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DNA-Binding and Transcriptional Regulatory Properties of Hepatic Leukemia Factor (HLF) and the t(17;19) Acute Lymphoblastic Leukemia Chimera E2A-HLF

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The t(17;19) translocation in acute lymphoblastic leukemias results in creation of E2A-hepatic leukemia factor (HLF) chimeric proteins that contain the DNA-binding and protein dimerization domains of the basic leucine zipper (bZIP) protein HLF fused to a portion of E2A proteins with transcriptional activation properties. An in vitro binding site selection procedure was used to determine DNA sequences preferentially bound by wild-type HLF and chimeric E2A-HLF proteins isolated from various t(17;19)-bearing leukemias. All were found to selectively bind the consensus sequence 5′-GTTACGTAAT-3′ with high affinity. Wild-type and chimeric HLF proteins also bound closely related sites identified previously for bZIP proteins of both the proline- and acidic amino acid-rich (PAR) and C/EBP subfamilies; however, E2A-HLF proteins were significantly less tolerant of certain deviations from the HLF consensus binding site. These differences were directly attributable to loss of an HLF ancillary DNA-binding domain in all E2A-HLF chimeras and were further exacerbated by a zipper mutation in one isolate. Both wild-type and chimeric HLF proteins displayed transcriptional activator properties in lymphoid and nonlymphoid cells on reporter genes containing HLF or C/EBP consensus binding sites. But on reporter genes with nonoptimal binding sites, their transcriptional properties diverged and E2A-HLF competitively inhibited activation by wild-type PAR proteins. These findings establish a spectrum of binding site-specific transcriptional properties for E2A-HLF which may preferentially activate expression of select subordinate genes as a homodimer and potentially antagonize expression of others through heteromeric interactions.

Homeostatic control of gene expression at the level of mRNA transcription is essential for normal cellular growth and differentiation (37). A remarkably wide range of diverse cellular proteins known as transcription factors has been implicated in this process. One major subfamily of transcription factors, the basic leucine zipper (bZIP) proteins, is characterized by the presence of a highly charged basic region responsible for DNA binding and an adjacent amphipathic α helical domain termed the leucine zipper that mediates protein dimerization (34). In addition to playing important roles in control of normal growth and differentiation, specific bZIP proteins have been implicated in malignant transformation. Fos and Jun, which heterodimerize to form the transcription factor AP-1, were originally identified as oncogenes transduced by transforming retroviruses (for a review, see reference 10). In addition, a chicken homolog of C/EBPβ, NF-M, has been implicated in the development of avian myelogeneous leukemia (5, 20). More recently, chromosomal translocations in acute leukemias (6) and solid tumors (3, 9, 24, 31, 38) have been shown to oncogenically activate various transcriptional proteins, including members of the bZIP family. For example, in human myxoid liposarcoma, the t(12;16) translocation creates a protein chimera composed of the dominant negative C/EBP-like protein CHOP fused to a portion of a novel protein referred to as TLS (9) or FUS (31). Another translocation in human cancer, t(12;22) in malignant melanoma of soft parts, fuses the *ATF-1* gene to *EWS*, which was previously identified on the basis of its involvement in the t(11;22) translocation in Ewing's sarcoma (38). Thus, structural alterations of bZIP proteins are a recurring theme in oncogenesis; however, the effects on their transcriptional regulatory properties remain undefined.

We and others have reported that a chimeric bZIP protein called E2A-hepatic leukemia factor (HLF) is created by the t(17;19)(q21-q22;p13) translocation in acute lymphoblastic leukemia (ALL) (15, 16). E2A-HLF chimeras consist of the amino-terminal two-thirds of E2A proteins, including two regions with transcriptional activation properties (2, 30), fused to the DNA-binding and dimerization domains of HLF, a previously unknown bZIP protein. HLF was shown to be closely related to bZIP proteins DBP (27) and TEF/VBP (11, 17), which comprise a distinct subgroup (the proline- and acidic amino acid-rich [PAR] subfamily) distinguished both structurally and functionally from the larger bZIP protein superfamily (11). These characteristic bZIP proteins have been implicated in the regulation of genes that display a tissue-restricted or developmental stage-specific expression profile, particularly in mature hepatocytes (DBP and VBP) or developing thyrotrophs (TEF) in the embryonic pituitary (11, 17, 21, 27, 35, 36). Similar to PAR and several other bZIP proteins, HLF expression is restricted in adult and fetal tissues (primarily liver, kidney, and lung), suggesting that it also plays a role in cell-type-specific transcription (15, 16). Notably, HLF is not expressed in normal or malignant hematolymphoid cells, suggesting that its redirected expression by chromosomal translocations in immature lymphoid cells is an important pathogenetic event.

In earlier studies, wild-type and chimeric HLF proteins were

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demonstrated to cross-bind to a TEF recognition site present in the rat growth hormone (GH) promoter (15). Binding was observed as homodimers or heterodimeric complexes with the related PAR proteins DBP and TEF. It was noted that DNA binding to the TEF GH site was measurably poorer by E2A-HLF compared with wild-type HLF, and a zipper mutation in E2A-HLF further exacerbated this difference. Since DNA binding by E2A-HLF heterodimers was less impaired, these findings suggested the intriguing possibility that chimeric HLF proteins could participate in heterodimeric interactions with other bZIP proteins in addition to a potential role as homodimeric transcription factors. More recently, the chimeric products from additional t(17;19)-ALLs have been characterized (13, 16). These studies show that all E2A-HLF chimeras include the same portion of HLF containing its basic and leucine zipper motifs and that somatic mutations of the zipper are not a general feature. However, amino acids juxtaposed with the HLF basic region, either from E2A or from random insertions of nontemplated nucleotides, can vary from one leukemia to another because of diverse genomic rearrangements underlying the t(17;19) translocation. The variable structural features of different E2A-HLF chimeras suggested that their DNA-binding and transcriptional properties might be heterogeneous, particularly since the sites of protein fusion occurred within or adjacent to the regions implicated in protein-DNA interactions (i.e., the basic region).

The current studies were undertaken to further characterize and establish the structural basis for differences observed previously in the DNA-binding capability of E2A-HLF and to establish their potential role in determining or restricting the transcriptional properties of the chimeric proteins. Using an in vitro binding site selection assay, we identified an identical dyad symmetric core consensus sequence to which both HLF and various E2A-HLF chimeras bound avidly. HLF and E2A-HLF also bound well to closely related sites, but altered binding to certain divergent sites was a consistent feature of E2A-HLF proteins and was directly attributable to loss of an HLF ancillary DNA-binding domain. In both lymphoid and nonlymphoid cells, HLF and E2A-HLF functioned experimentally as transcriptional activators of reporter genes containing a minimal promoter with adjacent HLF consensus binding sites or high affinity C/EBP sites, although their transcriptional properties diverged on reporters containing less-optimal sites. Thus, despite their altered DNA-binding properties, chimeric E2A-HLF proteins can function as transcriptional activators whose leukemogenic properties are likely mediated by dysregulation of target genes normally regulated by related bZIP proteins.

MATERIALS AND METHODS

EMSAs and consensus binding site selection. Electrophoretic mobility shift assays (EMSAs) were performed as described previously (15) with proteins translated in vitro (IVT) from expression plasmids, using a coupled reticulocyte lysate system (Promega, Madison, Wis.). In vitro translates from reactions programmed with vector (pSP64) alone served as negative controls for EMSA. To ensure that approximately equal amounts of IVT protein were present in binding reaction mixtures, 1/10 of each reaction mixture was translated in parallel with [^{35}S]methionine. Labelled proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and quantitated on a phosphoimager, and values were normalized to the number of methionine residues; these results were used to standardize the relative amounts of unlabelled translation mixtures added to each binding reaction mixture. Oligonucleotides containing all binding sites (see Table 1 for sequences) shared an identical backbone derived from the VBP site in the chicken vitellogenin II (VTG II) promoter (17). Single-stranded oligonucleotides [5′-GATCT CAAAAAGAGGAGT$(X)_{10}$CCTGATAAAAAAG-3′ and 5′-GATCCTTTTTTATCAGG$(Y)_{10}$ACTCCTCTTTTTGA-3′, where X and Y represent complementary nucleotides of the binding site of interest] were annealed and labelled by Klenow fill-in. For several nondyad symmetric sites the 10-nucleotide binding site core was analyzed in both orientations with respect to the backbone; no differences in binding were observed.

A modification of the selective amplification and binding (SAAB) assay (4) was used to determine the consensus HLF binding site. A single-stranded oligonucleotide containing 20 internal degenerate positions [ST1, 5′-GAGGATCCAGTC AGCATG-$(N)_{20}$-CTCAGCCTCGAGATCTCG-3′] was annealed to an oligonucleotide primer complementary to the 3′ arm (ST1-D, 5′-CGAGATCTCGAGGCTGAG-3′) and converted to double-stranded DNA with unlabelled nucleotides. The resultant double-stranded ST1 DNA was end labelled with [γ-^{32}P]ATP, using T4 polynucleotide kinase, and 0.15 nmol was used in binding reactions with IVT HLF. A faint shifted complex was observed in the HLF binding reaction but not in the negative control IVT (see Fig. 1). The area of the gel corresponding to this complex was excised, and the DNA was eluted in H_2O. Approximately 10% of the eluate was amplified by PCR with ST1-D and an oligomer homologous to the 5′ arm of ST1 (ST1-U, 5′-GAGGATCCAGTCAGCATG-3′). The amplification was performed as described previously (14) for 15 to 30 cycles with annealing at 52°C. The amplification product of the selected probe was end labelled, and approximately 1% was used for a subsequent round of selection. After five rounds of SAAB, the amplified product was digested with *Bam*HI and *Bgl*II to cleave sites present in the ST1 arms and cloned into pBluescript (Stratagene, San Diego, Calif.). Nucleotide sequences of independent clones were determined by using commercially prepared reagents (Sequenase; U.S. Biochemicals, Cleveland, Ohio) and visually aligned. Only sequences derived entirely from the internal degenerate positions were used to determine the consensus binding site. Consensus binding sites for various E2A-HLF proteins were determined in an identical manner, with the exception that six, rather than five, rounds of SAAB were employed.

Plasmid constructions. The in vitro expression plasmids pHLF, pHLF^{F}, pHLFΔN$^{3\text{-}155}$, pE2A-HLF^{I}, and pE2A-HLF have been described previously (15). In this report we use the terminology EH^{F526} to refer to the e13-Ins-e4 spliced protein isolated from the HAL-01 cell line which contains an I-526-to-F-526 somatic mutation and EH^{I526} to refer to a protein in which this position has been reverted to the wild-type isoleucine residue. Various expression plasmids were modified by standard PCR and cloning procedures to create additional constructs for in vitro expression of modified wild-type or fusion proteins. EH^{UOC} (a plasmid that expresses an e13-Ins-e4 spliced fusion protein which contains an insertion different from that in the HAL-01 chimera) was constructed by amplifying the 3′ portion of the fusion cDNA from the UOC-B1 t(17;19)-ALL cell line (16) with an oligomer homologous to sequences in *E2A* exon 11 (JN3, 5′-CGTCCAGC CCTTCTACCC-3′) and an oligomer complementary to *HLF* exon 4 sequences 3′ of the stop codon which contained an artificial *Eco*RI site (HLF 3′Eco, 5′-GCGAATTCAGCCAG CCTGCAAAAATGCC-3′). The PCR product was digested with *Xho*I (a natural *Xho*I site is present in *E2A* exon 11) and *Eco*RI and used to replace the corresponding *Xho*I-*Eco*RI fragment of pEH^{I}. An identical strategy was employed to

construct the expression plasmid pEH^{Type2} derived from a t(17;19)-ALL with an e12-e4 spliced mRNA (13). Two additional truncation mutants of HLF were used in these studies: HLF$^{\Delta 1\text{-}224}$, which contains only the portion of HLF (residues 225 to 295, encoded by exon 4) present in the E2A-HLF fusion protein preceded by a methionine residue, and HLF$^{\Delta 1\text{-}209}$ (residues 210 to 295), which includes 15 additional amino acids which compose the so-called basic region extension (BRE). These were constructed by PCR with primer HLF 3'Eco and specific upstream primers which contained artificial *Bam*HI sites for cloning purposes (HE4 Met-Bam, 5'-GCGGATCC GCGATGGATGACAAGTACTGGGCAAGG-3', and HBE Met-Bam, 5'-GCGGATCCGCGATGATCAAGAAAGCTC GCAAA-3'). Amplification products were digested with *Bam*HI and *Eco*RI and ligated into the pSP64 expression plasmid. The artificial fusion protein construct EH^{PAR} contained HLF residues comprising the PAR and BRE in addition to HLF exon 4-encoded sequences (amino acids 158 to 295), essentially the same portion of HLF found in HLF$^{\Delta 3\text{-}155}$. Plasmid pEH^{I526} was amplified with JN3 and a downstream oligomer complementary to the 3' end of the insertion with a 3' *Bam*HI overhang (INSENDBAM, 5'-CGCGGATCCCACCAGAAA TCTCAGGCG-3'), cut with *Xho*I and *Bam*HI and ligated into the *Xho*I- and *Eco*RI-cut pEH-I vector along with a portion of wild-type pHLF^{I} amplified with HLF 3'Eco and an upstream oligomer with a 5' *Bam*HI overhang (HLFPARBAM, 5'-GCG GATCCCGCAATACACCAAGTCCC-3'). The construct assembled in this manner includes E2A residues 1 to 477, the HAL-01-derived insertion, two residues inserted by the cloning process (glycine and serine), and HLF residues 158 to 295. EH$^{\Delta ZIP}$, which contains residues 1 to 524 of E2A-HLF with an artificial stop codon immediately preceding the HLF leucine zipper, was constructed by replacing the *Xho*I-*Eco*RI fragment of pEH^{I526} with a fragment generated by amplifying pEH^{I526} with oligomers JN3 and HDZ (5'-CCGGAATTCGAACTA GTTCTCTTTCAGCCTCC-3'). The amplified portions of all constructs were sequenced to ensure that no PCR-induced mutations had occurred.

Transient transfections and transcriptional activation assays. For transfection experiments the coding portions of various in vitro expression plasmids were cloned into the mammalian expression vector pCMV1. The expression vector pET3aTEF (11) was used to express rat TEF in dominant negative experiments. The pCMV1 vector without transactivator insert served as a negative control. Reporter constructs were assembled by digesting double-stranded oligonucleotide binding sites at *Bam*HI and *Bgl*II sites present in the invariant portions and concatamerizing and cloning them into the pBLCAT2 (23) vector adjacent to the herpes simplex virus thymidine kinase (TK) promoter which drives expression of a chloramphenicol acetyltransferase (CAT) gene. A CAT reporter driven by the liver, bone, and kidney alkaline phosphatase (AP) promoter with eight VBP VTG II sites was a generous gift of John Birch; for some experiments, the bank of VBP sites was excised and replaced by HLF consensus sites. Nomenclature used indicates the type and number of copies of the binding site and promoter; for example, $(HLF)_4$-TK-CAT.

Transient transfections in monkey kidney-derived CV-1 cells were performed by calcium phosphate-mediated transfection as described previously (22). Human B-precursor ALL cell lines REH and 697 were transfected by electroporation with DEAE-dextran as described previously (22). The total amount of DNA transfected within each experiment was kept constant with the use of carrier plasmid DNA. In each experiment a control plasmid expressing the luciferase gene (pRSV-luciferase or pSVtk-luciferase) was included to control for transfection efficiency. CAT and luciferase assays were performed as described (22). No significant CAT activity was detected in negative control experiments performed with various transactivators and reporter constructs lacking binding sites. For each experiment, transfections were performed in duplicate on at least three separate occasions with similar results.

RESULTS

HLF and E2A-HLF bind preferentially to an identical consensus DNA sequence. An HLF consensus DNA-binding site was determined by a modified SAAB procedure (4). IVT HLF was incubated with an oligonucleotide (ST1) containing 20 degenerate nucleotides flanked by PCR "handles." In the initial round of EMSA, using the fully degenerate ST1, a faint shifted complex was observed with HLF but not with control lysate proteins (Fig. 1A). With each successive round of selection this complex became more prominent; after five rounds, an intense shifted complex was evident. The DNA from this complex was amplified and cloned, and the nucleotide sequences of individual inserts were determined. Comparison and alignment of 18 individual sequences showed an obvious consensus binding site: 5'-GTTACGTAAT-3' (Fig. 1B). There was no consensus outside these 10 positions. This sequence was highly similar to binding sites previously identified for related PAR and C/EBP proteins—see Table 1 (7, 11, 17, 27, 34, 36). There was a minor preference (39%) for C at the +2 position such that 6 of 36 half-sites matched the C/EBP high-affinity site (ATTGC or GCAAT) (34); this contrasted to 10 of 36 matches for the HLF consensus half-sites.

To confirm that this consensus sequence represented a high-affinity HLF-binding site, EMSA was performed with an oligonucleotide containing the consensus site with and without 100-fold excess unlabelled competitor DNAs (Fig. 1C). The HLF complex was fully inhibited by an excess of unlabelled HLF consensus site and unaffected by a 100-fold excess of an unrelated binding site for the PBX1 protein (22). Complete or near-complete competition was also observed with excess, unlabelled C/EBP site or the TEF GH site used in our earlier studies (15). The albumin D and VTG II sites (which were used to clone DBP and VBP, respectively) were effective, but less efficient, competitors.

Our previous observation that E2A-HLF bound less avidly than wild-type HLF to the TEF GH site (15) raised the possibility that the fusion protein might have a different optimal recognition sequence than HLF. To address this, we performed the SAAB procedure with three different E2A-HLF chimeric proteins—the type I chimera (EH^{F526}) isolated from the HAL-01 cell line, a variant of this construct (EH^{I526}) in which the zipper mutation was reverted to the wild-type sequence, and a type II E2A-HLF fusion protein (Fig. 2). Following six rounds of SAAB, a total of 59 individual clones selected by the three E2A-HLF chimeras were sequenced. Each of the three fusion proteins bound selectively to the same 5'-GTTACGTAAT-3' consensus (Fig. 3) as had HLF. A total of 34 of 59 individual clones were either an exact match (n = 11) or contained only one mismatch (n = 23) from the 10-bp consensus. In general, sequences selected by the fusion proteins displayed a tighter adherence to the consensus than wild-type HLF although an additional round of SAAB was employed for the fusion proteins.

The central 8-bp core of the consensus site (−4 to +4) displays dyad symmetry, whereas flanking nucleotides at −5 and +5 are noncomplementary. However, 44% of the HLF-selected sites contained complementary nucleotides at these positions (the same as between −2 and +2 [Fig. 1]), as did

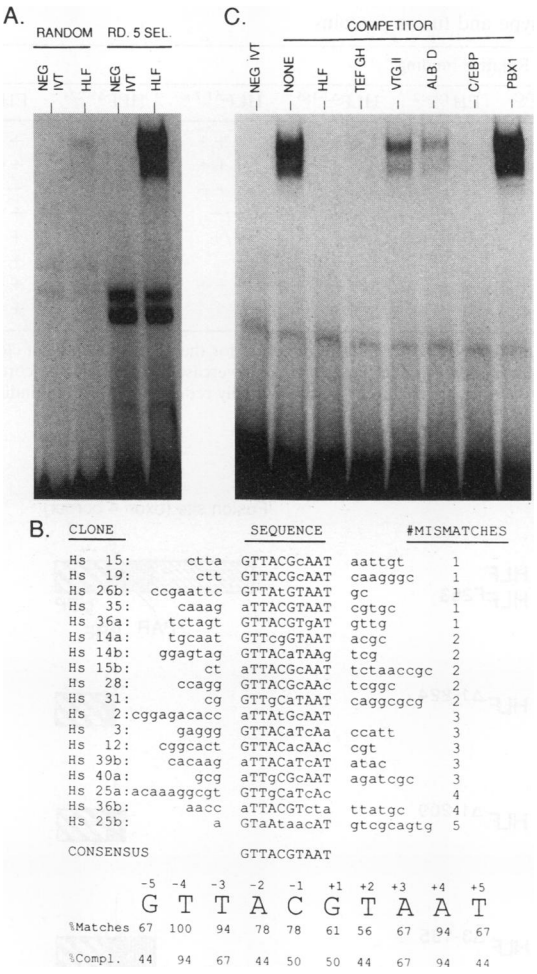


FIG. 1. Selection of a high-affinity HLF consensus binding site. (A) Wild-type HLF was translated in vitro and used in binding reactions with unselected random ST-1 oligonucleotide or oligonucleotides resulting from five rounds of SAAB. NEG IVT, translation reaction programmed with vector alone. (B) Alignment of internal sequences of 18 selected clones. The nucleotides used to derive the consensus site are offset in the center. Positions which match the consensus are in uppercase type, and those which differ are in lowercase type. For each clone the number of positions that do not match the consensus is given at the right. At the bottom the percentage of matches with the consensus at each position is listed. Complementarity (Compl.) refers to the percentage of symmetric positions (e.g., i.e., −1 and +1, etc.) which are complementary to one another in individual clones. (C) EMSA was performed with radiolabelled double-stranded HLF consensus binding site oligonucleotide with IVT HLF in the presence of a 100-fold molar excess of indicated unlabelled competitor oligonucleotides.

51% of the E2A-HLF-selected sites (data not shown), suggesting no strong selection against complementarity in individual clones. Another obvious feature of the consensus is the pair of Ts at −4 and −3 flanked by purines and the complementary As at +3 and +4 flanked by pyrimidines, suggesting a strong bias against three or more consecutive purine or pyrimidine nucleotides in each half-site. Whereas site selection for HLF did not reveal any consensus outside the 10-bp core, the fusion proteins displayed a trend toward a C in the +6 position and a strong selection against an A at −7.

The DNA-binding properties of E2A-HLF show site-specific differences with HLF. Potential differences in DNA binding by wild-type and chimeric HLF proteins (Fig. 2) were investigated by EMSA. The effect of the zipper mutation in one of the E2A-HLF proteins was assessed in constructs with or without this change (EH^{F526} versus EH^{I526}) and compared with analogous wild-type proteins (HLF^{F253} versus HLF). Binding studies employed IVT proteins that were quantitated to ensure that approximately equal amounts were added to the binding reaction mixtures. The binding sites were placed in the same oligonucleotide backbone to assess differences at positions which varied from the consensus (Table 1).

EMSA showed that DNA binding by each of the fusion proteins to the HLF consensus site was similar to that for HLF and the zipper mutation had little, if any, effect on binding by wild-type or fusion proteins (Fig. 4 and Table 1). However, binding of E2A-HLF fusion proteins EH^{I526}, EH^{UOC}, and $EH^{Type\ 2}$ to sites that deviated from the consensus varied from that of wild-type HLF in a manner that was binding site specific. For example, HLF bound well to the C/EBP high-affinity and TEF GH sites but significantly less well to the VBP VTG II and DBP albumin D sites, in agreement with their relative abilities to compete for binding to the HLF consensus site (Fig. 4 and Table 1). The chimeras, on the other hand, bound well to the C/EBP site but less well to the TEF GH site, and no consistent binding was detected to the VTG II and albumin D sites under these conditions. The binding properties of the three chimeras were identical, indicating that the amino acid differences at their fusion sites did not have a significant effect on DNA binding. Although binding of HLF to its consensus site was not significantly altered by a point mutation (I-253 to F-253) in its leucine zipper, significant effects were observed on more divergent sites. This mutation abolished binding to the VTGII and albumin D sites (Fig. 4 and Table 1). Similarly, the same zipper mutation in the fusion protein (EH^{F526}) further exacerbated its decreased tolerance of deviation from the consensus binding site, since binding to the less divergent TEF GH and C/EBP sites was further impaired compared with that to EH^{I526}.

It is notable that binding of wild-type and fusion proteins to the albumin D and VTG II sites was significantly different from that to the C/EBP high-affinity site, despite the fact that each matches the HLF consensus in 7 of 10 positions. Comparison of the nucleotide composition of these sites suggested at least two possible explanations for these differences. The C/EBP site is fully dyad symmetric, while the VTG II site contains one (−1 to +1) and the D box contains two (−1 to +1 and −2 to +2) positions of asymmetry. In addition, our site selection revealed a strong bias against three or more consecutive purine or pyrimidine nucleotides in either half-site, a feature present in both VTG II and albumin D but not C/EBP sites. To investigate whether either of these possibilities might explain the observed differences in DNA binding, we compared binding by various proteins to sites in which the HLF consensus nucleotides were changed to T at −2, −1, or both positions (Table 1). Binding to the HLF(−1T) site was similar to binding to the consensus site for all proteins, indicating that asymmetry is tolerated at this central position. Conversely, binding to both the HLF(−2T) site which contains three consecutive Ts and the HLF(−1,−2T) site with four consecutive Ts was substantially impaired. These data suggest that the observed alterations in binding to the VTG II and D box sites are largely due to the presence of three or more consecutive pyrimidines or purines in a half-site and not simply to loss of dyad symmetry.

Impaired binding of E2A-HLF is due to loss of HLF residues amino terminal to the basic region. The observed differences in DNA binding by E2A-HLF versus HLF occurred despite

TABLE 1. DNA-binding properties of wild-type and fusion proteins

Site	Sequence	Relative binding[a]									
		HLF	HLF^{F253}	EH^{I526}	EH^{F526}	EH^{UOC}	EH^{Type2}	HLF$^{\Delta 3\text{-}155}$	HLF$^{\Delta 1\text{-}209}$	HLF$^{\Delta 1\text{-}224}$	EH^{PAR}
HLF consensus	GTTACGTAAT	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
TEF GH	GTTACGcAAg	+++	+	++	+	++	++	+++	+++	++	+++
C/EBP	aTTgCGcAAT	+++	++	+++	++	+++	+++	+++	+++	+++	+++
VBP VTG II	tTTAtGTAAa	+	0	+/−	0	+/−	+/−	+	+/−	+/−	+
DBP albumin D	aTTttGTAAT	+	0	0	0	0	0	+	+/−	0	+
HLF (−2T)	GTTtCGTAAT	++	0	+/−	0	+/−	+/−	++	+	0	++
HLF (−1T)	GTTAtGTAAT	+++	++	++	++	++	++	+++	+++	++	+++
HLF (−1, −2T)	GTTttGTAAT	+	0	0	0	0	0	+	0	0	+

[a] Relative binding of proteins to naturally occurring and artificial sites. The sequence of each site is listed in a 5′-to-3′ direction for the strand which most closely matches the HLF consensus. Positions which match the consensus appear in uppercase type, and those which differ are shown in lowercase type. Binding is scored in relation to wild-type HLF binding to the HLF consensus site: +++, approximately equivalent; ++, clearly reduced; +, substantially reduced; +/−, faint binding is occasionally detected; 0, no binding detected in at least three duplicate experiments.

intact bZIP domains, suggesting that residues outside of the basic region play an important role in determining binding site specificity. To further investigate this possibility, DNA binding to different sites was compared by using variously truncated HLF proteins (Fig. 2). These studies focused on two regions of protein homology that distinguish the PAR subfamily from other bZIP proteins: an amino-terminal extension of the classic basic region (the BRE) and the adjacent PAR region (10). These highly conserved domains are not present in E2A-HLF, since each fusion protein contains only HLF residues encoded by exon 4, including the bZIP and 6 of 21 amino acids which compose the BRE (13). Binding by wild-type HLF was compared with that of different deleted forms of HLF, including HLF$^{\Delta 1\text{-}224}$, which contains only the residues included in E2A-HLF, HLF$^{\Delta 1\text{-}209}$ containing the additional 15 amino acids of the BRE, and HLF$^{\Delta 3\text{-}155}$ containing the BRE and PAR domains. HLF$^{\Delta 1\text{-}224}$ displayed DNA-binding properties analogous to those of the fusion proteins in that it bound well to the HLF consensus, TEF GH, and C/EBP sites but not to the VTG II and albumin D sites (Fig. 5 and Table 1). In contrast, HLF$^{\Delta 3\text{-}155}$ showed binding to this same panel of sites that was indistinguishable from wild-type HLF. HLF$^{\Delta 1\text{-}209}$ showed an intermediate pattern of binding, since it did not fully reconstitute wild-type binding, particularly with the less preferred sites. These data confirm and extend earlier observations (10) that residues outside the basic region are important for binding to less preferred sites and further demonstrate that residues within the PAR, in addition to those within the BRE, are necessary for full wild-type DNA binding.

To test this conclusion, we constructed an E2A-HLF protein (EH^{PAR}) wherein the entire HLF BRE and PAR domains were reinserted into EH^{I526} adjacent to the bZIP domains shared by HLF and E2A-HLF. In contrast to EH^{I526}, EH^{PAR} bound well to the VTG II and albumin D sites and its binding site preferences were identical to those of wild-type HLF as measured under our experimental conditions (Fig. 5 and Table 1). Thus, the observed differences in DNA binding by E2A-HLF did not result from properties conferred by E2A but were a direct consequence of loss of residues amino terminal to the classic basic region that are specifically conserved in the PAR subfamily of bZIP proteins and function as an ancillary DNA-binding domain.

HLF and E2A-HLF are transcriptional activators with divergent properties dictated by their DNA-binding preferences. The transcriptional regulatory properties of wild-type HLF and E2A-HLF were investigated by cotransfection of expression vectors into mammalian cells along with reporter constructs containing a CAT gene under control of either the

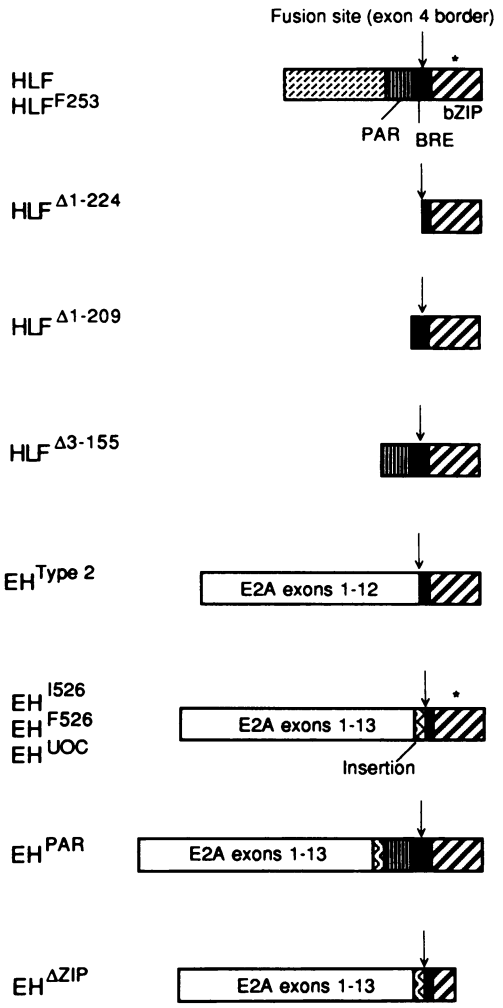
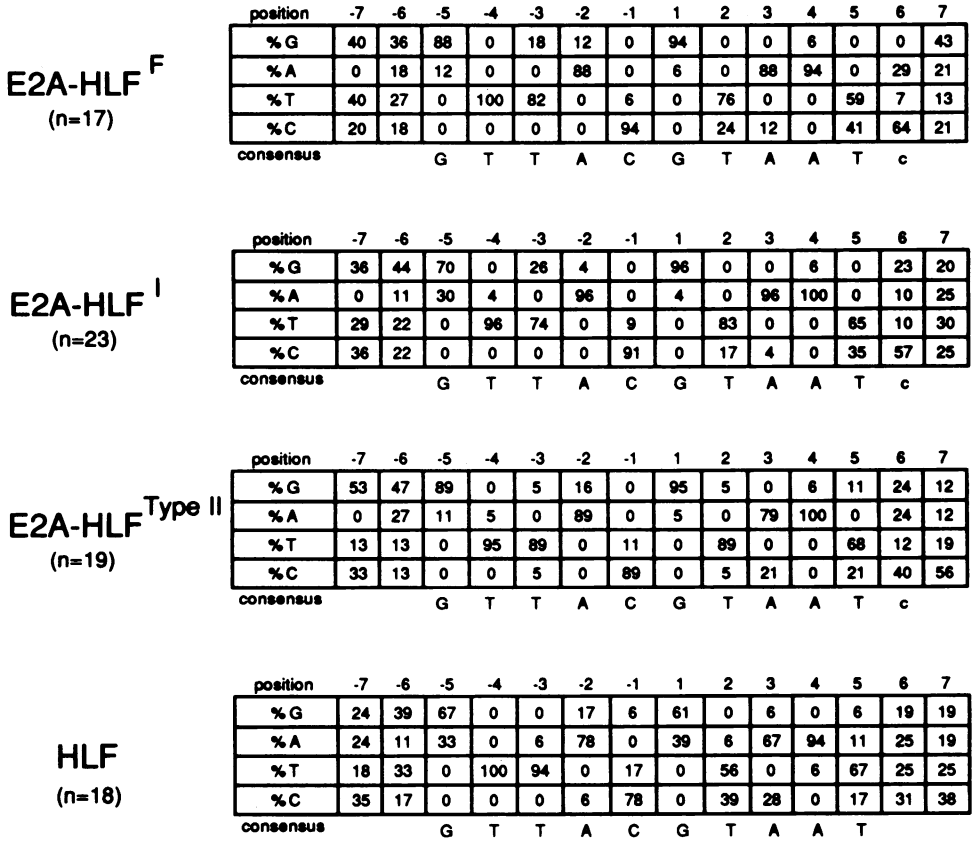


FIG. 2. Schematic diagram of mutant and wild-type HLF proteins. The fusion and wild-type protein constructions used in these studies are compared with wild-type HLF. An asterisk indicates the first heptad repeat position of the leucine zipper which is occupied by either an isoleucine (I) or phenylalanine (F) residue (HLF amino acid 253, E2A-HLF amino acid 526). The insertions found in EH$^{I/F526}$ and EH^{UOC} chimeras are composed of different amino acids. The site in HLF where fusion to E2A proteins occurs is indicated by an arrow. Hatched box, bZIP of HLF; filled box, extended basic region (BRE); vertically lined box, PAR.



E2A-HLF^{F} (n=17)

position	-7	-6	-5	-4	-3	-2	-1	1	2	3	4	5	6	7
%G	40	36	88	0	18	12	0	94	0	0	6	0	0	43
%A	0	18	12	0	0	88	0	6	0	88	94	0	29	21
%T	40	27	0	100	82	0	6	0	76	0	0	59	7	13
%C	20	18	0	0	0	0	94	0	24	12	0	41	64	21
consensus			G	T	T	A	C	G	T	A	A	T	c	

E2A-HLF^{I} (n=23)

position	-7	-6	-5	-4	-3	-2	-1	1	2	3	4	5	6	7
%G	36	44	70	0	26	4	0	96	0	0	6	0	23	20
%A	0	11	30	4	0	96	0	4	0	96	100	0	10	25
%T	29	22	0	96	74	0	9	0	83	0	0	65	10	30
%C	36	22	0	0	0	0	91	0	17	4	0	35	57	25
consensus			G	T	T	A	C	G	T	A	A	T	c	

E2A-HLF$^{Type\ II}$ (n=19)

position	-7	-6	-5	-4	-3	-2	-1	1	2	3	4	5	6	7
%G	53	47	89	0	5	16	0	95	5	0	6	11	24	12
%A	0	27	11	5	0	89	0	5	0	79	100	0	24	12
%T	13	13	0	95	89	0	11	0	89	0	0	68	12	19
%C	33	13	0	0	5	0	89	0	5	21	0	21	40	56
consensus			G	T	T	A	C	G	T	A	A	T	c	

HLF (n=18)

position	-7	-6	-5	-4	-3	-2	-1	1	2	3	4	5	6	7
%G	24	39	67	0	0	17	6	61	0	6	0	6	19	19
%A	24	11	33	0	6	78	0	39	6	67	94	11	25	19
%T	18	33	0	100	94	0	17	0	56	0	6	67	25	25
%C	35	17	0	0	0	6	78	0	39	28	0	17	31	38
consensus			G	T	T	A	C	G	T	A	A	T		

FIG. 3. Wild-type and chimeric HLF proteins bind preferentially to the same consensus site. The results of binding site selection with three different E2A-HLF chimeras are compared with selection with HLF. The number (n) of individual sequences on which the consensus is based appears below each protein. The percent frequency of each nucleotide at positions −7 to +7 is presented in tabular form, and the consensus binding site is listed below.

herpes simplex virus (HSV) TK or liver, bone, and kidney alkaline phosphatase promoters linked to concatamerized binding sites. In CV-1 cells, wild-type HLF strongly activated reporter gene transcription through HLF consensus binding sites and was a less potent activator of reporters containing VTG II sites (Fig. 6). HLF$^{\Delta 3\text{-}155}$ did not activate transcription significantly above background levels, indicating that portions required for activation of these reporters were located in the amino-terminal half of wild-type HLF. Basal activity was minimal on the $(VBP)_8$-TK-CAT reporter in the absence of cotransfected activator, whereas more abundant basal activity was consistently observed for the $(HLF)_4$-TK-CAT reporter, perhaps reflecting the presence of endogenous HLF, or related proteins, in monkey kidney cells.

E2A-HLF chimeric proteins strongly activated transcription of the $(HLF)_4$-TK-CAT reporter in CV-1 cells (Fig. 6). Similar levels of activation, ranging from 100 to 150% of wild-type HLF activity, were observed for all forms of the chimera analyzed (EH^{I526}, EH^{F526}, EH^{UOC}, and EH^{Type2}). This activation was dependent on the presence of HLF bZIP domains since E2A-PBX1, which contains the same portion of E2A, did not increase CAT expression above background levels. In marked contrast to the results seen with HLF, when the fusion proteins were cotransfected with the $(VBP)_8$-TK-CAT reporter, CAT activity was minimally above background levels seen in the absence of cotransfected activator. Similar results were observed with $(HLF)_4$ and $(VBP)_8$ reporters under control of the alkaline phosphatase rather than TK promoter (data not shown). Thus, both wild-type HLF and wild-type E2A-HLF were capable of potent transcriptional activation of reporters containing high-affinity HLF consensus binding sites. However, transcriptional activation by E2A-HLF differed from HLF when assayed on more divergent, nonoptimal binding sites such as that found in the VTG II promoter.

The observed differences in transcriptional activation paralleled the DNA-binding differences of the wild-type and chimeric HLF proteins and likely reflected the loss of critical HLF residues following fusion to E2A. To test this, the transactivation potential of a fusion construct (EH^{PAR}) with restored DNA-binding properties was tested on reporter genes under control of either consensus or nonoptimal binding sites. EH^{PAR} strongly activated transcription of both the $(HLF)_4$- and $(VBP)_8$-TK-CAT reporters and in each case was a substantially more potent activator than wild-type HLF (Fig. 6B). These data indicated that the transcriptional properties of wild-type and chimeric HLF proteins may be markedly divergent and dictated by specific binding sequences because of loss of HLF ancillary DNA-binding regions that occurs following the t(17;19) translocation.

Both HLF and E2A-HLF are transcriptionally competent in lymphoid cells. To determine whether the transactivation properties of wild-type HLF might be cell type specific and to investigate the properties of E2A-HLF in human lymphoid cells phenotypically similar to those in which the t(17;19) translocation is observed, transfections were performed with the $(HLF)_4$-TK-CAT reporter in the B-precursor ALL cell line

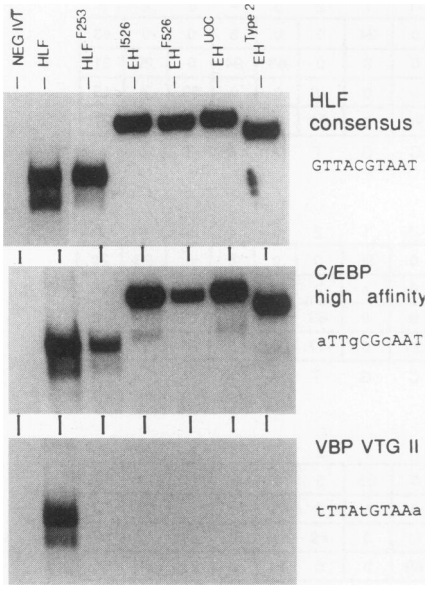


FIG. 4. Comparison of site-specific DNA binding by HLF and E2A-HLF proteins. Mobility shift analyses demonstrate DNA-binding complexes containing HLF and E2A-HLF proteins on selected sites. EMSAs with three sites are aligned (see Table 1 for results of binding to additional sites); the protein in each lane is denoted at the top, and the binding site and sequence appear at the right. Positions which match the consensus are shown in uppercase type and those which differ are in lowercase type. The exposure of autoradiographs differs for each binding site; relative binding is indicated in Table 1. NEG IVT, translate programmed with vector alone.

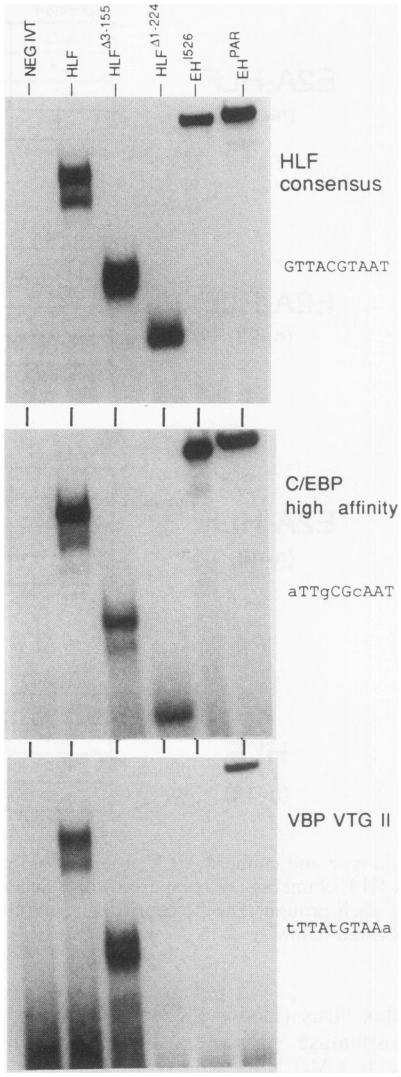


FIG. 5. DNA-binding alterations are due to loss of an HLF ancillary DNA-binding domain. Mobility shift analyses show DNA binding by various HLF and E2A-HLF proteins to selected sites. The protein in each lane is denoted at the top, and the binding site and sequence appear at the right. Positions which match the consensus are in uppercase type, and those which differ are in lowercase type. The exposure of autoradiographs differs for each binding site; relative binding is indicated in Table 1. NEG IVT, translate programmed with vector alone.

REH (Fig. 7). Wild-type HLF activated CAT expression in REH cells approximately fivefold over background levels, analogous to results seen with CV-1 cells. Significant basal activation of the $(HLF)_4$-TK-CAT reporter was observed in the absence of cotransfected activator. This background activation was dependent on the presence of HLF-binding sites, as it was not seen with reporters lacking binding sites or with reporters containing C/EBP-binding sites. E2A-HLF proteins were also strong transcriptional activators of the $(HLF)_4$-TK-CAT reporter in REH cells. The isoleucine-containing chimeras were consistently more potent activators than wild-type HLF, while EH^{F526} was similar in potency to HLF. These data demonstrate that wild-type HLF is not restricted in its transactivation capability to cell lineages in which its mRNA is normally expressed (liver, kidney, and lung) and that the mechanism for oncogenic activation of HLF by fusion with E2A is not a simple consequence of converting a nonlymphoid activator into one that is transcriptionally competent in lymphoid cells.

HLF wild-type and chimeric proteins transactivate C/EBP reporter constructs. As wild-type HLF and E2A-HLF bound well to the high-affinity C/EBP site in EMSA, we also investigated whether these proteins could activate a reporter containing concatamerized C/EBP sites (Fig. 7). Both wild-type and fusion proteins activated CAT expression from $(C/EBP)_4$-TK-CAT. Consistently less basal activation of the $(C/EBP)_4$-TK-CAT reporter in the absence of cotransfected activator was seen compared with results with $(HLF)_4$-TK-CAT, thus accounting for a higher fold induction of the $(C/EBP)_4$-TK-CAT reporter. In contrast to results seen with the $(HLF)_4$-TK-CAT reporter, EH^{F526} was less potent than wild-type HLF or the other fusion proteins, compatible with differences observed in protein binding to this site (Fig. 4 and Table 1).

E2A-HLF is capable of dominant-negative inhibition of transcription mediated by wild-type TEF. The disparate transcriptional properties of HLF and E2A-HLF observed on nonoptimal binding sites suggested that interactions between E2A-HLF and wild-type PAR proteins could potentially occur and significantly alter the normal transcriptional activity of the latter. Indeed, in previous studies we have shown that E2A-HLF can heterodimerize with each of the wild-type PAR proteins (15). To investigate the transcriptional properties of such heterodimers, we performed cotransfections with TEF expression constructs. TEF was chosen for these experiments because its mRNA is expressed in human lymphoid cells and

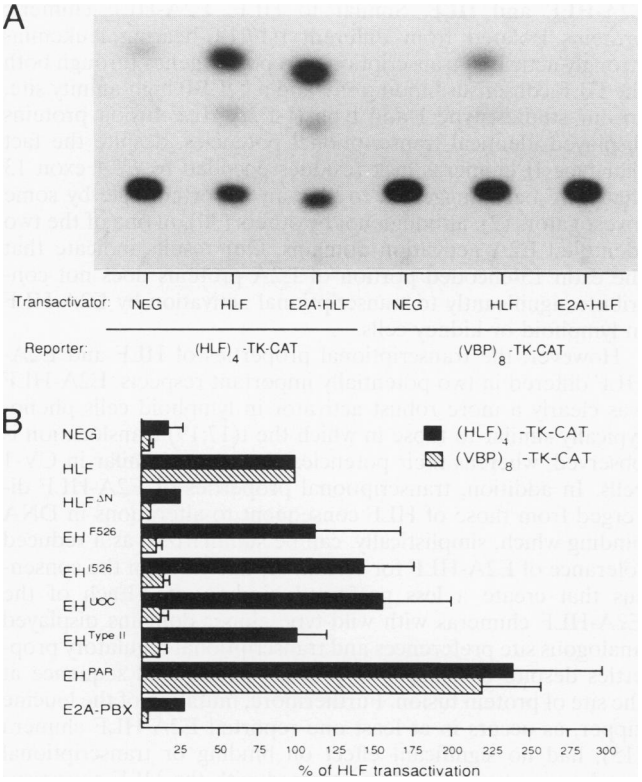


FIG. 6. Transactivation properties of wild-type and chimeric HLF proteins on optimal and nonoptimal binding sites in CV-1 cells. (A) Representative results of CAT assays assessing relative transcriptional properties of HLF and E2A-HLF^{I526} on reporter genes containing the herpes simplex virus TK minimal promoter linked to optimal (HLF) or nonoptimal (VBP) binding sites. NEG, reporter cotransfected with pCMV1 expression vector lacking transactivator insert. (B) Histogram showing relative transactivation properties of wild-type and fusion HLF proteins on reporter constructs containing optimal (HLF) or nonoptimal (VBP) binding sites. Results shown are averages of duplicate determinations and standard deviations for six [$(HLF)_4$-TK-CAT] or three [$(VBP)_8$-TK-CAT] independently performed experiments expressed as a percentage of activation observed for wild-type HLF. NEG, reporter without cotransfected activator construct. HLF$^{\Delta N}$ denotes the HLF$^{\Delta 3\text{-}155}$ protein.

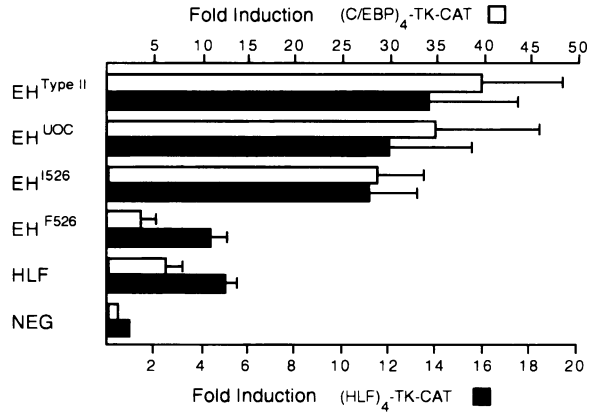


FIG. 7. Wild-type and chimeric HLF proteins display transcriptional activation properties on optimal binding sites in lymphoid cells. Transcriptional activation of chimeric and wild-type HLF proteins was assessed on reporter genes following cotransfections into B-precursor ALL REH cells. Results shown are averages of duplicate determinations and standard deviations of four independently performed experiments expressed as fold induction of activation seen with pCMV1 expression vector lacking activator insert (NEG). Reporter constructs and relative scales are indicated above and below the bar graph.

its DNA-binding and transcriptional regulatory properties are essentially identical to those of HLF when assayed with the binding sites utilized in this report (12). For these studies reporters containing the nonoptimal VTG II sites were used since they enabled the transcriptional properties of E2A-HLF and TEF to be readily distinguished. When TEF was transfected with the $(VBP)_8$-AP-CAT reporter into CV-1 or 697 (human pre-B ALL) cells, robust transactivation of CAT expression was observed. However, cotransfection of increasing amounts of E2A-HLF with a fixed amount of TEF led to a progressive decline in TEF-mediated transactivation (Fig. 8). Qualitatively similar reductions were observed with either EH^{I526} or EH^{F526} chimeras in both CV-1 and 697 cells, with a reduction to levels seen with E2A-HLF alone when 5- to 10-fold more E2A-HLF than TEF expression construct was transfected. The observed inhibition did not appear to be specific for the E2A portion of the chimera since HLF^{F253} also had significant dominant-negative effects. To ensure that nonspecific squelching did not account for the observed dominant-negative effects, identical experiments were performed with a mutant fusion protein (EH$^{\Delta ZIP}$ [Fig. 2]) unable to dimerize with TEF because of deletion of its leucine zipper. Western blot (immunoblot) analysis of transfected cells indicated that similar amounts of EH$^{\Delta ZIP}$ and other E2A-HLF proteins were

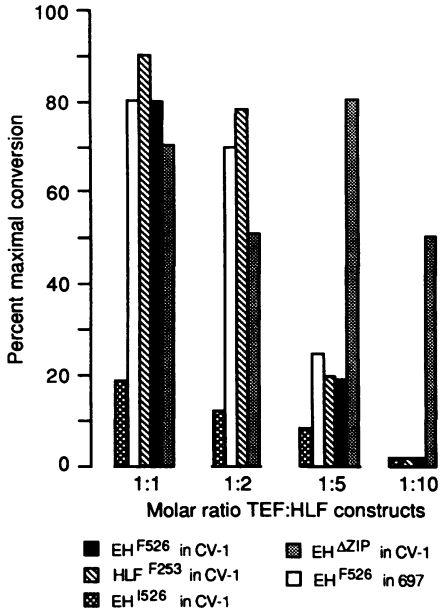


FIG. 8. E2A-HLF displays dominant negative transcriptional properties on nonoptimal binding sites. Histogram showing effects on TEF-mediated transactivation of $(VBP)_8$-AP-CAT in the presence of increasing molar ratios of various chimeric and mutant HLF proteins. Results shown are averages of duplicate determinations and expressed as the percentage of activation observed for TEF alone. Each construct was analyzed in at least two different experiments on separate occasions. Constructs and cells employed for each experiment are as follows: ■, EH^{F526} in CV-1; ▨, HLF^{F253} in CV-1; ▩, EH^{I526} in CV-1; ▧, EH$^{\Delta ZIP}$ in CV-1; □, EH^{F526} in 697.

present (12). TEF-mediated transcriptional activation was not significantly affected by excess $EH^{\Delta ZIP}$.

DISCUSSION

The studies described in this report were undertaken to investigate the transcriptional consequences of E2A-HLF fusion which occurs in a subset of ALLs bearing a t(17;19)(q21-q22;p13) translocation. Structural features predict that E2A-HLF should function as a chimeric transcription factor that binds to cognate HLF sites in target gene promoters and activates transcription via specific E2A effector domains. Our studies demonstrate experimentally that E2A-HLF does indeed possess such transcriptional regulatory properties. However, while both HLF and E2A-HLF bind avidly to, and activate transcription via, the consensus sequence 5′-GTT ACGTAAT-3′, significant differences in both DNA-binding and transcriptional activation are seen on certain closely related sites. In light of the fact that physiologic binding sites for transcription factors are not generally exact matches with in vitro-selected binding sites, these differences may have significant consequences with respect to leukemogenic target genes regulated by the chimera. In addition, these studies have important implications for understanding the transcriptional properties of HLF and the PAR subfamily of bZIP proteins.

HLF is a transcriptional activator in several cell types. Our studies demonstrate that HLF functions experimentally as a transcriptional activator of reporter constructs containing appropriate binding sites upstream of minimal promoters. HLF was transcriptionally competent in both CV-1 monkey kidney cells which express wild-type *HLF* mRNA and in human B-precursor ALL cells in which *HLF* mRNA is normally absent. In addition, we have observed that HLF can activate transcription in NIH 3T3 cells which also do not express *HLF* mRNA (12). This property clearly distinguishes HLF from the closely related PAR protein DBP which is transcriptionally competent only in hepatocytes or hepatocyte-derived cell lines (21, 27, 36). In this respect HLF is more functionally analogous to TEF/VBP; in fact, in these and other studies (12) we have observed no differences between HLF and TEF with respect to either binding site preferences or transcriptional regulatory properties.

It is apparent from the DNA-binding studies presented herein and earlier by others that PAR proteins are capable of substantial cross-recognition of various binding sites (11, 15, 17). The ability of PAR proteins to heterodimerize with one another introduces an additional layer of complexity. The situation is further complicated by the binding of PAR proteins to sites recognized by C/EBP proteins and vice versa, although heterodimerization between proteins in the different classes has not been observed and structural constraints predict that it should not occur (33). However, it is possible that PAR and C/EBP proteins can interact by other mechanisms, as DBP has been suggested to synergize with C/EBP in postpubertal activation of the factor IX promoter that occurs in hemophilia B Leyden (29). The mechanism of this synergy is undefined but does not involve direct DNA binding by heterodimers. Thus, PAR proteins by themselves and in combination with C/EBP proteins potentially comprise a complex regulatory network, particularly in the liver, where many of these proteins appear to be expressed simultaneously. It will be technically challenging to experimentally sort out the relative contributions of individual proteins to the expression of specific genes; however, such combinatorial regulatory networks are also a common feature of other transcription factor families.

Divergence in transcriptional regulatory properties between E2A-HLF and HLF. Similar to HLF, E2A-HLF chimeric proteins isolated from different t(17;19)-bearing leukemias strongly activated transcription of reporter genes through both the HLF consensus binding site and a C/EBP high-affinity site. In our studies, type I and type II E2A-HLF fusion proteins displayed identical transcriptional potencies, despite the fact that type II chimeras lack residues encoded by *E2A* exon 13 that have been suggested to play an important role by some investigators (2), although not by others (30), in one of the two identified E2A activation domains. Our results indicate that the exon 13-encoded portion of E2A proteins does not contribute significantly to transcriptional activation by E2A-HLF in lymphoid or kidney cells.

However, the transcriptional properties of HLF and E2A-HLF differed in two potentially important respects. E2A-HLF was clearly a more robust activator in lymphoid cells phenotypically similar to those in which the t(17;19) translocation is observed, whereas their potencies were more similar in CV-1 cells. In addition, transcriptional properties of E2A-HLF diverged from those of HLF consequent to alterations in DNA binding which, simplistically, can be summarized as a reduced tolerance of E2A-HLF for certain deviations from the consensus that create a less preferred binding site. Each of the E2A-HLF chimeras with wild-type zipper domains displayed analogous site preferences and transcriptional regulatory properties despite differences in primary amino acid sequence at the site of protein fusion. Furthermore, mutation of the leucine zipper, as occurs in at least one reported E2A-HLF chimera (15), had no significant effect on binding or transcriptional regulatory properties when assessed with the HLF consensus site but exacerbated the divergences observed on less preferred sites. It is currently unclear whether this mutation may direct the E2A-HLF chimera away from or toward select binding sites in vivo or simply was tolerated as functionally insignificant in the leukemia cells in which it arose. Distinguishing between these two possibilities will require an in vitro transformation assay.

The most dramatic differences between the transcriptional effects of wild-type and chimeric HLF proteins were observed with reporters containing nonoptimal binding sites such as VBP VTG II sites. Despite decreased binding to this site in EMSA, wild-type HLF and TEF each activated transcription from reporters containing concatamers of this site; in contrast, E2A-HLF displayed no significant ability to bind to this site in EMSA or to activate transcription of VTG II site-containing reporters. In fact, using exogenously transfected activator constructs, we found that E2A-HLF was capable of abolishing TEF-mediated transcriptional activation of VTG II site-containing reporters in a dominant-negative manner. We attempted to test the physiologic significance of the observed repression by transfecting VTG II site-containing reporters into cell lines with and without the t(17;19) translocation and relying on endogenous, rather than exogenous, levels of E2A-HLF for repression. However, basal levels of expression from both VBP-TK-CAT and VBP-AP-CAT reporters were virtually undetectable (1 to 2% CAT conversion) and no different from reporters lacking binding sites. This lack of VTG II site-dependent baseline transcriptional activation made it technically impossible to address this question by relying on endogenous levels of protein expression. It should be emphasized that the repression observed in cotransfection experiments occurred with fairly modest ratios of E2A-HLF to TEF (5:1), suggesting that gross overexpression of E2A-HLF is not required. It is likely that the observed inhibition occurred through sequestration of TEF in E2A-HLF–TEF heterodimeric complexes that are significantly impaired in binding

to the nonoptimal VTG II site. In support of this, we found that while E2A-HLF–TEF heterodimers bound well to the TEF GH and HLF consensus sites, binding to the VTG II site was barely detectable in EMSA (12, 15). Although *TEF* mRNA is present in human lymphoid cells, including t(17;19)-ALL cell lines (12), it is currently uncertain whether or not TEF or other endogenous proteins are available for in vivo dimerization with E2A-HLF. However, our findings suggest the potential for heteromeric interactions that may alter expression of a subset of subordinate genes with nonoptimal binding sites.

Transcriptional differences result from loss of an HLF ancillary DNA-binding domain. PAR proteins each possess an amino-terminal extension of the basic region that contains a highly conserved cluster of basic amino acids and the adjacent PAR domain. Drolet et al. (11) have previously demonstrated that experimental substitution of alanines for pairs of lysine residues within this cluster substantially altered TEF binding to a recognition element in the prolactin gene promoter, but not the GH site. Our studies confirm and extend these observations to underscore the importance of regions outside the classic basic region for binding to select, nonoptimal sites. These differences were a consistent feature of E2A-HLF fusion proteins and experimentally truncated HLF constructs. Furthermore, our data show that residues within the PAR also contribute to DNA binding, since restoration of wild-type binding required both the PAR and BRE within the backbone of the E2A-HLF fusion protein. By analogy, Jun contains a functionally similar ancillary DNA-binding domain which is also located immediately amino terminal to the basic region (1). Similar to the PAR, this region is proline rich, but there is no obvious structural homology between Jun and HLF in this region.

Broad spectrum of potential E2A-HLF target genes. In this report we have (at least partially) defined the spectrum of sites to which HLF binds, the subset of those sites bound by E2A-HLF, and the transcriptional effects of wild-type and chimeric HLF proteins on reporter genes containing concatamers of optimal or nonoptimal binding sites. Each of the sites employed in experiments described herein contained complementary pairs of thymidine and adenosine nucleotides at positions −4 and −3 and +3 and +4. This feature is characteristic of high-affinity binding sites for the PAR and C/EBP subclasses of bZIP proteins. In contrast, high-affinity binding sites for members of the Fos/Jun and ATF/CREB bZIP subfamilies do not contain such pairs (18). We have also observed that both HLF and E2A-HLF are capable of binding to sites composed of one PAR- or C/EBP-like half-site and one Fos/Jun- or ATF/CREB-like half-site (12). Thus, potential leukemogenic target genes regulated by the chimera include not only cognate targets of HLF and related PAR proteins but also those normally regulated by other bZIP proteins. One such protein to consider is E4BP4, which is highly homologous to HLF within the basic region and binds preferentially to a site, (G/A)T(G/T)A(C/T)GTAA(C/T), that is essentially identical to the HLF consensus site (8). Interestingly, E4BP4 functions experimentally as a transcriptional repressor.

Two additional bZIP chimeras are known to be created by chromosomal translocations in human malignancies—TLS-(FUS)/CHOP-10 (9, 31) and EWS/ATF-1 (38). Similar to E2A-HLF, each of these chimeras consists of a bZIP region fused to a portion of the structurally related proteins EWS or TLS(FUS) that contain potential transactivation domains. Given this structural similarity, it will be important to determine whether or not the transforming properties of these different bZIP chimeras are mediated by aberrant transcriptional regulation of an overlapping set of target genes.

Functional consequences of E2A-HLF fusion. We have demonstrated previously that endogenous E2A-HLF-containing complexes are present in the HAL-01 t(17;19)-ALL cell line (15). Similar complexes are observed when EMSA is performed with HAL-01 nuclear extracts and the HLF consensus site, and these complexes comigrate with IVT E2A-HLF homodimers (12). Thus, our studies are consistent with a model in which the leukemogenic effects of E2A-HLF are mediated by its ability to activate transcription of target genes as a homodimer. In addition, possible heteromeric interactions with potential dominant-negative effects on transcription of select subordinate genes cannot be excluded, but their in vivo significance remains to be determined.

E2A-HLF chimeras resulting from t(17;19) translocation are structurally analogous to E2A-PBX1 proteins in t(1;19)-bearing leukemias (19, 28). In both cases, the amino-terminal portion of E2A including its transactivation domains is fused to a heterologous DNA-binding domain donated by genes not normally expressed in lymphoid cells. PBX1 and closely related PBX2 and PBX3 proteins (26) bind a specific DNA sequence but do not activate reporter genes containing this site, in contrast to E2A-PBX1, which is a robust activator on the same reporters (22, 32). Similarly, wild-type PBX proteins are incapable of transforming NIH 3T3 cells and require fusion with E2A to activate their oncogenic potential (25). Thus, t(1;19)-induced chimeras appear to have unique transcriptional regulatory and transforming properties not possessed by either of the constituent wild-type proteins. By comparison, the transcriptional differences between HLF and E2A-HLF appear more subtle, as both functioned as activators in our experimental assays. This raises the issue of whether protein fusion is essential for activation of the oncogenic potential of HLF or whether wild-type HLF may also function as an oncoprotein in the correct cellular context. It is not clear from our studies whether there is a specific role for the E2A transactivation domains as opposed to others in conferring oncogenic potential to the HLF bZIP domains. Establishment of an in vitro transformation assay for E2A-HLF will allow an experimental assessment of these issues. However, our studies have established the spectrum of possible transcriptional regulatory properties of t(17;19)-encoded chimeras and will serve as a foundation for future studies aimed at determining which of these properties is essential for transformation and to help identify leukemogenic target genes.

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