Hlf, a novel hepatic bZIP protein, shows altered DNA-binding properties following fusion to E2A in t(17;19) acute lymphoblastic leukemia

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Oncogenic conversion of transcription factors by chromosomal translocations is implicated in leukemogenesis. We report that the t(17;19) in acute lymphoblastic leukemia produces a chimeric transcription factor consisting of the amino-terminal portion of HLH proteins E12/E47 (products of the E2A gene) fused to the basic DNA-binding and leucine zipper dimerization motifs of a novel hepatic protein called hepatic leukemia factor (Hlf). Hlf, which is not normally transcribed in lymphoid cells, belongs to the recently described PAR subfamily of basic leucine zipper (bZIP) proteins, which also includes Dbp and Tef/Vbp. Wild-type Hlf is able to bind DNA specifically as a homodimer or as a heterodimer with other PAR factors. Structural alterations of the E2a–Hlf fusion protein markedly impair its ability to bind DNA as a homodimer compared with wild-type Hlf. However, E2a–Hlf can bind DNA as a heterodimer with other PAR proteins, suggesting a novel mechanism for leukemogenic conversion of a bZIP transcription factor.

[Key Words: Chromosomal translocation, transcription factor, bZIP protein, HLH protein, chimeric protein, oncogene]

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The largest group of proto-oncogenes identified to date codes for proteins implicated in regulation of transcription and shares with known transcription factors common structural motifs that mediate cognate interactions with DNA or other components of the transcription complex (Cleary 1991). Molecular studies have suggested several general mechanisms whereby transcriptional proteins may be converted to oncogenic factors in leukemia. One mechanism results in dysregulated expression of wild-type proteins following translocation to a transcriptionally dominant locus such as the immunoglobulin or T-cell receptor (TCR) genes. Dominant transforming properties may also result from protein structural changes in the form of truncations or protein fusions. In acute leukemias, chimeric transcription factors such as E2a–Pbx1 and Pml–Rara have been described in t(1;19)-carrying pre-B cell acute lymphoblastic leukemia (ALL) and t(15;17) acute promyelocytic leukemia, respectively (Mellentin et al. 1989; Kamps et al. 1990; Nourse et al. 1990; Cleary 1991; de Thé et al. 1991; Kakizuka et al. 1991; Pandolfi et al. 1991). Structural features of E2a–Pbx1 fusion proteins suggest that transcriptional activation domains may be oncogenic depending on their specific protein contexts. E2a–Pbx1 chimeras consist of the DNA-binding homeo domain of Pbx1 fused to a portion of helix-loop-helix (HLH) proteins E12/E47, which includes a domain with trans-activation properties (Henthorn et al. 1990). Consistent fusion of E2a and Pbx1 has been observed between different pre-B cell t(1;19) leukemias, suggesting that collaboration of specific motifs in each protein determines leukemogenic properties of the chimera (Mellentin et al. 1990; Hunger et al. 1991; Privitera et al. 1992). E2a-Pbx1 proteins transform NIH-3T3 cells (Kamps et al. 1991), but mutagenesis studies have not yet established which portions of the chimera are required. Wild-type Pbx1 is not normally expressed in lymphoid cells (Kamps et al. 1990, Nourse et al. 1990; Monica et al. 1991), raising the possibility that E2a–Pbx1 deregulates target genes normally regulated by Pbx1 in nonlymphoid cells or genes regulated by highly homologous proteins such as Pbx2 or Pbx3 in pre-B cells (Monica et al. 1991). These models presuppose that the DNA-binding characteristics of E2a–Pbx1 recapitulate those of wild-type Pbx1, an assumption that has not yet been tested experimentally.

To further characterize the role of E2a in leukemogenesis and to establish the spectrum of transcriptional pro-

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proteins that may be oncogenically activated by protein fusion, we have investigated potential variant translocations involving E2A in acute leukemias. A newly recognized nonrandom translocation involving the general chromosome 19 region of E2A, the t(17;19) (q21-q22;p13), has been described recently in childhood ALL (Raimondi et al. 1991) but molecular studies have provided conflicting results as to the involvement of the E2A gene (Inaba et al. 1991; Ohyashiki et al. 1991; Yamada et al. 1991). In the current study, we demonstrate that the t(17;19) results in fusion of the E2A gene to the gene for a novel basic leucine zipper (bZIP) protein [hepatic leukemia factor (Hlf)] resulting in expression of a chimeric transcription factor. The t(17;19) fusion protein shows several unique structural and functional features that distinguish it from previously described translocated proteins in ALL. These features are responsible for a marked difference in the DNA-binding properties of the E2a–Hlf fusion protein when compared with wild-type Hlf. These data suggest a novel mechanism for oncogenic conversion of a bZIP protein and may have significant consequences with respect to target genes affected by a chimeric HLH–bZIP transcription factor.

Results

Cloning of E2A fusion cDNAs from the HAL-01 cell line

To assess whether t(17;19) breakpoints involve the E2A gene on chromosome 19, Southern blots were performed on the HAL-01 cell line, which was established previously from a patient with t(17;19) ALL (Ohyashiki et al. 1991). Hybridization with a probe that detected rearrangements in all cases of t(1;19) ALL (Mellentin et al. 1989, 1990) showed that HAL-01 DNA contained E2A gene rearrangements (Fig. 1A), indicating that the t(17;19) breakpoint was located in the E2A gene similar to previously reported t(1;19) breakpoints.

Northern blot analyses showed aberrant-sized E2A transcripts (~4.1 and 4.3 kb) in HAL-01 in addition to the expected wild-type E2A transcripts of 3 and 4.4 kb (Fig. 1A). E2A homologous cDNAs were isolated from a HAL-01 cDNA library by use of a different screening procedure (see Materials and methods) that selected for potential fusion cDNAs containing 5' E2A sequences. Nucleotide sequence analyses indicated that all 10 clones contained 5' portions of E2A up to nucleotide 1519 [Nourse et al. 1990], at which point they diverged from E2A. This point of divergence coincided precisely with the fusion site between E2A and PBX1 sequences in t(1;19) ALL transcripts and is known to be the 3' border of E2A exon 13 (J. Nourse and M.L. Cleary, unpubl.). The predicted E2A amino acid sequence of the HAL-01 cDNAs was identical to that reported previously (Henthorn et al. 1990; Kamps et al. 1990; Nourse et al. 1990) except for a nonconservative glycine-to-serine substitution (GGC → AGT) in codon 425 (Fig. 2A).

The sequence 3' of nucleotide 1519 was not homologous to E2A sequences nor to PBX1 or closely related family members PBX2 and PBX3 (Monica et al. 1991). However, an open reading frame (ORF) was maintained for 91 additional codons beyond the fusion site (Fig. 2A). The protein predicted by these additional codons was not identical to previously reported proteins but shared significant similarity to the hepatic bZIP transcription factor Dbp (D-binding protein) involved in albumin gene expression.

Figure 1. Blot analyses of the HAL-01 cell line demonstrating involvement of E2A and HLF by t(17;19). (A) Southern and Northern blots hybridized with an E2A probe [E47M]. (Left) Southern blot of EcoRI-digested DNA from HAL-01 and control genomic DNA (C). Dashes indicate germ-line bands of 23 and 11 kb. E2A gene rearrangements are denoted by arrowheads. (Right) Northern blot of RNA from the t(1;19) ALL cell line 697, HAL-01, and a B-lineage lymphoma cell line (FL18). Wild-type E2A transcripts of 3.0 and 4.4 kb are indicated by dashes. aberrantly sized E2A transcripts are observed HAL-01 (4.3 and 4.1 kb), and E2A–PBX1 transcripts of 8.3 kb are present in 697 cells. (B) Northern blots hybridized with an HLF-specific probe. (Right) Transcripts that hybridize with the HLF probe are observed only in HAL-01 and comigrate with the aberrantly sized E2A transcripts. (Left) Wild-type HLF transcripts are detected in the HepG2 hepatocellular carcinoma cell line.
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**Figure 2.** Sequence analyses of the E2A–HLF fusion protein and wild-type HLF. (A) Amino acid sequence predicted from HAL-01 fusion cDNA shown in single-letter amino acid code. The 20-amino-acid insertion between E2a and Hlf sequences is underlined. Amino acid numbering is according to Nourse et al. (1990). Of 57 amino acids, 41 were identical between Dbp and the E2a fusion partner in a region corresponding to the Dbp basic and leucine zipper motifs (on the basis of the corrected Dbp sequence in Drolet et al. 1991; Iyer et al. 1991). The novel Dbp-related protein fused to E2a in HAL-01 therefore has features of a bZIP protein and will hereafter be referred to as Hlf.

**Tissue-specific expression and cloning of wild-type HLF**

Tissue-specific expression of the HLF gene was determined by Northern blot analyses on RNA isolated from human cell lines and tissues of both adult and fetal origin by use of an HLF-specific probe. Expression of HLF was quite restricted (Table 1), as no expression was detected in normal peripheral blood mononuclear cells or in any hematolymphoid cell line examined other than HAL-01. The abundant HLF transcripts detected in HAL-01 comigrated with aberrant-sized E2A transcripts, confirming the fusion nature of these RNAs (Fig. 1B). The nontranslocated, wild-type HLF gene in HAL-01 appeared to be transcriptionally silent, as all transcripts detected with the HLF probe also hybridized to the E2A probe. Abundant transcription of wild-type HLF RNA was observed in adult liver and the HepG2 hepatocellular carcinoma cell line (Table 1; Fig. 1B). Low levels of HLF expression were also detected in adult kidney and lung. Of several fetal tissues examined, HLF expression was detected only in fetal liver, at levels significantly lower than that seen in adult liver. Low levels of HLF expression were also detected in adult kidney and lung. Of several fetal tissues examined, HLF expression was detected only in fetal liver, at levels significantly lower than that seen in adult liver. To further characterize HLF, cDNAs for wild-type HLF were isolated from a HepG2 cDNA library. Nucleotide sequence analyses of a 3-kb cDNA predicted an ORF of 885 nucleotides that overlapped at its 3' end with the 20-amino-acid insertion between HLF and the E2a fusion partner in a region corresponding to the Dbp basic and leucine zipper motifs (on the basis of the corrected Dbp sequence in Drolet et al. 1991; Iyer et al. 1991). The novel Dbp-related protein fused to E2a in HAL-01 therefore has features of a bZIP protein and will hereafter be referred to as Hlf.
Table 1. Expression of HLF

| Cell line/tissue                      | HLF mRNA expression |
|--------------------------------------|---------------------|
| **Hematolymphoid cells**             |                     |
| lymphoid cell lines                  |                     |
| HAL-01                               | ++                  |
| mature B-cell lines                  |                     |
| [607B, FL18, DUL5]                   | −                   |
| precursor B-cell lines               |                     |
| [697, KJ, HB, RCH-ACV]               | −                   |
| mature T-cell lines [Molt4]          | −                   |
| early T-cell lines                   |                     |
| [8402T, NL, JH]                      | −                   |
| nonlymphoid cell lines               |                     |
| myeloid [HL60, U937]                 | −                   |
| erythroid [K562]                     | −                   |
| indeterminate lineage                | −                   |
| (DHL1, TS, DUS28)                    | −                   |
| normal peripheral blood              |                     |
| lymphocytes                          | −                   |
| **Nonhematolymphoid cell lines**     |                     |
| fibroblast (2938)                    | −                   |
| epithelial [PA-1, A431, MCF7]        | −                   |
| neuroectodermal (HS683, A172)        | −                   |
| hepatocellular carcinoma             |                     |
| [HepG2]                              | ++                  |
| **Adult tissues**                    |                     |
| liver                                | + +                 |
| lung                                 | + / −               |
| kidney                               | + / −               |
| ovary                                | −                   |
| placenta                             | −                   |
| heart                                | −                   |
| brain                                | −                   |
| pancreas                             | −                   |
| **Fetal tissues (18–22 weeks gestational age)** | + / − |
| liver                                | + / −               |
| intestine                            | −                   |
| brain                                | −                   |
| lung                                 | −                   |
| kidney                               | −                   |

HLF transcript expression was measured by Northern blot analysis. The integrity of all RNAs was confirmed by hybridization with a human actin cDNA probe. Symbols: [ + + ] Abundant mRNA visible after 2–4 hr of exposure, [ + / − ] mRNA detected only after prolonged exposure, [ − ] no mRNA detected after at least 3 days of exposure.

*aAbundant E2A–HLF fusion mRNA but no wild-type HLF mRNA.

served in all three reading frames in the 100 nucleotides upstream of the initiating ATG. Several different poly(A) sites were observed in both wild-type HLF and E2A–HLF fusion cDNAs (data not shown), presumably accounting for the different-sized RNA species detected by Northern blot analysis [Fig. 1].

Comparison of E2A, HLF, and E2A–HLF sequences showed two important features that distinguished them, in addition to the previously mentioned glycine-to-serine substitution. First, there were 57 nucleotides in E2A–HLF between the E2A and HLF portions that did not match either of the corresponding wild-type sequences, indicating that a 20-amino-acid insertion of unknown origin was present in the fusion protein (underlined in Fig. 2A). Second, wild-type HLF contained an isoleucine at amino acid 253 (Fig. 2B) compared to a phenylalanine at the equivalent position in E2α–HLF [amino acid 526 in Fig. 2A]. This missense mutation was confirmed by sequencing multiple independently isolated E2A–HLF and wild-type HLF cDNAs. Isoleucine appeared to be the wild-type amino acid, as an isoleucine is also found at this position in Dbp and Tef/Vbp (Mueller et al. 1990; Drolet et al. 1991; Iyer et al. 1991), which are significantly similar to HLF in this region (see below).

HLF shows sequence similarity to a subfamily of bZIP transcription factors

Data base searches indicated that HLF is most similar to three previously described bZIP proteins: Dbp, a recently described transcription factor that binds to the D box in the albumin promoter (Mueller et al. 1990); Tef, a bZIP factor involved in thyrotroph differentiation (Drolet et al. 1991); and Vbp, involved in vitellogenin II gene expression, which appears to be the chicken homolog of Tef (Iyer et al. 1991). The region of highest similarity between these proteins lies within a basic region involved in mediating sequence-specific DNA binding and a dimerization motif with features of a modified leucine zipper containing isoleucine and cystine residues in place of leucines in the first and fifth heptad repeats (Fig. 3). However, Dbp and Tef/Vbp are also extensively similar to each other in a proline and acidic amino-acid-rich region (called PAR) that distinguishes this subfamily from other bZIP proteins (Drolet et al. 1991). Because a PAR homology domain is also present in HLF, it constitutes a third member of the PAR subfamily. The carboxy-terminal 138 amino acids of each protein, which include the entire bZIP region and the upstream PAR domain (as defined by Drolet et al. 1991), are 73% identical between HLF and Tef, 68% identical between HLF and Dbp, and 65% identical between Dbp and Tef. The homology between PAR family members within the bZIP region is far greater than is the similarity with other bZIP proteins such as C/EBP, c-Fos, c-Jun, CREB, and Gcn4 (cf. Drolet et al. 1991). Amino-terminal to the PAR region, the sequences diverge (data not shown); however, another region of significant homology exists from amino acid 42 to 101 of HLF. Within this region, HLF is much more similar to Tef/Vbp (62% identical) than it is to Dbp.

A 57-nucleotide insertion in E2A–HLF fusion RNA originates from the breakpoint introns of E2A and HLF

Nucleotide sequence analysis of germ-line chromosome 19 DNA, where the HAL-01 t(17;19) breakpoint occurred, indicated that the first 20 nucleotides of the cDNA insertion originated from the E2A breakpoint intron (Fig. 4A). These 20 nucleotides are flanked on their 5' side by sequences characteristic of a 3' intron/5' exon.
Figure 3. Homology of Hlf to the PAR subfamily of bZIP proteins. The region of homology between Hlf, Tef, Vbp, and Dbp is subdivided into a PAR region as defined by Drolet et al. (1991), a PAR-specific basic region extension, the bZIP basic region as defined by Vinson et al. (1989), the “hinge” which separates the basic and zipper regions, and a leucine zipper domain. Sequences are from (Vinson et al. 1989): (B) basic amino acid; (N) asparagine; (A) alanine; (R) arginine; (L) leucine. Spaces indicate that there is no consensus by an AG dinucleotide (Mount 1982). Immediately 3’ of the basic region is a pyrimidine-rich region followed by a heptamer border, consisting of a pyrimidine-rich region followed by an AG dinucleotide (Mount 1982). Immediately 3’ of these 20 nucleotides, 7 nucleotides showed features similar to heptamer motifs that serve as signal sequences for the immunoglobulin/TCR (IG/TCR) recombinase (Apelan et al. 1990; Brown et al. 1990). These data suggested that at least a portion of the insert originated from a cryptic exon present in the E2A breakpoint intron and suggested a possible role for aberrant IG/TCR recombinase activity in causation of t(17;19).

A polymerase chain reaction (PCR) approach was used to show that the 57-bp E2A–Hlf insertion constitutes the junction of chromosome 17 and 19 sequences at the t(17;19) breakpoint. Oligonucleotide primers homologous to portions of the insertion (I5’ and I3’ in Fig. 4A) and chromosome 19 genomic DNA flanking the insertion (INS-US and INS-DS) were employed. With I5’/I3’ primers, a 57-bp amplification product was observed in both the E2A–Hlf fusion cDNA and HAL-01 genomic DNA but not in other genomic DNAs (Fig. 4B). The INS-US/I3’ primer pair amplified a product of the predicted size (141 bp) only when HAL-01 genomic DNA was used as template. A 275-bp product was amplified from all genomic DNAs; primer pairs in the INS-US/INS-DS primer pair homologous to intron sequences surrounding the breakpoint. These data indicated that the 57-bp insertion directly spans the t(17;19) breakpoint in HAL-01 DNA. Although not directly demonstrated by these studies, the 3’ 37 bp of the insertion are likely derived from Hlf intron sequences at the breakpoint or, perhaps, from a combination of Hlf sequences and potential “N” nucleotide insertions frequently present at recombinase-mediated translocation breakpoints (Apelan et al. 1990, Brown et al. 1990). The junction of chromosome 17 and 19 sequences is apparently recognized as a cryptic exon during RNA splicing of the E2A–Hlf fusion transcript.

Hlf binds DNA specifically as a homodimer or as a heterodimer with other PAR proteins

Because Hlf is highly homologous to Tef/Vbp and Dbp within the basic region that mediates sequence-specific DNA binding in other bZIP proteins (Agre et al. 1989), we hypothesized that Hlf might also bind DNA sequences recognized by Tef/Vbp or Dbp. Electrophoretic mobility-shift assays (EMSAs) were performed with in vitro-translated Hlf with a radiolabeled oligonucleotide containing the Tef recognition element present in the rat growth hormone (GH) promoter as described by Drolet et al. (1991). Hlf bound to the GH site resulting in a major shifted DNA–protein complex (Fig. 5A); minor faster migrating bands were presumed to result from translation initiation at alternative sites of degradation. The binding was specific because it was effectively competed with excess unlabeled GH oligonucleotide and, to a lesser extent, by the albumin D site (Mueller et al. 1990) or an optimized Vbp site (Iyer et al. 1991), which are binding sites for similar PAR proteins. The Fos serum response element (SRE), which has recently been shown to contain a functional C/EBP site (Metz and Ziff 1991), competed much less effectively. No competition was observed with two unrelated oligonucleotides. Although the GH TEF site does not necessarily represent the optimal site, the results indicated that Hlf specifically and preferentially bound the GH site under these experimental conditions. Thus, subsequent DNA-binding studies were carried out under similar conditions.

As PAR and bZIP proteins bind DNA as dimers (Kouzarides and Ziff 1988; Gentz et al. 1989; Turner and Tjian 1989; Vinson et al. 1989; Drolet et al. 1991; Iyer et al. 1991), the dimerization potential of Hlf was assayed by EMSA following cotranslation of wild-type Hlf and a truncated version of Hlf (HlfΔN3, 155). When assayed individually, Hlf and HlfΔN demonstrated different mobil-
bZIP fusion protein in acute leukemia

Figure 4. Genomic origin of insertion sequences present in E2A–HLF fusion cDNA. (A) The E2A–bcr intron between exons (solid boxes) 13 and 14 is depicted at the top. [S, H] SacI and HindIII restriction sites, respectively. A schematic illustration of the E2A–HLF fusion cDNA appears at the bottom. Sequences show nucleotides comprising insertion (uppercase letters) in chromosome 19 germ-line DNA (top) and HAL-01 fusion cDNA (bottom). The consensus 3’ splice site and heptamer are overlined. The relative positions of the PCR amplification and detection oligonucleotides are indicated by arrows and lines, respectively. (B) PCR analysis demonstrating juxtaposition of germ-line chromosome 19 and nonchromosome 19 DNA sequences at the t{17;19} breakpoint in HAL-01. Input DNA consisted of E2A–HLF fusion cDNA (cDNA), HAL-01 genomic DNA (HAL-01), or control genomic DNA (control). Amplification and detection oligonucleotides are indicated below panels. The sizes (in bp) of the PCR products are indicated at left.
Figure 5. DNA-binding analyses of wild-type Hlf homodimers and heterodimers. (A) Mobility-shift analysis of in vitro-translated Hlf with a radiolabeled double-stranded oligonucleotide corresponding to the rat GH promoter TEF site. One hundred-fold molar excesses of the indicated oligonucleotides were added to each binding reaction as indicated. (B) Mobility-shift analysis of in vitro-translated wild-type Hlf (Hlf), an Hlf deletion mutant containing the PAR, basic, and leucine zipper domains (HlfΔN), or wild-type Hlf cotranslated with HlfΔN (Hlf + HlfΔN). (C) Interaction of Hlf and Tef on the TEF GH recognition element. Wild-type Tef (TEF) or a truncated Tef (HlfΔN) was translated independently or cotranslated in vitro and analyzed for DNA binding by EMSA. The positions of Hlf, Dbp, and Hlf–Dbp are indicated at right. (100× GH) A binding reaction with cotransfected Tef and HlfΔN in the presence of 100-fold molar excess of unlabelled probe. (D) Interaction of Hlf and Dbp on the TEF GH recognition element. Wild-type Hlf (Hlf), or Dbp (Dbp) were translated independently or cotranslated in vitro and analyzed for DNA binding by EMSA. The positions of Hlf, Dbp, and Hlf–Dbp are indicated at right.

Figure 6. DNA-binding analyses of E2a–Hlf. (A) Interaction of E2a–Hlf with Tef on the GH promoter TEF site. Mobility-shift analysis of E2a–Hlf [lane 1] translated individually was compared with Tef [lane 2] or cotranslation of E2a–Hlf and Tef [lanes 3–6]. Migrations of E2a–Hlf : Tef heterodimer (HD) and Tef homodimer (TEF) complexes are indicated. Binding reactions were performed with an excess of unlabelled TEF site (lane 4), in the presence of nonimmune sera (lane 5) or anti-E2a sera (lane 6). Cotranslations of E2a–Hlf with Dbp or wild-type Hlf (Fig. 6B,C) also showed slower migrating complexes whose formation was specifically inhibited in the presence of anti-E2a sera. These data indicate that E2a–Hlf binds DNA in a heterodimeric complex with other PAR proteins. Mini-
A zipper mutation alters the DNA-binding properties of E2a–Hlf

Sequence comparison of E2a–Hlf with other PAR proteins including wild-type Hlf [Figs. 2A and 3] showed that a missense mutation had converted an isoleucine to phenylalanine in the first heptad repeat of the leucine zipper of E2a–Hlf. As previous studies have shown that mutations in the leucine zipper can have profound consequences on dimerization and DNA-binding properties of bZIP proteins, the effect of this nonconservative amino acid alteration in E2a–Hlf was assessed further. Various forms of Hlf and E2a–Hlf [Fig. 7A] were translated in vitro and tested for DNA binding by EMSA. A mutant fusion protein [E2a-HlfI] in which F526 was converted to an isoleucine by site-directed mutagenesis showed significantly greater binding to the TEF site than E2a–Hlf under these conditions [Fig. 7B, lanes 1,2]. The reduced DNA binding observed for E2a–Hlf was not the result of less protein in the reaction, as equal amounts of input protein were confirmed by SDS-PAGE of radiolabeled in vitro translates [data not shown]. The contribution of the I→F526 change to DNA binding was assessed further by analysis of a mutant Hlf protein containing an I→F233 substitution in the analogous zipper residue of wild-type Hlf. The HlfI mutant showed significantly poorer binding than wild-type Hlf, as evidenced by at least a 100-fold reduction in the amount of shifted complex on EMSA [Fig. 7B, lanes 3,4]. However, HlfI retained an ability to bind DNA in a complex with HlfAN containing a wild-type zipper, although the shifted HlfI: HlfAN complex was reduced about fourfold compared with binding by an Hlf: HlfAN complex with no zipper mutations [Fig. 7B, lanes 5–7]. These data indicated that the I→F mutation in the first position of the zipper had a significant effect on DNA binding as assessed by EMSA.

E2a–Hlf shows altered DNA-binding affinity compared with wild-type Hlf independent of the zipper mutation

In addition to the basic region conserved among the entire bZIP family, Hlf shares with other PAR proteins an upstream cluster of basic amino acids [Fig. 3] that has been shown to contribute to the DNA-binding specificity of Tef [Drolet et al. 1991]. However, this PAR-specific basic cluster was not present in the E2a–Hlf fusion protein, possibly contributing to additional alterations in the DNA-binding properties of E2a–Hlf compared with wild-type Hlf. To evaluate the relative DNA-binding affinities of Hlf and the t(17;19) fusion product, competition analyses with mobility-shift assays were performed with in vitro-translated proteins. For these studies the E2a–HlfI fusion protein was employed to assess DNA-binding properties independent of the effects of the leucine zipper mutation. The affinity of E2a–HlfI for the GH DNA recognition sequence was decreased ~10-fold compared with wild-type Hlf, as demonstrated by the greater competitor DNA concentrations required for equivalent reductions in maximal binding by E2a–HlfI [Fig. 8]. The observed reduction in binding affinity is similar to that reported for Tef PAR-specific basic region mutants and supports a similar role for this region in the DNA-binding specificity of Hlf. These data showed that fusion of Hlf to E2a resulted in altered DNA-binding properties.
least for the GH TEF site) independent of the effect of the zipper mutation. The potential additive contribution of the I → F substitution to reduced DNA-binding affinity could not be measured directly as E2a–Hlf homodimers did not bind DNA sufficiently to quantitate in EMSA competitor assays.

An endogenous E2a–Hlf complex is present in HAL-01 cells

Gel-shift analysis of nuclear proteins isolated from the HAL-01 cell line showed several shifted complexes with the GH TEF site whose binding was specifically competed with an excess of unlabeled probe (Fig. 6D). The possible presence of E2a–Hlf in the shifted complexes was assessed by using anti-E2a antibodies. EMSA of binding reactions that contained E2a-specific antibodies showed loss of a major shifted complex when compared with reactions performed with nonimmune sera (Fig. 6D, lanes 3,4). This complex showed a migration comparable to those observed with in vitro-translated heterodimers of E2a–Hlf and other PAR proteins. Most of the complexes that bound the GH TEF site in HAL-01 nuclear extracts showed no alteration in their mobilities or abundance in the presence of anti-E2a antibodies. These data indicated that HAL-01 contains an endogenous complex consisting, at least in part, of E2a–Hlf capable of binding to the GH TEF site in addition to several other complexes that appear to be unassociated with the fusion protein.

Discussion

Although the t(17;19) translocation is relatively uncommon, current studies show that it has unusual features with important implications for the role of transcription factors in leukemogenesis. In the HAL-01 cell line, t(17;19) results in fusion of the E2A gene with a previously undescribed gene that codes for a novel bZIP protein called Hlf. These features are similar to the E2A–Pbx1 fusion resulting from t(1;19) in childhood pre-B ALL (Kamps et al. 1990, Nourse et al. 1990). In both types of translocations, the chimeric proteins contain identical portions of E12/E47 that experimentally function as a transcriptional activation domain when fused to a heterologous DNA-binding element (Henthorn et al. 1990). The translocation partner in each case contributes a DNA-binding domain: a basic region/zipper in the case of Hlf and a homeo domain in the case of Pbx1. However, E2a–Hlf is distinguished from E2a–Pbx1 and other translocation-associated proteins in that it has measurably altered DNA-binding properties compared with its wild-type equivalent. Although the t(15;17) Pml-Raro protein has altered trans-activation properties (de The et al. 1991; Kakizuka et al. 1991; Pandolfi et al. 1991), there is no indication that these result from differences in DNA binding by the chimera.

An E2a–Hlf insertion mutation results from inclusion of a cryptic breakpoint-containing exon in the fusion RNA

A feature of the t(17;19) fusion RNA that has not been described previously in leukemic translocations is the inclusion of intron sequences surrounding the genomic breakpoint. The insertion is apparently recognized as a cryptic exon during splicing of the fusion RNA. No wild-type E2A RNAs have been isolated in which splicing occurred at this cryptic site (Henthorn et al. 1990, Kamps et al. 1990, Nourse et al. 1990 and unpubl.). The reason for this differential recognition is unclear, perhaps it allows for the proper spacing of E2a and Hlf protein domains or maintains an open translational reading frame in the chimeric RNA. The remaining portion of the insertion (37 nucleotides) is not a feature of wild-type Hlf, however, we cannot exclude the possibility that alternative splicing of HLF may occur in other tissues, as all cDNAs were isolated from the same source (HepG2).

Sequences in the E2A–breakpoint cluster region (bcr) intron flanking the HAL-01 breakpoint show homology to heptamer signal sequences consistent with the possibility that the t(17;19) resulted from aberrant recombination activity. In support of this, HAL-01 cells show features of precursor B cells arrested at a point in B-cell maturation when recombination is expressed because they
contain rearranged immunoglobulin heavy-chain genes but no detectable rearrangements of Igκ or TCRβ genes. The specific genomic sequence constraints observed in HAL-01 t(17;19), that is, crossover at a consensus heptamer adjacent to a cryptic splice site, may account for the infrequent occurrence of this translocation in ALL.

**Hlf is a new liver-associated member of the PAR subfamily of bZIP proteins**

The PAR proteins exhibit structural and functional properties establishing them as a distinct subfamily of bZIP proteins. They bind to specific target DNA sequences as homodimers or as heterodimers with other PAR family members, but not with non-PAR bZIP proteins (Drolet et al. 1991; Iyer et al. 1991). Unlike other bZIP proteins, experimental data indicate that the specificity of DNA binding of PAR family members is not only dependent on the integrity of the classic basic region itself but also on sequences located both in the extended basic region (Drolet et al. 1991) and within or amino-terminal to the PAR region (Iyer et al. 1991). Hlf exhibits properties analogous to those of the other PAR proteins; namely, it specifically binds to the TEF GH site as a homodimer or as a heterodimer with either Tef or Dbp.

Hlf represents the second example of a PAR protein expressed preferentially in hepatocytes. Despite widespread mRNA expression, the Dbp protein is present predominantly in adult liver and is down-regulated during hepatic regeneration (Mueller et al. 1990). In contrast to Hlf, TEF/VBP mRNA is widely expressed in adult rat/chicken tissues; however, protein expression has not yet been determined (Drolet et al. 1991; Iyer et al. 1991). The role of Tef/Vbp may differ during embryogenesis, as mRNA expression appears to be much more spatially limited (Drolet et al. 1991; Iyer et al. 1991). The expression pattern of Hlf, and the known role of Dbp and Vbp in regulation of liver-specific gene expression, suggests that PAR proteins may play a specific role in hepatic metabolism. Additional studies are required to determine whether these proteins interact to regulate expression of the same, or different, target genes and whether Hlf constitutes the liver-specific factor necessary for efficient Dbp activity (Mueller et al. 1990).

**Altered DNA binding occurs with fusion of Hlf to E2a in t(17;19) ALL**

In contrast to wild-type Hlf, E2a–Hlf encoded by the HAL-01 fusion cDNA bound extremely poorly to the GH site as a homodimer but readily when heterodimerized with either wild-type Hlf or other PAR proteins. This marked difference in DNA-binding properties was partially attributable to the isoleucine-to-phenylalanine mutation in the first heptad repeat position of the leucine zipper. The effect of phenylalanine on DNA binding was shown by reversion of F → I, which improved DNA binding by the E2a–HlfI homodimer. Similarly, the I → F mutation of wild-type Hlf significantly reduced its ability to bind DNA as a homodimer. Our data do not indicate whether the zipper mutation is essential for oncogenesis; however, circumstantial evidence suggests that it provided a growth advantage to the subclone in which it arose.

Our data also show that E2a–Hlf demonstrates altered DNA binding to the TEF site independent of the I → F zipper mutation. t(17;19)-mediated fusion resulted in loss of wild-type Hlf sequences that encode the basic region extension and regions farther amino-terminal of the classic basic region. The effects of E2a fusion on DNA binding were assessed in the F → I revertant E2a–HlfI, which showed significantly lower affinity for the GH TEF site compared with wild-type Hlf. These results are very similar to the altered binding of Tef mutants (in which pairs of alanines were substituted for pairs of lysines in the extended basic region of Tef) for the pro lactin TEF recognition site (Drolet et al. 1991), suggesting that absence of the extended Hlf basic region in the fusion protein changes its binding properties. Additional studies are required to show whether this effect is the result of alterations in DNA-binding specificity, which may only affect interactions of E2a–Hlf with a subset of its potential binding sites or change its binding site preferences completely. These studies do not indicate whether the markedly impaired DNA binding of E2a–HlfI homodimers is the result of a direct effect on protein–DNA interaction or is secondary to altered dimerization properties of the phenylalanine-containing chimera.

**Potential models for the role of a chimeric bZIP protein in leukemia**

A model for the role of E2a–Hlf in ALL needs to account for three primary observations. First, t(17;19) resulted in ectopic lymphoid expression of the Hlf bZIP domains that mediate dimerization and DNA binding. Second, in E2a–Hlf the amino-terminal portion of Hlf just upstream of the classic bZIP basic region was replaced with a portion of E2a that has properties of a trans-activation domain (Henthorn et al. 1990). Third, at least two structural alterations in E2a–Hlf reduce its DNA binding as a homodimer (at least to the TEF site). The most straightforward model is that E2a–Hlf homodimers deregulate target genes through binding to Hlf (or other PAR)-binding sites. This presupposes that the observed alterations in DNA binding are not functionally relevant or are overcome by high intracellular concentrations of E2a–Hlf. Conversely, binding alterations might expand the spectrum of target sites recognized by E2a–Hlf homodimers, leading to deregulation of a new set of target genes. We cannot rule out these possibilities as the functional relevance of altered DNA-binding affinity is undefined; furthermore, our studies employed a single binding site. Future investigations need to address the potential binding preferences of E2a–Hlf homodimers.

The alternative is that E2a–Hlf functions in vivo as a heterodimer. This requires that endogenous proteins capable of heterodimerizing with E2a–Hlf are present in lymphoid cells, a possibility supported by the detection...
of endogenous complexes in HAL-01 cells under conditions where in vitro-translated homodimers bind extremely poorly. A potential dimerization partner is Tef/Vbp as it is known to be transcribed in lymphoid tissues of rat and chicken, respectively. It is also possible that the PAR family contains additional, as yet undiscovered, members, and perhaps one or more of these is involved in regulation of lymphoid gene transcription. Dimerization might also occur with non-PAR bZIP proteins such as Jun, Fos, or C/EBP family members present in lymphoid cells. However, previous in vitro studies suggest that heterodimerization of PAR proteins is restricted as Tef/Vbp is incapable of binding to DNA as a dimer with C/EBP [Drolet et al. 1991; Iyer et al. 1991]. A potential role for the E2a–Hlf zipper mutation in relaxing dimerization specificity and allowing binding with non-PAR bZIP proteins needs to be addressed. Because of the altered DNA-binding affinity of E2a–Hlf, an endogenous dimerization partner may play an important role in directing the complex to target genes whose expression is deregulated by the E2a trans-activation domain. Alternatively, E2a--Hlf might function as a dominant-negative factor by dimerizing with and sequestering endogenous bZIP proteins. We consider the latter unlikely on the basis of the known trans-activation characteristics of the retained portion of E2a, although the transcriptional properties of E2a--Hlf remain to be determined.

Our studies show that the potential role of E2a fusion in activating transcriptional proteins in ALL is not restricted to homeo domain proteins but extends to the bZIP family. A specific oncogenic role for portions of E2a retained in both E2a-Pbx1 and E2a--Hlf is suggested, but additional studies are required to define the contributions of E2a to potential transcriptional and transforming properties of these chimeras. Such investigations should provide further insights into the mechanisms by which structural and/or regulatory mutations in transcription factors induce neoplastic transformation.

Materials and methods

Cell lines

Establishment of the cell line HAL-01 has been described previously, along with its morphological, immunophenotypic, and cytogenetic characteristics [Ohyashiki et al. 1991]. Karyotype analyses of HAL-01 indicated that the cytogenetic alterations involving chromosomes 17 and 19 have remained stable with long-term culture. Additional cell lines utilized for Northern analysis have been described previously [Nourse et al. 1990, Monica et al. 1991].

Cloning of E2a--HLF and HLF cDNAs

To isolate fusion cDNAs from HAL-01, 3 μg of poly(A)+ RNA was converted to double-stranded cDNA by use of the Superscript kit [BRL-GIBCO], as recommended by the supplier with minor modifications. Approximately 1 million recombinant phage were subjected to a differential screening procedure with E2A probes, as described previously [Nourse et al. 1990]. Ten plaques that met the criteria for fusion cDNAs [hybridized with a 5’ (B) but not a 3’ (C) probe] were purified and characterized by nucleotide sequence analyses. For one fusion cDNA clone the nucleotide sequence was completely determined from -50 nucleotides 5’ of the E2A start site [nucleotide 26 in Nourse et al. 1990] to ~200 nucleotides beyond the HLF stop codon. In five other fusion cDNA clones, the E2A--HLF fusion sites were determined and shown to be identical. Six of six independently isolated fusion cDNAs contained an identical A→T missense mutation converting isoleucine [ATC] to phenylalanine [TTC] in the first heptad repeat of the Hlf leucine zipper [amino acid 526 in Fig. 2A]. Wild-type HLF cDNAs were isolated from a cDNA library in λZAP prepared from mRNA purified from IL1-stimulated HepG2 cells [Stratagene]. Approximately 2 million plaques were screened at high stringency with an HLF-specific probe as described below for Northern blot hybridizations. Twelve phages were purified to homogeneity, and the phage inserts were excised and converted to double-stranded pBlue-Script. Portions of several clones were sequenced independently, and the entire ORF of one clone, pBSH11A, was sequenced completely (Fig. 2B). The wild-type isoleucine in the first heptad repeat of the leucine zipper was present in six independently isolated clones of differing lengths.

Plasmid constructions and mutagenesis

The in vitro expression plasmid pE2A--HLF was constructed by replacing the 3’ Xhol–EcorI fragment of pSP64-E2A–Pbx3A [Monica et al. 1991] with a 3’ Xhol–EcorI fragment of the HAL2 cDNA. A two-step overlap extension PCR procedure [Zaret et al. 1990] was used to construct in vitro expression plasmid pE2A–HLF1 in which the phenylalanine in the first heptad repeat of E2a–Hlf was converted to an isoleucine as is found in the wild-type Hlf. All constructions were confirmed by restriction enzyme analysis, and those involving PCR were analyzed by nucleotide sequencing to ensure that no mutations had occurred during construction.

A construct for optimal in vitro expression of wild-type HLF was constructed by PCR with pBSH11A as a template and primers DB-1 (5’-CGAAAGCTACGCTGGAAATGGGTTAATGCAGGCACC-3’) and HAL4 (5’-GAAGCTTGCCTGGAGGAAATGCC-3’), and the PCR product was cloned into the HindIII and XbaI sites of pSP64. An Hlf mutant (ΔN9155HLF) lacking amino acids 3–155 was constructed by PCR with pBSH11A as a template and primers DB-1 and HLF1 (5’-GAAGCTTGCCTGGAGGAGCCACACGCAATACACCGAAGTC-3’), and the PCR product was inserted into HindIII and XbaI sites of pSP64. The in vitro expression plasmid pHLF2 was constructed by overlap extension PCR to convert the isoleucine of the first heptad repeat in the leucine zipper of wild-type HLF to a phenylalanine. In vitro expression plasmids for TEF [pMET3–TEF] and DBP [pCMV–DBP] have been reported previously [Mueller et al. 1990, Drolet et al. 1991].

PCR

Amplifications of specific sequences were performed in a Perkin-Elmer programmable thermocycler with commercially prepared reagents [Perkin-Elmer Cetus]. For some analyses an aliquot of the PCR reaction was size fractionated by electrophoresis in agarose gel, transferred onto nylon membranes, and hybridized with radiolabeled internal oligonucleotides as described previously [Hunger et al. 1991]. Oligonucleotides employed included IS5 (5’-GCCAGGGCATCTCAG-3’), I3 (5’-GCACCAGAATCTCAG-3’), INS-INT (5’-CAGAGGACC-
GGAGTCCG GCC-3′), INS-DS [5′-GAAACTGCTGACCTGTGGTGTC-3′], and INS-US [5′-CTGGCCCTGTCCCTTACC-3′].

Blot analyses
Southern blot analyses of HAL-01 cell line DNA were performed on 10 μg of DNA by use of an E2A-specific probe [E47M] under conditions described previously [Mellentin et al. 1989]. Poly(A)-selected RNAs were purified from cell lines and tissues with commercial reagents [Invitrogen] under conditions recommended by the supplier. Adult human tissues were obtained from autopsy specimens, and human fetal tissues were obtained from a fetus of 18-22 weeks gestational age. All human tissue usage was approved by the human subjects committee of Stanford University. RNA was size-fractionated in denaturing formaldehyde-0.8% agarose gels, transferred to nylon membranes, and hybridized under conditions described elsewhere (Cleary et al. 1986). Blots were washed under high-stringency conditions (0.1x SSC, 0.1% SDS at 63°C) and exposed for 24-72 hr at −80°C with intensifying screens. The HLF probe for Northern blot analyses consisted of an ~1 kb Scal fragment of HLF cDNA containing the bZIP domain and a portion of the 3′-untranslated region. The E2A probe used for Northern blots [probe B] is described above; a human γ-actin probe was used to ensure that adequate RNA had been isolated and equal amounts were loaded. A commercially prepared Northern blot (CloneTech) was utilized for some analyses of adult tissues.

Protein preparations
Nuclear extracts of lymphoid leukemia cell lines were prepared as described previously [Kuo et al. 1991] with 1 × 10^7 to 2 × 10^7 logarithmically growing cells as starting material. Mobility-shift assays were performed with 3–5 μg of nuclear protein. In vitro transcriptions were performed in a 100-μl volume by use of an in vitro transcription system [Promega] employing either T7 or SP6 polymerase and 2 μg of appropriate linearized template DNA. In vitro translations were performed in a 25-μl reaction containing 70% nuclease-treated rabbit reticulocyte lysate [Promega] and 0.5 μg of in vitro-transcribed RNA under conditions recommended by the supplier. Mobility-shift assays were performed with 1–1.5 μl of the in vitro translation reactions.

DNA-binding studies
Standard mobility-shift assays were performed as described previously [Drolet et al. 1991], with minor modifications. Gel shift oligonucleotides have been described previously for the growth hormone TEF site [Drolet et al. 1991], albumin D site [Mueller et al. 1990], and chicken VBP [Iyer et al. 1991]. Binding reactions were performed at room temperature for 20 min in a 20-μl volume of 20 mM HEPES (pH 7.8), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 15% glycerol, 3 μg of poly[dIl-Cl], 0.5 μg of bovine serum albumin, and 0.25% dry nonfat milk with ~1 mM double-stranded oligonucleotide. Oligonucleotide probes were labeled by fill-in with the Klenow fragment of DNA polymerase and [γ-32P]dCTP. One-fifth of each reaction was analyzed on a 5% nondenaturing 0.5 x TBE-polyacrylamide gel, electrophoresed at 200 V for 2 hr. Autoradiography was performed on the dried gel for 6-18 hr at −80°C. For supershift assays, binding reactions were performed as described above, followed by the addition of 1 μl of appropriate serum and further incubation at 4°C for 16 hr. The anti-E2a antisemum has been described elsewhere [Murre et al. 1989].

Computer analyses
The University of Wisconsin Genetics Computer Group software package was used on a Digital Equipment VAX 8550 computer. Peptide sequences predicted from nucleotide analyses of the HLF cDNA were analyzed by use of the search algorithm FASTA at ktup = 1.

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Note added in proof
Sequence data described in this paper have been submitted to the EMBL/GenBank data libraries.

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