E2A-HLF-Mediated Cell Transformation Requires both the trans-Activation Domains of E2A and the Leucine Zipper Dimerization Domain of HLF

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The E2A-HLF fusion gene, formed by the t(17;19)(q22;p13) translocation in childhood acute pro-B-cell leukemia, encodes a hybrid protein that contains the paired trans-activation domains of E2A (E12/E47) linked to the basic region/leucine zipper DNA-binding and dimerization domain of hepatic leukemia factor (HLF). To assess the transforming potential of this novel gene, we introduced it into NIH 3T3 murine fibroblasts by using an expression vector that also contained the neomycin resistance gene. Cells selected for resistance to the neomycin analog G418 formed aberrant colonies in monolayer cultures, marked by increased cell density and altered morphology. Transfected cells also grew readily in soft agar, producing colonies whose sizes correlated with E2A-HLF expression levels. Subclones expanded from colonies with high levels of the protein reproducibly formed tumors in nude mice and grew to higher plateau-phase cell densities in reduced-serum conditions than did parental NIH 3T3 cells. By contrast, NIH 3T3 cells expressing mutant E2A-HLF proteins that lacked either of the bipartite E2A trans-activation domains or the HLF leucine zipper domain failed to show oncogenic properties, including anchorage-independent cell growth. Thus, both of the E2A trans-activation motifs and the HLF leucine zipper dimerization domain are essential for the transforming potential of the chimeric E2A-HLF protein, suggesting a model in which aberrant regulation of the expression pattern of downstream target genes contributes to leukemogenesis.

A variety of chimeric proteins, many involving transcription factors, are produced by chromosomal translocations in specific types of leukemias and soft-tissue sarcomas (20, 25). The sequence identity of such proteins with master proteins that regulate pattern formation during the earliest stages of embryogenesis in Drosophila melanogaster (reviewed in reference 20) suggests that their normal counterparts play critical regulatory roles in mammalian development (26). Thus, truncation and fusion of transcription factor genes as a result of interchromosomal rearrangements are a prominent theme in human oncogenesis. One example is the E2A-PBX1 fusion gene of pre-B-cell acute lymphoid leukemia (ALL) (16, 23), in which E2A (E12/E47), a basic region/helix-loop-helix (bHLH) gene, is fused to the PBX1 homeobox gene by a t(1;19)(q23:p13) translocation. E2A-PBX1 encodes two proteins, p77E2A-PBX1 and p85E2A-PBX1 (15), which bind to DNA in a sequence-specific manner through the PBX1 homeodomain and trans activate reporter gene expression in constructs containing the binding site (19, 21, 30). Other studies have shown that E2A-PBX1 efficiently transforms NIH 3T3 cells (15), causes acute myeloid leukemia when present in mouse bone marrow progenitor cells transplanted to lethally irradiated recipients (14), and reproducibly induces T-cell leukemia/lymphoma in transgenic mice (5). These results indicate that E2A-PBX1 can mediate oncogenic conversion in a range of murine cell types, while in humans its transforming ability has been associated exclusively with pre-B-cell ALL.

We and others identified a second fusion gene, resulting from a t(17;19)(q22;p13) translocation in human pro-B-cell ALL (11, 12), in which E2A sequences are joined with those of HLF, which encodes hepatic leukemia factor (HLF), a member of the basic region/leucine zipper (bZIP) superfamily of transcription factors (31). HLF belongs to the proline- and acidic amino acid-rich bZIP subfamily (6, 17), which also includes DBP (22), an albumin gene promoter D-box-binding protein, and thyrotroph embryonic factor, a protein expressed concurrently with the thyroid-stimulating hormone β gene during anterior pituitary development (6). HLF and E2A-HLF recombinant proteins produced in vitro bind to the consensus sequence 5'-GT TACGTAAT-3', as do E2A-HLF proteins in nuclear extracts of a leukemic cell line harboring the t(17;19) (10, 13). E2A-HLF functions through this sequence motif as a potent trans activator of reporter gene expression in a wide variety of cell types (10, 13). In contrast, the unrearranged HLF protein is active in 293 human (13) and CV-1 (10) monkey kidney cells but not in NIH 3T3 murine fibroblasts or HepG2 hepatocarcinoma cells (13), suggesting tissue-specific regulation of the HLF effector domain. These results suggest that E2A-HLF may be able to subvert transcriptional programs that normally control the growth, differentiation, and survival of cells of diverse lineages (13).

Here we show that the E2A-HLF fusion protein induces anchorage-independent growth of NIH 3T3 mouse fibroblasts and renders these cells tumorigenic in nude mice. Proteins lacking either of the trans-activation domains of E2A or the leucine zipper dimerization domain of HLF are inactive, suggesting that both dimerization and the ability to trans activate gene expression are essential components of E2A-HLF-induced tumorigenesis.

MATERIALS AND METHODS

Construction of eukaryotic expression vectors. Expression plasmids containing wild-type and mutated E2A-HLF cDNAs, as well as the neomycin resistance...
genetic, were constructed with the pRc/RSV (pRc) vector (Invitrogen, San Diego, Calif.). Mutant cDNAs (Fig. 1) included (i) a ΔAD1 mutant that lacks a 450-bp fragment including the initiator codon (activation domain 1 [AD1]), which encodes a mutant protein originating at Met-143, (ii) a ΔHL1 mutant that lacks a 405-bp PvuI-NarI restriction fragment (Leu-278 to Ala-412) (loop helix [LH]), and (iii) a ΔZIP mutant that was truncated by translation of the E2A-HLF cDNA at a BouHI restriction site, which encodes a mutant protein lacking leucine zipper domain amino acids from Ala-535 to the carboxyl terminus of the E2A-HLF protein. The ΔAD1 and ΔHL1 mutant proteins each lack one of the E2A trans-activation domains, while the ΔZIP mutant lacks most of the leucine zipper domain.

Cell culture and transformation assay. Subconfluent mouse NIH 3T3 fibroblast cells, kindly provided by Douglas Lowy (Dermatology Branch, National Cancer Institute, Bethesda, Md.), were stored at low passage number and used in all experiments. The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal calf serum. A total of 5 × 10^4 cells were plated in 100-mm-diameter tissue culture dishes the day before transfection, and 30 μg of plasmid DNA was added to the monolayer cells after the DNAs were coprecipitated with calcium phosphate (4). Focus formation assays were performed by culturing the transfected cells for 2 to 4 weeks in medium with or without the neomycin analog G418 (800 μg/ml) and examining cell morphology by phase microscopy; cell monolayers were also analyzed after fixation in methanol and staining with Wright-Giemsa stain. Soft-agar assays were performed by resuspending the cells (5 × 10^3 or 2 × 10^4) in Iscove’s modified Dulbecco’s medium containing 15% fetal calf serum and 0.3% Noble agar (Difco, Detroit, Mich.), after which this mixture was plated on a base layer containing 0.6% Noble agar. Colonies were scored 2 to 4 weeks after plating. To determine cell growth curves, we picked subclones from agar, expanded them by monolayer culture, and plated 2 × 10^3 cells in 35-mm-diameter dishes, and then cultured the cells in Dulbecco’s modified Eagle’s medium with 5% fetal calf serum. The cell number was determined by counting viable cells per dish from triplicate cultures harvested by trypsinization and then expressed as the number of viable cells per dish.

Preparation of bacterially expressed E2A protein and production of anti-serum. We used the pGEX-3X plasmid vector (Pharmacia, Piscataway, N.J.) to subclone a 633-bp EcoRI/Ncol E2A cDNA restriction fragment (amino acids 16 to 226). Recombinant glutathione S-transferase fusion proteins were purified from bacterial cell lysates with use of glutathione beads and digested with factor Xa to release the E2A protein (29). Rabbits were injected with recombinant protein every 4 weeks to raise polyclonal rabbit antisera. An HLF(C) antisera prepared by injection of the recombinant bZIP domain of the HLF protein used in this study was characterized and described elsewhere (13).

Metabolic labeling and immunoprecipitation. Cells were metabolically labeled by incubation in methionine-free medium for 30 min. [35S]methionine (New England Nuclear, Boston, Mass.) was added (0.5 μCi/ml) and the cell were incubated for an additional 30 min. The medium was then removed, and cells were lysed in dissociation buffer (0.5% sodium dodecyl sulfate [SDS], 1 mM dithiothreitol, 50 mM Tris-HCl [pH 7.4], 1 mM EDTA) and immediately boiled for 5 min. The boiled lysates were diluted with a 4 × volume of radioimmunoprecipitation assay buffer without SDS (130 mM NaCl, 1% NP-40, 50 mM Tris-HCl [pH 8.0], 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA). The cell lysates were clarified by centrifugation at 100,000 × g for 30 min. Lysates containing the same amount of protein were then mixed with designated rabbit antisera for 60 min at 4°C, and immune complexes were collected by incubation with protein A-Sepharose (Pharmacia) for 30 min. The proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by autoradiography. The level of protein expression was determined by use of a PhosphorImager (Molecular Dynamics) as instructed by the manufacturer.

In vitro transcription-translation and electrophoretic mobility shift analysis. Linearized pBluescript plasmids (Stratagene, La Jolla, Calif.) containing human cDNAs were transcribed with an mCap mRNA capping kit (Stratagene). In vitro transcription-translation was performed in rabbit reticulocyte lysate (Promega) containing 15% fetal calf serum and 0.3% Noble agar (Difco, Detroit, Mich.), as instructed by the manufacturer. Electrophoretic mobility shift assay binding reactions were performed by adding a 32P-end-labeled DNA oligonucleotide probe (2 × 10^5 cpm) containing an HLF consensus binding site sequence (5′-GGAATACCATTTATTCGCCGATGTT-3′) in 10 μl of binding buffer (12% glycerol, 1 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES; pH 7.9], 4 mM Tris [pH 7.9], 133 mM KCl, 1.5 μg of sheared calf thymus DNA, 300 μg of bovine serum albumin per ml) to 5 μl of unlabeled in vitro transcription and translation products previously described (13), and incubating this mixture at 30°C for 15 min. The relative amounts of each translated product were determined in duplicate reactions from the results of SDS-PAGE analysis of [35S]methionine-labeled protein. Nondenaturing polyacrylamide gels containing 4% acrylamide and 2.5% glyceral were prerun at 4°C in a high-ion-strength Tris-glycine buffer for 30 min, loaded with the samples containing protein-DNA complexes, run at 35 mA for approximately 90 min, dried under vacuum, and analyzed by autoradiography (3).

Transactivation assay. To construct reporter gene plasmids, we inserted sites of either the authentic (pCATpro-CS) or altered (pCATpro-M4) HLF binding consensus sequence into the BglII site immediately upstream of the minimal simian virus 40 early gene promoter driving the chloramphenicol acetyltransferase (CAT) gene (pCATpromoter; Promega). The sequences of the inserts are 5′-AGATGTCATGATACGTAGAAGTCGATGACGGATCT-3′ for pCATpro-CS and 5′-AGATGTCATGACGTACGATAGGATCTTGGAGATCGATCT-3′ for pCATpro-M4 (mismatches are in boldface type). Transfections with calcium phosphate and CAT assays were performed as previously described (28). Percent acetylation was measured with a PhosphorImager as instructed by the manufacturer. CAT assay results were corrected for transfection efficiency on the basis of secreted alkaline phosphatase activity obtained by cotransfection with a pCMV-SP6 vector; secreted alkaline phosphatase activity was measured as described by Bram et al. (2).
cellular and four morphological flat colonies were chosen at random and expanded for analysis of protein expression. Metabolically labeled lysates were immunoprecipitated with an HLF(C) antiserum that recognizes the bZIP domain of the HLF protein. The 62-kDa E2A-HLF chimera was readily detected in a mixed pool of G418-selected, pReE2A-HLF-transfected NIH 3T3 cells (Fig. 2C, lanes 2 and 4) and expressed at the same or higher levels in four subclones established from hypercellular colonies (F1 to F4; lanes 5 to 8). In subclones established from four morphologically flat, contact-inhibited colonies (L1 to L4; lanes 9 to 12), the protein was either undetectable or present in only slight amounts, suggesting that higher levels of E2A-HLF expression are related to the hypercellularity and morphologic changes in transfected cells.

E2A-HLF imparts anchorage-independent cell growth and tumorigenicity. NIH 3T3 cells transfected with pReE2A-HLF and selected with G418 reproducibly formed cells in soft agar, although at relatively low efficiency (55 to 87 colonies per 2 \times 10^5 cells plated). The pRe vector-transfected cells, by contrast, did not exhibit anchorage-independent growth (Table 1; Fig. 3A and B). The contribution of the E2A-HLF protein to this finding was demonstrated by expanding six subclones from soft agar colonies (SA1 to SA6) and immunoprecipitating [35S]methionine-labeled lysates of each cell population with the HLF(C) antiserum. The cells consistently expressed approximately 3 (SA3) to 10 (SA1, SA2, and SA4 to SA6) times more E2A-HLF protein than did the mixed G418-selected cell population (Fig. 3G, lanes 2 to 8).

To test whether the anchorage-independent colony size and number (cloning efficiency) of each subclone were related to the expression level of E2A-HLF protein, we plated the pReE2A-HLF-transfected cell subclones in soft agar, together with NIH 3T3 cells expressing v-fms as a positive control. Three of the subclones derived from morphologically flat monolayer colonies with low levels of E2A-HLF expression (L1, L3, and L4; Fig. 2C) failed to grow in soft agar (Table 2), while three subclones derived from agar colonies that overexpressed E2A-HLF (SA1, SA2, and SA4) showed a cloning efficiency and colony size equivalent to those of v-fms-transformed cells (Table 2; Fig. 3C, D, and F). The SA3 cell subclone that expressed intermediate levels of the E2A-HLF protein formed smaller colonies with lower efficiency than did the subclones expressing higher levels of E2A-HLF (Table 2; Fig. 3E). These observations suggest that higher levels of expression of the E2A-HLF protein are required to promote anchorage-independent growth of NIH 3T3 cells in soft agar.

The tumorigenicity of two of the subclones overexpressing E2A-HLF (SA1 and SA2) was tested by subcutaneous injection of 5 \times 10^6 cells per site into nude mice. Six to eight weeks later, SA1 cells formed tumors in two of three mice, while SA2 cells were tumorigenic in one of three mice at the injection site. Tumors did not arise in three mice injected with negative control (pRe-transfected) cells after more than 12 weeks of observation. Tumor cells that were transferred back into tissue culture overexpressed the E2A-HLF protein (data not shown).

Morphologic features and growth profile of E2A-HLF-transformed NIH 3T3 cells. The subclones that overexpressed E2A-HLF showed a transformed morphology, characterized by small, spindle-shaped cells that overgrew one another (SA1 and SA2; Fig. 4A and B), in contrast to subclones derived from flat monolayer colonies (L1 and L3; Fig. 4C and D). Results with the SA2 subclone demonstrate that overexpression of E2A-HLF allows cells to partially overcome contact inhibition and achieve approximately twofold-higher concentrations at plateau phase. Such cells grew to a density of 2.71 \times 10^6 cells per cm^2, compared with 1.17 \times 10^5 cells per cm^2 for the nontransfected NIH 3T3 line and 1.22 \times 10^5 cells per cm^2 for the control pRe-transfected cells. Nevertheless, the transformed growth properties of cells overexpressing E2A-HLF were clearly less pronounced than those of v-fms-transformed cells, which did not exhibit contact inhibition or reach plateau phase (Fig. 5).

E2A-HLF proteins with altered functional domains. In experiments to elucidate the mechanisms by which E2A-HLF contributes to the transformation of NIH 3T3 cells, we relied on three functionally distinct mutants (Fig. 1). The ΔAD1 and ΔLH vectors expressed mutant proteins lacking either the AD1 or LH domain of the E2A protein; each domain contributes independently to its trans-activation properties (1, 24). The ΔZIP vector expresses a mutant protein without the leucine zipper domain of the HLF protein, which is necessary for protein dimerization.

Figure 6 shows the DNA-binding abilities of the mutant proteins by electrophoretic mobility shift analysis and trans-activating potential in transient transfections of CAT reporter plasmids. Equal amounts of proteins produced by in vitro transcription and translation were mixed with a [32P]-end-labeled, double-stranded oligonucleotide probe containing a 10-bp HLF consensus sequence (GTACGCTAAT) (13) and then analyzed by electrophoresis in a nondenaturing gel. Two DNA-protein complexes were detected in reaction mixtures containing wild-type E2A-HLF proteins made by in vitro transcription and translation (Fig. 6A, lane 2), as we have previously reported (13). A faster-migrating complex was detected in binding reactions containing the ΔAD1 and ΔLH proteins, while no complexes were observed in those containing the ΔZIP protein (lanes 3 to 5), suggesting that the ΔZIP mutant protein no longer binds to the consensus sequence.

We next tested the trans-activation potential of the mutated proteins in CAT reporter assays. Eukaryotic expression vectors designed to express wild-type or mutant E2A-HLF proteins were cotransfected into NIH 3T3 cells with reporter plasmids containing either two HLF consensus DNA-binding sites (pCATpro-CS) or two mutant HLF consensus DNA-binding sites with four mismatched base pairs (pCATpro-M4), upstream of a minimal simian virus 40 promoter (see Materials and Methods). In cells transfected with constructs producing the wild-type E2A-HLF protein together with the HLF consensus sequence, CAT activity was four- to fivefold higher than in control cells in which the reporter plasmid contained a mutated binding site. Although expressed at equivalent levels, none of the mutant E2A-HLF proteins were able to transactivate CAT expression above the background level (Fig. 6B), as expected from their inability to form transcriptionally active
FIG. 2. E2A-HLF-transformed NIH 3T3 cell colonies in monolayer culture. Cells were transfected with either the empty pRc vector (A) or the pRcE2A-HLF expression construct (B) and cultured in selection medium containing G418 for 2 weeks. Cells attached to the dishes were fixed and stained with Wright-Giemsa stain. E2A-HLF-transfected colonies were scored as either flat or hypercellular; flat colonies resembled those produced by transfection with the empty vector. (C) NIH 3T3 cell subclones were expanded from flat monolayer (L) or hypercellular (F) colonies, and metabolically labeled lysates were immunoprecipitated with the preimmune serum (lane 1) or HLF(C) antiserum (lanes 2 to 12). Results are shown for cells transfected with the pRc (lane 3) or pRcE2A-HLF (lanes 1, 2, and 4) vector and for single-cell subclones from hypercellular (F1 to F4; lanes 5 to 8) or flat monolayer (L1 to L4; lanes 9 to 12) colonies. The mobility of the 62 kDa E2A-HLF protein is indicated by an arrowhead.
dimers (ΔZIP) or to act as a functional activator (ΔAD1 and ΔLH).

The E2A-HLF LH and leucine zipper domains are required for transformation. Mutant E2A-HLF expression vectors were transfected into low-passage-number NIH 3T3 cells by the calcium phosphate method, and the cells were selected in medium containing G418. Metabolically labeled lysates of each G418-resistant cell population were immunoprecipitated with either HLF(C) or E2A antiserum (Fig. 7). E2A-HLF wild-type (lanes 2, 5, and 8), ΔAD1 (lane 3), ΔLH (lane 6), and ΔZIP (lane 9) proteins were readily detected in these stably transfected cell lines. In addition, both wild-type and the indicated mutant E2A-HLF proteins were analyzed by immunofluorescence staining, and each was expressed in the nuclei of transfected NIH 3T3 cells (data not shown). Despite expression levels of the mutant E2A-HLF cDNAs comparable to wild-type levels, these mutants failed to form hypercellular colonies in monolayer culture and were unable to generate colonies in soft agar (Table 1). Thus, both the functional trans-activation domain from the E2A portion of the protein and the leucine zipper protein-protein interaction domain of HLF are essential for the ability of the chimeric protein to alter the growth properties of NIH 3T3 cells.

DISCUSSION

At the outset of this study, we had shown that the E2A-HLF hybrid protein could bind to DNA in a sequence-specific manner, recognizing a bZIP-related core dyad symmetric motif, and could trans activate the expression of artificial reporter genes (13). Although suggestive of a role in transcriptional regulation, these properties did not establish the oncogenic potential of the protein or address whether both the trans-activation domain of E2A and the leucine zipper dimerization domain of HLF are required for malignant conversion. Here we demonstrate that the ability of E2A-HLF to induce anchorage-independent cell growth depends on dimerization mediated through the leucine zipper domain and the capacity to
trans-activate transcription through the E2A portion of the hybrid protein. In addition, cells overexpressing E2A-HLF formed tumors in nude mice, demonstrating that the protein contributes to the oncogenic conversion of NIH 3T3 cells. The inability of specific mutant proteins, such as ΔAD1, ΔZIP, and ΔLH, to transform NIH 3T3 cells suggests that E2A-HLF acts as a dimer formed through the leucine zipper domain and trans activates oncogenic target genes through the combined action of the AD1 and LH motifs, in a manner consistent with our initially proposed model (13).

E2A-HLF-mediated transformation appears similar in several important ways to that mediated by the E2A-PBX1 oncprotein (15), whose chimeric gene produces two alternatively

| Transfectant                                      | Expt 1 | Expt 2 |
|--------------------------------------------------|--------|--------|
| pRe vector (mixed population)                    | 1      | 0      |
| pRe/E2A-HLF (mixed population)                   | 10     | 14     |
| pRe/E2A-HLF subclones                           |        |        |
| From agar colonies                               |        |        |
| SA1                                              | 2,264  | 3,424  |
| SA2                                              | 3,144  | 2,672  |
| SA3                                              | 33     | 24     |
| SA4                                              | 3,144  | 3,000  |
| From flat monolayer colonies                     |        |        |
| L1                                               | 1      | 0      |
| L3                                               | 0      | 0      |
| L4                                               | 1      | 0      |
| v-fms                                            | 5,150  | 5,580  |

* In agar plates seeded with $5 \times 10^4$ cells.
spliced mRNAs encoding leukemogenic transcription factors. E2A-PBX1 and E2A-HLF have identical amino-terminal E2A amino acids but differ in their DNA-binding domains. NIH 3T3 cells expressing the p77E2A-PBX1 chimera form primary transformed foci in monolayer culture, similar to those induced by \( v-fms \) and other transforming oncogenes, whereas transfectants expressing either p62E2A-HLF or p85E2A-PBX1 do not. Rather, they induce hypercellular colonies only when the untransfected cells are first abolished with the antibiotic G418, implying that they are contact inhibited by neighboring NIH 3T3 cells not expressing the chimeric transcription factors. This interpretation is supported by the ability of E2A-HLF-expressing cells to grow to higher density than control cells in reduced serum (Fig. 5), although this relative loss of contact inhibition does not approach the complete loss of growth arrest, at confluence, observed with cells expressing the \( v-fms \) oncogene (27).

The most reliable assay of the transforming potential of E2A fusion proteins in NIH 3T3 cells is their ability to induce growth in soft agar. In all experiments attempted, E2A-HLF expression was associated with colony formation in agar, the colony size and cloning efficiency being directly related to the abundance of the transforming protein as was previously observed for both the p77 and p85 forms of E2A-PBX1 (15). These observations indicate that high intracellular levels of E2A-HLF are required for optimal anchorage-independent cell growth.

Focus formation and loss of contact inhibition tend to be linked with anchorage-independent cell growth, especially in cells transformed with activated retroviral oncogenes. Recent studies by Assoian and coworkers, however, demonstrate the separability of these properties in NIH 3T3 and normal rat kidney fibroblasts overexpressing cyclin A (7) and in cell lines generated by random mutagenesis, which acquired anchorage independence but retained normal morphology and contact inhibition and did not form foci in monolayer cultures (8). Our results with NIH 3T3 cells transformed by E2A-HLF indicate an intermediate phenotype, in that the cells grew to an increased density at confluence, exhibited anchorage-independent growth, and formed tumors in nude mice but were unable to form primary foci in monolayer cultures. A similar uncoupling of transformed growth properties was observed for

FIG. 6. Characterization of E2A-HLF mutant proteins. (A) Electrophoretic mobility shift analysis of complexes formed with an HLF consensus sequence oligonucleotide in lysates of rabbit reticulocytes programmed to express the E2A-HLF mutant proteins. Lysates containing no RNA (control; lane 1), wild-type (WT) E2A-HLF (lane 2), ΔAD1 (lane 3), ΔLH (lane 4), and ΔZIP (lane 5) were analyzed. The mobilities of specific DNA-protein complexes are shown by a bracket, and the unbound labeled oligonucleotide probe is indicated by a circle. (B) Trans activation of reporter gene expression by the E2A-HLF mutant proteins. The percent conversion of \( \Delta^{[14]C} \) chloramphenicol to its acetylated products is shown for NIH 3T3 cells. Cells were cotransfected with the wild-type or mutant E2A-HLF cDNAs, together with a reporter plasmid, either pCATpro-CS (containing two authentic HLF binding sites; CS) or pCATpro-M4 (containing two altered HLF binding sites; M4). Mean values are shown for duplicate independent transfections in a representative experiment; similar results were obtained in three replicates of this series of transfections.

FIG. 7. Expression of wild-type E2A-HLF and its mutants. Metabolically labeled cell lysates were immunoprecipitated with either the HLF(C) antiserum (lanes 1 to 6) or the E2A antiserum (lanes 7 to 9). Lysates were obtained from G418-resistant NIH 3T3 cells stably transfected with the pRc vector (lanes 1, 4, and 7), or the pRc vector expressing wild-type (WT) E2A-HLF (lanes 2, 5, and 8) or the ΔAD1 (lane 3), ΔLH (lane 6), and ΔZIP (lane 9) mutants.
p85E2A-PBX1, despite the difference in DNA sequence elements recognized by the binding domains of HLF and PBX1: GT TAGCTAATT (10, 13) versus ATCAAATCA (21, 30). Although the two E2A-containing chimeric proteins would appear to regulate different targets, they may influence genes in common pathways leading to oncogenic conversion.

Results of the soft-agar colony assay for E2A-HLF-mediated anchorage-independent cell growth allowed us to begin to address key questions regarding the mechanism by which this protein induces transformation. At least three models exist to explain the association of the chimeric protein with leukemia: (i) direct regulation by E2A-HLF homodimers of genes that contain binding sequence motifs in their promoters; (ii) direct alteration of gene expression by heterodimeric complexes formed between E2A-HLF and other closely related bZIP proteins, such as thyrotroph embryonic factor or DBP; and (iii) dominant negative interference mediated by E2A-HLF with a normally expressed and compatible bZIP protein that is trapped within a nonfunctional heterocomplex.

The trans-activating properties of E2A were originally described by Henthorn et al. (9), and the LH domain was identified by Quong et al. (24), on the basis of site-directed mutagenesis and the recognition that critical residues were shared with the E2-2, HEB, and daughterless bHLH transcription factors. An additional trans-activation motif called AD1 was subsequently identified in the amino-terminal region of E2A (1). In our experiments, the AD1 and ΔLH E2A-HLF mutants retained their DNA binding specificity, implying intact bZIP domains, but lost their ability to trans activate reporter gene expression or induce anchorage-independent cell growth. Thus, a dominant negative model underlying transformation seems unlikely, as the intact leucine zipper would be able to interact with and inactivate other bZIP proteins, even when the trans-activating functions mediated by E2A had been disabled.

Our previous study of E2A-HLF expression by leukemic cells (13) supports a model in which the protein acts as a homodimeric complex to inappropriately trans activate the expression of target genes with oncogenic potential. Failure of the AZIP E2A-HLF mutant to render cells anchorage independent is consistent with this proposal, although we cannot rule out an alternative model in which E2A-HLF acts as part of a heterodimeric complex with thyrotroph embryonic factor, DBP, or other bZIP proteins whose leucine zipper domains are able to interact with HLF. A major task will be to reconcile the growth-promoting effects of E2A-HLF with any differentiation abrogating effects the protein might have in maturing cells. Direct transformation of early B lymphocytes in mice is needed to strengthen the argument that E2A-HLF has a leading role in the development of human ALL with the t(17;19) chromosomal translocation.

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REFERENCES

1. Aroheim, A., R. Shiram, A. Rosen, and M. D. Walker. 1993. The E2A gene product contains two separable and functionally distinct transcription activation domains. Proc. Natl. Acad. Sci. USA 90:8063–8067.
2. Bram, R. J., D. T. Hung, P. K. Martin, S. L. Schreiber, and G. R. Crabtree. 1993. Identification of the immunohistochemically detectable angiotensin II type receptor in adult rat lung. J. Mol. Biol. 234:457–459.
3. Chader, G. J., and O. H. Okayama. 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. 7:2745–2752.
4. Dedera, D. A., E. K. Waller, D. P. LeBrun, A. Sen-Majumdar, M. E. Stevens, G. S. Barsh, and M. L. Cleary. 1993. Cisogenic homeobox gene E2A-PBX1 induces proliferation, apoptosis, and malignant lymphomas in transgenic mice. Cell 74:833–843.
5. Dorel, D. W., K. M. Scully, D. M. Simmons, M. Wegner, K. T. Chu, L. W. Swanson, and M. G. Rosenfeld. 1991. TEF, a transcription factor expressed specifically in the anterior pituitary during embryogenesis, defines a new class of leucine zipper proteins. Genes Dev. 5:1739–1753.
6. Guadagno, T. M., M. Ohtsubo, J. M. Roberts, and R. K. Assoian. 1993. A link between cyclin A expression and adhesion-dependent cell cycle progression. Science 262:1572–1575.
7. Han, E. K. H., T. M. Guadagno, S. L. Dalton, and R. K. Assoian. 1993. A cell cycle and mutational analysis of anchorage-independent growth: cell adhesion and TGF-β1 control G1/S transit specifically. J. Cell Biol. 122:461–471.
8. Henthorn, P., M. Kiledjian, and T. Kadesch. 1990. Two distinct transcription factors that bind the immunoglobulin enhancer E5/E2 motif. Science 247:467–470.
9. Hunger, S. P., R. Brown, and M. L. Cleary. 1994. DNA-binding and transcriptional regulatory properties of hepatic leukemia factor (HLF) and the (t(17;19)) acute lymphoblastic leukemia chimera E2A-HLF. Mol. Cell. Biol. 14:5986–5996.
10. Hunger, S. P., K. Ohyashiki, K. Toyama, and M. L. Cleary. 1992. HLF, a novel hepatic bZIP protein, shows altered DNA-binding properties following fusion to E2A in (t(17;19)) acute lymphoblastic leukemia. Genes Dev. 6:1606–1620.
11. Inaba, T., W. M. Roberts, L. H. Shapiro, K. W. Jolly, S. C. Raimondi, S. D. Smith, and A. T. Look. 1992. Fusion of the leucine zipper gene HLF to the E2A gene in human acute B-lineage leukemia. Science 257:531–534.
12. Inaba, T., L. H. Shapiro, T. Funahiti, E. K. Waller, M. G. Jones, R. A. Ashmun, and A. T. Look. 1994. DNA-binding specificity and trans-activating potential of the leukemia-associated E2A-hepatic leukemia factor fusion protein. Mol. Cell. Biol. 14:3403–3413.
13. Kamps, M. P., and D. Baltimore. 1993. E2A-Pbx1, the t(1;19) translocation protein of human pre-B-cell acute lymphocytic leukemia, causes acute myeloid leukemia in mice. Mol. Cell. Biol. 13:351–357.
14. Kamps, M. P., A. T. Look, and D. Baltimore. 1991. The human t(1;19) translocation in pre-B cell ALL produces multiple nuclear E2A-Pbx1 fusion proteins with differing transforming potentials. Genes Dev. 5:358–368.
15. Kamps, M. P., C. Murre, X. H. Sun, and D. Baltimore. 1990. A new homeobox gene contributes the DNA binding domain of the t(1;19) translocation protein in pre-B ALL. Cell 60:547–555.
16. Katsch, Z. A., T. Inaba, M. Valentine, and A. T. Look. 1994. Chromosomal localization and cDNA cloning of the human DBP and TEF genes. Genomics 23:344–351.
17. Lassar, A. B., R. L. Davis, T. Kadesch, C. Murre, A. V. Voronova, D. Baltimore, and H. Weintraub. 1991. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. Cell 66:305–315.
18. LeBrun, D. L., and M. L. Cleary. 1994. Fusion with E2A alters the transcriptional properties of the homeodomain protein PBX1 in t(1;19) leukemias. Oncogene 9:1641–1647.
19. Look, A. T. 1994. Pathobiology of the acute lymphoid leukemia cell. p. 1047–1086. In R. Hofmann (ed.), Hematology, 2nd ed. Churchill Livingstone, New York.
20. Lu, Q., D. D. Wright, and M. P. Kamps. 1994. Fusion with E2A converts the PBX1 homeodomain protein into a constitutive transcriptional activator in human leukemias carrying the t(1;19) translocation. Mol. Cell. Biol. 14:3938–3948.
21. Mueller, C. R., P. Maire, and U. Schibler. 1990. DBP, a liver-enriched transcriptional activator, is expressed late in ontogeny and its tissue specificity is determined posttranscriptionally. Cell 61:279–291.
22. Nourse, J., J. D. Melletent, N. Galili, J. Wilkinson, E. Stanbridge, S. D. Smith, and M. L. Cleary. 1990. Chromosomal translocation (t(1;19)) results in synthesis of a homeobox fusion mRNA that codes for a potential chimeric transcription factor. Cell 60:535–545.
23. Quong, M. W., M. E. Massari, R. Zwart, and C. Murre. 1993. A new transcriptional-activation motif restricted to a class of helix-loop-helix proteins is functionally conserved in both yeast and mammalian cells. Mol. Biol. Cell 13:792–800.
24. Rabbitts, T. H. 1991. Translocations, master genes, and differences between the origins of acute and chronic leukemias. Cell 67:641–644.
25. Roberts, C. W. M., J. R. Shutter, and S. J. Korsmeyer. 1994. Hox11 controls the genesis of the spleen. Nature (London) 368:747–749.
26. Roussel, M. F., and C. J. Sherr. 1988. Mouse NIH 3T3 cells expressing human colony-stimulating factor 1 (CSF-1) receptors overgrow in serum-free
medium containing human CSF-1 as their only growth factor. Proc. Natl. Acad. Sci. USA 86:7924–7927.

28. Shapiro, L. H., R. A. Ashmun, W. M. Roberts, and A. T. Look. 1991. Separate promoters control transcription of the human aminopeptidase N gene in myeloid and intestinal epithelial cells. J. Biol. Chem. 266:11999–12007.

29. Smith, D. B., and L. M. Corcoran. 1993. Expression and purification of glutathione-S-transferase fusion proteins. p. 1–8. In F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. Wiley Interscience, New York.

30. Van Dijk, M. A., P. M. Voorhoeve, and C. Murre. 1993. Pbx1 is converted into a transcriptional activator upon acquiring the N-terminal region of E2A in pre-B-cell acute lymphoblastoid leukemia. Proc. Natl. Acad. Sci. USA 90:6061–6065.

31. Vinson, C. R., P. B. Sigler, and S. L. McKnight. 1989. Scissors-grip model for DNA recognition by a family of leucine zipper proteins. Science 246:911–916.