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26. The 131 hybridoma (*4*) secretes an All-reactive, Ab3 MAb (γ1, κ); the Ab1 hybridomas 110 and 133 (*3*) (both γ1,κ) were generated against keyhole limpet hemocyanin (KLH)–coupled All. Both were obtained by fusion of immune BALB/c spleen cells to the nonsecreting myeloma line NS-1. Immunoglobulin class and subclass were determined by Ouchterlony analysis of supernatants with commercial antibodies (Bionetics, Kensington, MD). Total RNA was prepared from 5 × 10^8 hybridoma cells by the guanidinum isothiocyanate–CsCl method (*27*) or by organic extraction (*28*). Polyadenylated RNA was purified by oligo(dT) cellulose chromatography (*29*). Minus-strand cDNA synthesis was carried out with Mo-MLV reverse transcriptase as described (*30*); primers were random hexanucleotides or the oligonucleotides V_H1FOR and V_K1FOR (*31*), modified to include Not I restriction sites. Amplification of V region sequences was carried out in a 20-μl reaction mixture containing 50 ng of the cDNA-RNA hybrid, 25 pmol of 3′ (V_H1FOR or V_K1FOR) and 5′ (V_H1BACK or V_K1BACK) primers, 200 μM each deoxynucleotide-5′-triphosphate, 67 mM tris-Cl (pH 8.8), 10 mM $MgCl_2$, and 2 units of Taq polymerase (Cetus). Light chain sequences were amplified in 30 cycles of 1 min at 95°C, 1 min at 30°C, and 2 min at 72°C. Heavy chain sequences were amplified in 30 cycles of 1 min at 95°C, 1 min at 30°C, and 2 min at 55°C. Amplified DNA was ligated into pBluescript (Stratagene) and nucleotide sequences were determined by the dideoxynucleotide chain termination method (*32*) with Sequenase (USB). The products of three independent amplification reactions were sequenced for each chain.
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Fusion of the Leucine Zipper Gene *HLF* to the *E2A* Gene in Human Acute B-Lineage Leukemia

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A t(17;19) chromosomal translocation in early B-lineage acute leukemia was shown to result in chimeric transcripts that contain sequences from the *E2A* basic helix-loop-helix transcription factor gene on chromosome 19, fused to sequences from a previously unidentified gene (*HLF*) on chromosome 17 that encodes a hepatic leukemia factor. The chimeric protein consisted of the amino-terminal transactivation domain of E2A linked to the carboxyl-terminal basic region-leucine zipper domain of HLF. HLF was normally expressed in liver and kidney, but not in lymphoid cells, and was found to be closely related to the leucine zipper–containing transcription factors DBP (albumin D-box binding protein) and TEF (thyrotroph embryonic factor), which regulate developmental stage–specific gene expression.

Molecular analysis of chromosomal translocations in human leukemic cells has led to the identification of new cellular proto-oncogenes that contribute to leukemogenesis (*1*). Genes encoding transcription factors that regulate cell growth and differentiation are frequent targets for such rearrangements (*2*). For example, the chromosomal translocation t(1;19)(q23;p13) in human pre-B cell acute lymphoblastic leukemia (ALL) fuses a basic helix-loop-helix (bHLH) gene (*E2A* or *E12/E47*) on chromosome 19 with a homeobox-containing gene (*PBX1*) on chromosome 1 (*3*). The protein product of the rearranged locus retains the NH_2-terminal activator domain of E2A, but its DNA-binding region is replaced by the homeobox domain of PBX1. Hybrid E2A-PBX1 proteins are expressed in the nucleus and induce malignant transformation when introduced into murine fibroblasts (*4*).

Other nonrandom chromosomal translocations occurring in leukemic cells at band p13 of chromosome 19 might also involve the *E2A* gene. Using an *E2A* cDNA probe, we analyzed the DNAs of t(17;19)(q22;p13)-positive leukemic lymphoblasts from two patients with early B cell precursor ALL and disseminated intravascular coagulation, which is rare in ALL cases lacking this translocation (*5*). Southern blot analysis revealed altered *E2A* restriction fragments in each of these patients, indicating rearrangement within the gene (*5*).

We prepared a genomic library with leukemic cell DNA from one of the patients (patient 1) and isolated two recombinant phages (LT1 and LT2) that contained altered restriction fragments surrounding the breakpoint within overlapping inserts that spanned 17 kb of genomic DNA (Fig. 1A). Probe A hybridized to *E2A* restriction fragments from chromosome 19, while probe B hybridized to human sequences in a mouse-human hybrid cell line that contained only human chromosome 17. Nucleotide sequencing revealed that *E2A* exons were oriented 5′ to 3′ relative to the breakpoint on chromosome 19, which occurred in the same intron of the *E2A* gene that contains the breakpoints of the 1;19 chromosomal translocation (*6*). Two cosmid clones, cosmids 17 and 19, isolated from a library prepared from unrearranged human DNA, were mapped by in situ hybridization with a fluorescent marker to chromosome bands 17q22 and 19p13, respectively (*7*).

We analyzed RNAs from a cell line

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(UOC-B1) that was established from the leukemic cells of patient 1 and from several leukemic cell lines that lacked this translocation. When hybridized with an *E2A* cDNA probe, UOC-B1 cells expressed two mRNAs of 4.4 and 4.8 kb, in addition to the family of normal *E2A* transcripts detected in the other cell lines (Fig. 2A). The abnormal mRNAs were also detected with a probe (designated C in Fig. 1A) prepared from an evolutionarily conserved genomic fragment located near the breakpoint on chromosome 17 (Fig. 2C). Thus, the 17;19 translocation resulted in transcripts comprising sequences from the *E2A* gene fused to sequences from an unidentified gene on chromosome 17.

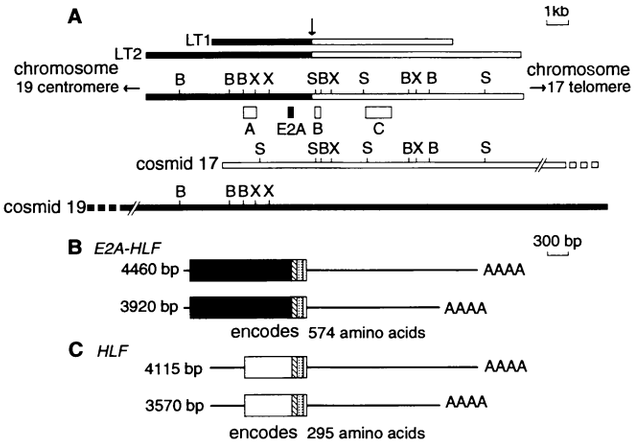

Fig. 1. (**A**) Restriction map of genomic clones from the chromosomal translocation t(17;19) breakpoint regions. Two recombinant phage clones (LT1 and LT2) were isolated with an *E2A* cDNA probe from a genomic library of leukemic cells (patient 1) with a t(17;19). The 17.3-kb LT2 insert contains all of the sequences found in the LT1 insert. Sequences within these clones were derived from either human chromosome 19 (shaded box) or chromosome 17 (open box); an arrow indicates the t(17;19) breakpoint. The black box under the restriction map outlines the chromosome 19 region, which contains three exons and hybridizes with the *E2A* cDNA probe. Open boxes A and B show the positions of genomic restriction fragments that were used as probes to map sequences on each side of the breakpoint to either human chromosome 17 (fragment B) or 19 (fragment A), with a human-mouse somatic cell hybrid panel (*18*). A genomic Spe I–Eco RV subclone from the region labeled C contains sequences that are conserved in mouse genomic DNA. Also shown are restriction maps of cosmid clones 17 and 19, which were isolated from a human genomic library with probes B and A, respectively. Sites of restriction for Xba I (X), Bgl II (B), and Spe I (S) are indicated. (**B**) The *E2A-HLF* fusion cDNA clones from the UOC-B1 cell line. The *E2A* coding sequences are indicated (shaded box), followed by a joining region (thin stripes), and *HLF* coding sequences, including the basic domain (wide stripes) and the leucine zipper domain (dotted box). (**C**) The *HLF* cDNA clones from the HepG2 cell line.

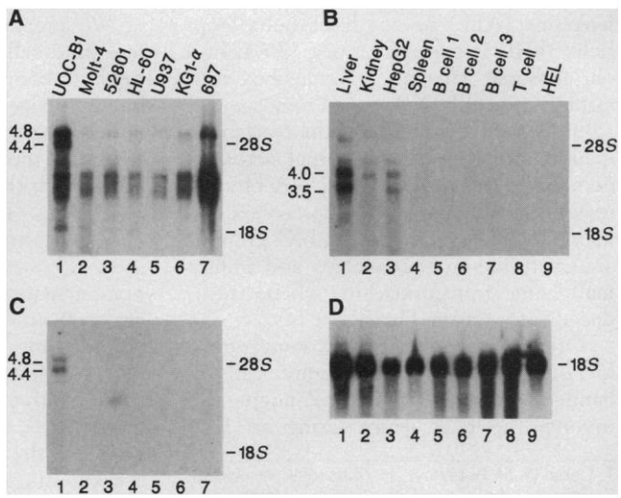

Fig. 2. Northern (RNA) hybridization analysis of human cell lines and tissues. The RNAs (1 μg) were hybridized with either (**A**) an *E2A* cDNA probe or (**C**) a conserved genomic restriction fragment from chromosome 17 (labeled C in Fig. 1A). Lane 1, the UOC-B1 t(17;19)-positive human B cell progenitor ALL cell line; lanes 2 and 3, the Molt-4 and 52801 human T cell leukemia cell lines; lanes 4 to 6, the HL-60, U937, and KG1-α human myeloid leukemia cell lines; and lane 7, the 697 human pre-B leukemia cell line, which has a t(1;19). Separate Northern blots were hybridized with (**B**) an *HLF* cDNA probe or (**D**) a β-actin probe. The results represent RNAs from human liver (lane 1), human kidney (lane 2), the HepG2 hepatocarcinoma cell line (lane 3), human spleen (lane 4), three separate EBV-induced human lymphoblastoid cell lines (lanes 5 to 7), phytohemagglutinin-stimulated T lymphocytes (lane 8), and the HEL erythroleukemia cell line (lane 9). Positions of ribosomal RNAs (28*S* and 18*S*) and the sizes of the mRNAs detected with each probe are shown.

To characterize the newly recognized fusion gene, we prepared a cDNA library from polyadenylated RNA of the UOC-B1 cell line. Two classes of cDNA clones that hybridized both to an *E2A* cDNA probe and to genomic fragment C (Fig. 1B) were identified. These corresponded to the chimeric 4.4- and 4.8-kb fusion mRNAs detected with the same two probes in this cell line (Fig. 2, A and C). The 5′ end of these cDNA clones matched the 5′ end of *E2A* cDNA clones (*3*, *8*), whereas the 3′ ends arose from the unidentified gene on chromosome 17. The two chimeric transcripts differed only in their 3′ nontranslated sequences. The predicted fusion protein contained the NH_2-terminal end of the E2A protein linked to a small open reading frame contributed by the unidentified gene on chromosome 17.

Analysis of a variety of human tissues and cell lines with a probe that contained the 3′ cDNA sequences identified mRNAs of 3.5 kb and 4.0 kb in normal liver cells and in a human hepatocarcinoma cell line (HepG2); a single mRNA of 4.0 kb was found in kidney (Fig. 2B). Transcripts were not detected in normal spleen, B cell lymphoblastoid cell lines, phytohemagglutinin-stimulated T lymphocytes, or leukemic cell lines lacking a 17;19 translocation. Thus, the gene does not appear to be normally expressed in lymphoid cells. Because the protein encoded by this gene is expressed by hepatocytes, it has been named hepatic leukemia factor (HLF).

To characterize normal *HLF* mRNAs, we screened a cDNA library from the HepG2 cell line and isolated four independent *HLF* clones, each of which contained identical open reading frames and, as shown for the fusion mRNAs, differed only in their 3′ untranslated regions (Fig. 1C). Nucleotide sequencing of *HLF* cDNA clones revealed a consensus initiation codon (*9*) at nucleotide 325, which initiated an open reading frame that encoded a polypeptide of 295 amino acids (Fig. 3A) with a predicted molecular size of 33.2 kD. In vitro transcription and translation of the full-length cDNA yielded a protein of about 43 kD (*10*). The difference between the predicted and observed molecular sizes of HLF proteins may reflect the high proline content of this molecule (11.4%), as observed for the related proteins DBP and GCN4 (*11*, *12*).

The predicted E2A-HLF fusion protein in leukemic cells from patient 1 contained 574 amino acids, of which the NH_2-terminal 483 residues were contributed by E2A (Fig. 3B). Comparison with published *E2A* sequences (*3*, *8*) revealed a single amino acid substitution ($Gly^{431} \rightarrow Ser^{431}$) resulting from two nucleic acid substitutions in the corresponding codon

(GGC → AGT). The 71 COOH-terminal amino acids were identical to those of HLF and were preceded by a joining region of 20 amino acids not found in either of the E2A or HLF products. Analysis of chimeric transcripts from leukemic cells of patient 2 (*13*) showed identical E2A and HLF amino acids (Fig. 3B), separated by a joining region of 29 amino acids, of which only the first 7 were identical to those in patient 1.

To determine the origin of the joining amino acids in these chimeric proteins, we compared nucleotide sequences of genomic subclones that contained the breakpoints with sequences of the corresponding genomic subclones that contained the unrearranged loci from cosmids 17 and 19. This comparison demonstrated that nucleic acids in the joining regions of *E2A-HLF* cDNA clones were derived from genomic sequences surrounding the breakpoint regions, with the exception of 25 bp (patient 1) or 20 bp (patient 2) of extra nucleotides that were not identified in genomic sequences from either chromosome 17 or chromosome 19 (Fig. 3C). These nucleotides represent N-region or filler DNA, of the type identified at about half of reciprocal translocation junctions analyzed in malignant cells; such nucleotide additions may result from terminal deoxynucleotidyl transferase activity or other mechanisms (*14*). These sequences form 59-bp (patient 1) or 86-bp (patient 2) exons that originate from an identical cryptic splice acceptor site in the *E2A* gene, extend through the breakpoints, and end in different cryptic splice donor sites on chromosome 17 (Fig. 3C). Sequences from these joining exons, together with the last nucleotide (G) of exon 13 of *E2A*, encode the joining amino acids identified in predicted E2A-HLF proteins (Fig. 3B) and merge NH_2-terminal coding sequences from *E2A* in frame with COOH-terminal sequences from *HLF*.

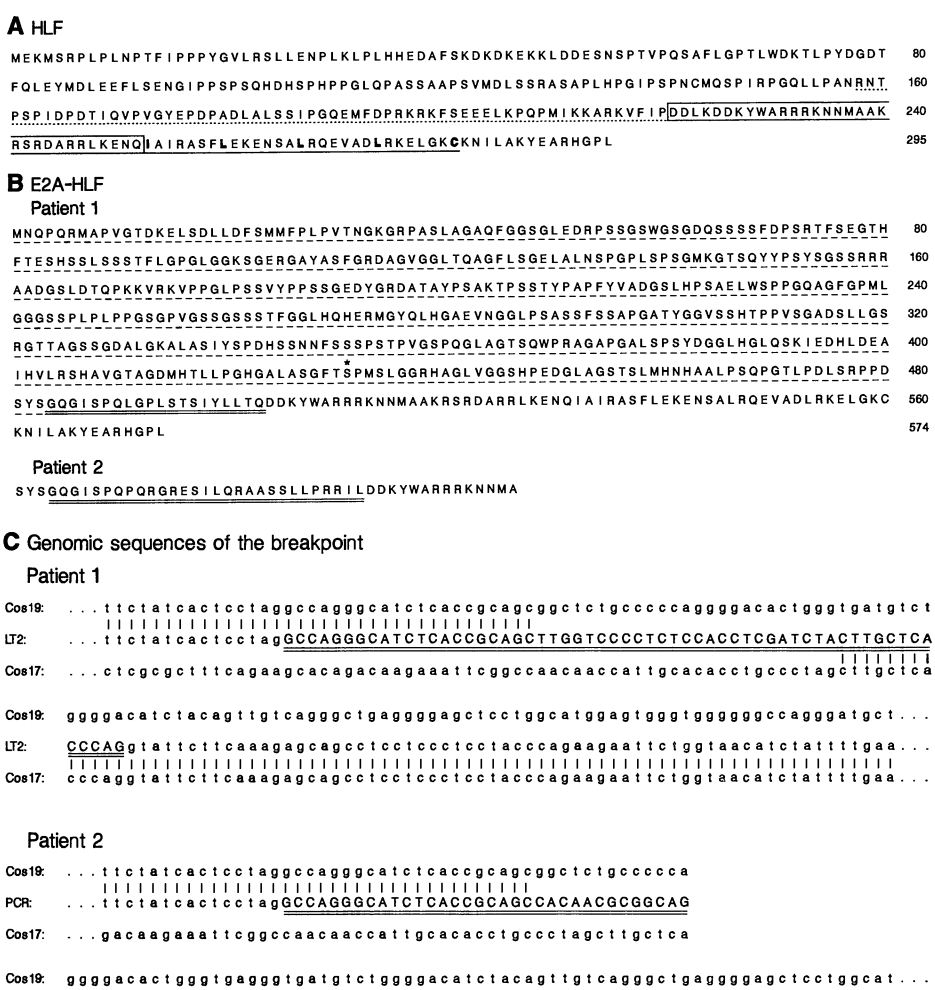

Fig. 3. The deduced amino acid sequences of (A) HLF and (B) E2A-HLF polypeptides, and (C) the nucleotide sequences of the genomic t(17;19) breakpoints in patients 1 and 2. (**A**) The HLF basic domain is shown as an open box. An upstream region with a high level of amino acid sequence identity with TEF and DBP is underscored with dots, the potential leucine zipper domain is underlined (solid), and the critical leucine residues are shown in bold. (**B**) In the E2A-HLF sequence from patient 1, NH_2-terminal E2A amino acids are underscored with dashes, and an amino acid substitution (S^{431} for G^{431}) in the published E2A sequence (*3, 8*) is indicated by an asterisk. Joining regions encoded by exonic sequences formed at the breakpoints are double underlined in the sequences from patients 1 and 2 (*13*). Amino acids 226 to 296 of HLF are located COOH-terminal to the joining region in hybrid proteins in both patients. (**C**) Comparison of the partial nucleotide sequence of a restriction fragment from phage clone LT2 (patient 1) or a PCR-generated genomic fragment [patient 2 (*13*)] that contained the breakpoints, shown with the corresponding unrearranged sequences from restriction fragments isolated from cosmids 19 and 17. Sequences shown in uppercase for each patient constitute exons that encode sequences in the joining regions of the corresponding hybrid E2A-HLF proteins. The abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp, E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

HLF is a member of the basic region leucine zipper (bZip) family of transcription factors (Fig. 4). In both the conserved basic and leucine zipper domains of the protein, the amino acid similarity was greatest with DBP (*11*) and TEF (*15*) (72% and 80% identity, respectively). An additional region of identity with DBP and TEF was found in the 65 amino acids immediately upstream of the basic domain (83% for each comparison), which is not conserved in other known leucine zipper transcription factors (*15*).

Insight into the function of HLF may be provided by the properties of DBP and TEF. DBP is responsible for high, tissue-specific expression of albumin in fully differentiated hepatocytes, is expressed by adult but not fetal liver cells, and is rapidly down-regulated in proliferating

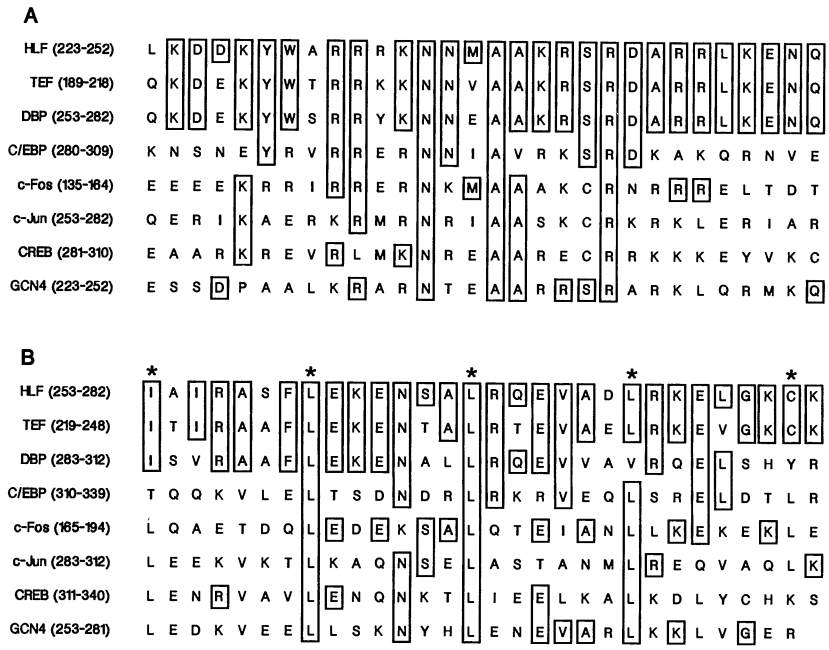

Fig. 4. The (**A**) basic and (**B**) leucine zipper domains of HLF and other members of the bZip superfamily. Identities between HLF and other proteins are boxed. All sequences shown are derived from the rat except HLF (human) and GCN4 (yeast); the coordinates of the first and last amino acids shown for each domain are given for each protein (in parentheses). The positions of critical leucines within the leucine zipper region are designated by asterisks. Abbreviations are HLF, hepatic leukemia factor; TEF, thyrotroph embryonic factor (*15*); DBP, albumin D-box binding protein (*11*); C/EBP, CCAAT/enhancer binding protein (*19*); c-fos and c-jun, components of the AP-1 site binding protein (*20*); CREB, cyclic AMP responsive element binding protein (*21*); and GCN4, a general control protein of yeast amino acid biosynthesis (*12*).

hepatocytes (*11*). In contrast, TEF is expressed by thyrotrophs in the anterior pituitary during embryologic development. It binds to and transactivates the thyroid-stimulating hormone β promoter, contains an NH_2-terminal activator domain, and forms homodimers and heterodimers with DBP through its leucine zipper region (*15*). Thus, HLF may regulate gene expression in hepatocytes and renal cells where it is normally expressed; its unscheduled expression as a chimeric protein in leukemic cells might alter normal regulatory circuits controlling early B lymphocyte growth and differentiation.

Although v-*fos* and v-*jun* are prominent retroviral oncogenes in experimental animal systems (*16*), bZip proteins have not been implicated in human cancer. We suggest that E2A-HLF hybrid proteins could contribute to neoplasia in early B cell progenitors. The bZip domain of HLF is predicted to mediate binding of the chimeric protein to the promoters of genes normally regulated by HLF. The regulatory sequences of the *E2A* gene, which is expressed in B cell progenitors, may account for expression of the chimeric protein in leukemic cells. The NH_2-terminal region of E2A contains a transcription activation domain (*17*) and may thus contribute a transactivating function to the chimeric protein. Anomalous induction or repression of a critical gene program may be the mechanism through which the chimeric protein contributes to leukemogenesis.

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13. The *E2A-HLF* breakpoint regions in RNA and DNA samples from leukemic cells of patient 2 were amplified using the polymerase chain reaction (PCR), and the products were subcloned in pBluescript SK+ plasmid vectors and sequenced. Random hexamers were used to prime *E2A-HLF* cDNA synthesis from leukemic cell RNA. The sequence of oligonucleotides used for amplification of this cDNA (5′-TGCACAACCACGCGGCCCTC-3′ and 5′-TGCCATGTTGTTCTTTCTGCG-3′) flanked the breakpoint based on cDNA sequences determined for patient 1. The sequence of oligonucleotides used for DNA PCR (5′-AGACTTTCCAAGTACCTTGA-3′ and 5′-GAGGTCACATGGGTTGGG-3′) matched intronic sequences of the *E2A* and *HLF* genes that spanned the genomic breakpoint in patient 1.
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