 and E12 and that the resultant *tal-1*/E47 and *tal-1*/E12 heterodimers recognize the E-box DNA motif in a sequence-specific fashion. At this level, the biochemical properties of *tal-1* are reminiscent of those described for the tissue-specific HLH proteins MyoD1 and achaete-scute T3 (36). Since the E-box motif is a functional component of numerous eucaryotic transcriptional enhancers, it is plausible that HLH heterodimers involving *tal-1* serve in vivo as transcriptional regulatory factors. In this report we have described *tal-1* interactions with μE2 and μE5, two well-characterized E-box sequences associated with the immunoglobulin heavy-chain gene enhancer (22, 32). The immunoglobulin enhancer is unlikely to be a relevant target of transcriptional regulation by *tal-1* during T-ALL formation. To fully understand the role of *tal-1* in human T-ALL, it will be necessary to identify those genes whose expression is modulated in vivo by *tal-1*. Thus, future studies will focus on potential subordinate targets of *tal-1* regulation, including the c-*myb* proto-oncogene and the T-cell receptor β- and δ-chain genes, all of which are expressed during T-cell development and harbor E-box motifs within their associated transcriptional regulatory sequences (8, 27, 30, 37, 38).

Human T-ALL is characterized by the accumulation of immature lymphoblasts in bone marrow and peripheral blood. Hence, malignant transformation appears to disrupt the normal pattern of T-cell development such that maturing T-ALL cells are arrested, and consequently accumulate, at an early stage of lymphoid differentiation (24). The interpretation of T-ALL as a developmental disorder is especially intriguing in view of its strong association with *tal-1* gene alteration. In this report we demonstrate that *tal-1* shares biochemical properties with the tissue-specific HLH proteins MyoD1 and achaete-scute. In view of their biochemical similarities, it is conceivable that each of these proteins performs an analogous function within the cellular lineage in which it is expressed. The MyoD1 and achaete-scute genes were identified on the basis of their ability to control cell type determination during myogenesis and neurogenesis, respectively. We propose, therefore, that the normal function of *tal-1* is to regulate cell type determination at certain stages of hematopoiesis. This hypothesis is consistent with the notion that *tal-1* gene dysfunction can be a critical factor in T-cell leukemogenesis. Hence, the *tal-1* alterations observed in human T-ALL might impair differentiation along the T-cell lineage and thereby elicit the accumulation of immature T lymphoblasts that typifies this malignancy.

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