Characterization of ABF-1, a Novel Basic Helix-Loop-Helix Transcription Factor Expressed in Activated B Lymphocytes

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Proteins of the basic helix-loop-helix (bHLH) family are required for a number of different developmental pathways, including neurogenesis, lymphopoiesis, myogenesis, and sex determination. Using a yeast two-hybrid screen, we have identified a new bHLH transcription factor, ABF-1, from a human B-cell cDNA library. Within the bHLH region, ABF-1 shows a remarkable conservation with other HLH proteins, including tal-1, NeuroD, and paraxis. Its expression pattern is restricted to a subset of lymphoid tissues, Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines, and activated human B cells. ABF-1 is capable of binding an E-box element either as a homodimer or as a heterodimer with E2A. Furthermore, a heterodimeric complex containing ABF-1 and E2A can be detected in EBV-immortalized lymphoblastoid cell lines. ABF-1 contains a transcriptional repression domain and is capable of inhibiting the transactivation capability of E47 in mammalian cells. ABF-1 represents the first example of a B-cell-restricted bHLH protein, and its expression pattern suggests that ABF-1 may play a role in regulating antigen-dependent B-cell differentiation.

The basic helix-loop-helix (bHLH) family of transcription factors is composed of a large number of proteins involved in a wide array of developmental processes, including cellular proliferation and differentiation (40, 41). These proteins share a common sequence motif consisting of a basic region and an adjacent helix-loop-helix (HLH) structure (42, 43). The basic region has been shown to be important for DNA binding, while the HLH domain mediates dimerization (42, 72). The DNA binding sites for bHLH proteins, known as E boxes, consist of the consensus sequence CANNTG. E-box elements were first identified in the immunoglobulin heavy-chain (IgH) intronic enhancer and have since been found in a large number of pancreatic-, lymphoid-, and muscle-specific promoter and enhancer elements (7, 11, 20, 40, 41, 74, 75). Mutational analysis of the E-box sites, in particular, the E2 box ([G/A]CAGNTG [T/G]), present in a variety of different regulatory elements has demonstrated their importance in regulating cell-type-specific gene transcription (40, 41).

The bHLH proteins have been categorized into different classes based on dimerization specificity and tissue distribution. Class I HLH members, also known as E proteins, include E12, E47, HEB, and E2-2 (40). Each is capable of homo- and/or heterodimerization and is widely expressed (40). Class II HLH proteins, including MyoD, myogenin, NeuroD, and members of the achaete-scute complex in *Drosophila melanogaster*, display a tissue-restricted expression pattern and bind DNA only as heterodimers with the class I HLH proteins (40, 41, 43). The class I HLH protein daughterless, for example, forms heterodimers with achaete-scute family members to activate common target genes that control sex determination and neurogenesis (9, 10, 12, 16).

A number of myogenic-specific bHLH proteins, including

MyoD, Myf-5, and myogenin, interact with class I HLH proteins to activate genes that are required for proper vertebrate muscle development (25, 45, 56, 57, 74). NeuroD, a neuronal- and pancreatic-specific bHLH protein, has been shown to regulate terminal differentiation of neurons in *Xenopus laevis* (35). Surprisingly, NeuroD knockout mice display a dramatic pancreatic defect and develop diabetes, yet neuronal development appears unaffected (46). Studies with knockout mice have demonstrated that hematopoiesis is also regulated, in part, by bHLH proteins. Recently, it was shown that mice lacking the *SCL/tal*-1 bHLH gene exhibit a block in early hematopoiesis, affecting multipotent progenitors (49). The *HEB* gene has been shown to be required for proper thymocyte development, and the *E2A* gene, which encodes both E12 and E47, is absolutely essential for proper B- and T-cell development (1a, 3, 78, 79). In E2A-deficient mice, B-lineage development is blocked prior to the stage in which Ig rearrangements are normally initiated (3). Furthermore, B-cell development is inhibited in transgenic mice that overexpress the E2A inhibitor, Id1 (66). E2A-like molecules have also been suggested to play a role in Ig switching, since overexpression of Id1 in B-cell lines interfered with the ability of these cells to undergo isotype switching (22).

During thymocyte development, both E2A and HEB gene products which bind as heterodimers to the E2 box site are expressed (1a, 59). In B-lineage cells, both E2A and E2-2 gene products are expressed (2). In pre-B cells, both E47 and E2-2 bind the E2-box site. In mature B cells, E47 homodimers are the predominant E2-box-binding species (2, 44, 63). Interestingly, relatively high levels of *E2A* transcripts can be detected in germinal centers, suggesting that E2A may play a role later in B-lymphocyte development (52).

Several class II bHLH genes have been shown to be expressed in the hematopoietic compartment (4, 34, 71). The *lyl-1* bHLH gene, for example, is expressed in a number of erythroid, myeloid, and B-cell lines (34, 71). To date, however, there has been no evidence supporting the existence of a B-cell-restricted bHLH protein. Here we report the isolation of a novel bHLH transcription factor, called ABF-1, from a B-cell cDNA library. ABF-1 displays a remarkable conservation within

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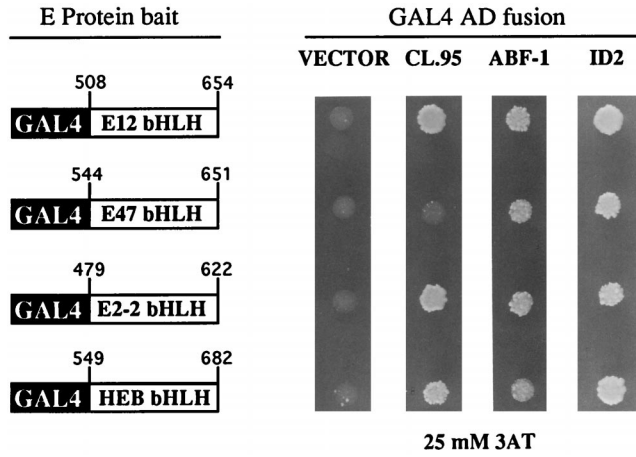
FIG. 1. ABF-1 is capable of interacting with the E proteins in the two-hybrid system. Expression plasmids encoding the indicated GAL4 DNA binding domain fusions (E-protein bait) and GAL4 AD fusions were cotransformed into the reporter yeast strain y190, which contains an integrated *GAL1-HIS3* reporter gene (19). Transformants were initially selected on synthetic medium lacking tryptophan and leucine and then spotted onto synthetic medium lacking histidine and containing 25 mM 3AT. Growth on the selective medium indicates an interaction between the E-protein bait and the GAL AD fusion protein (19). Yeast cells were allowed to grow for 4 days at 30°C and then photographed. Vector, pACT2; CL.95, clone 95; ABF-1, pACT2-ABF-1; ID2, pACT-ID2. The GAL4 AD–ABF-1(FL) fusion conferred a mild slow-growth phenotype in yeast strain y190.

the tissue-specific class II bHLH proteins. We demonstrate that ABF-1 is a transcriptional repressor which is expressed in a number of B-cell lines, lymphoid tissues, and, strikingly, activated primary B cells. Like other class II bHLH proteins, we find that ABF-1 is capable of binding to an E-box hexanucleotide element as a heterodimer with the E2A proteins in nuclear extracts derived from immortalized lymphoblastoid cell lines (LCLs). The data presented here suggest that ABF-1 is a downstream target of signalling through the antigen receptor.

MATERIALS AND METHODS

Yeast two-hybrid screening. Two-hybrid screening was performed with the reporter *Saccharomyces cerevisiae* strain y190 essentially as described previously (19). Details on construction of the human E-protein bait plasmids are available upon request.

Electrophoretic mobility shift assays (EMSA). DNA binding assays with the µE5 probe were performed essentially as described previously (2). Complexes were resolved on 5% native polyacrylamide gels in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA). For gel shift assays using the µE4-OCT (octamer) and µE4 probes, the following reaction conditions were used: 12 mM HEPES (pH 7.9), 100 mM KCl, 10 µM ZnCl₂, 12% glycerol, 0.05% Nonidet P-40, 50 µg of bovine serum albumin per ml, 25 µg of poly(dI-dC) per ml, 3 µl of in vitro-translated protein, 50,000 cpm of ^{32}P-end-labeled probe, and 1 µl of antiserum, when appropriate. Proteins were translated in vitro by using a coupled transcription-translation kit (TNT) as instructed by the manufacturer (Promega). Nuclear extracts were prepared as previously described (17). The oligonucleotide probes used in this study were µE5 (42), µE4-OCT (5′-CCG AAT TCA CAC CAC CTG GGT AAT TTG CAT TTC-3′), and µE4 (5′-TCG AGA CAC CAC CTG GGT AAG-3′).

cDNA library screening, sequencing, and plasmid construction. Several cDNA clones encoding the entire open reading frame of ABF-1 were isolated by screening the λACT B-cell library with the partial cDNA, clone 95, using standard techniques (58). The longest cDNA, designated 95-1A, was excised as an *Xho*I fragment and subcloned into pBSK+ (Stratagene) at the *Sal*I site to generate pBSK-ABF-1. The ABF-1 cDNA was sequenced on both strands by using a Sequenase version 2.0 DNA sequencing kit as instructed by the manufacturer (Amersham). The entire coding region of ABF-1 was amplified by PCR with the primers ABF FLAG F1 (5′-ATA GGG ATC CGA GCT TCG GGG GCT GCA G-3′) and ABF FLAG R1 (5′-ATA GGA ATT CTT AAC GAA TAA TCC CAT CAA G-3′). The PCR product was ligated into pSP64FLAG (48) digested with *Bam*HI and *Eco*RI to generate pFLAG-ABF-1. pGST-ABF-1

CTCGAGGCCACGAAGGCCCCCGGGGTCAGCAGAGGGCGCTCACGCTCTCGCCACCCACC 60
TCGCTTTCTCACCCCGGCGTTCCCGGCCTGGGTTTTTAGTCTTCCTTGGAGCGGCTCTCTG 120
GCCTCCGCCTCCGCCAGGGAGCGGAAGGCGGAGACAGCGGAGACTGGCCAGGGGGGAGGAA 180

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AGAGGACGCGTGTGGGCAAGGGGGACAACGGGATG TCC ACG GGC TCG GTG AGT   233
1                                    M   S   T   G   S   V   S

GAT CCG GAG GAG ATG GAG CTT CGG GGG CTG CAG CGG GAG TAC CCG   278
8   D   P   E   E   M   E   L   R   G   L   Q   R   E   Y   P

GTC CCC GCC TCC AAG AGG CCG CCC CTC CGC GGC GTA GAG CGC AGC   323
23  V   P   A   S   K   R   P   P   L   R   G   V   E   R   S

TAC GCC TCG CCC AGT GAC AAC TCG TCG GCA GAG GAG GAG GAC CCC   368
38  Y   A   S   P   S   D   N   S   S   A   E   E   E   D   P

GAC GGC GAG GAG GAG CGC TGC GCT CTG GGC ACA GCC GGC AGC GCG   413
53  D   G   E   E   E   R   C   A   L   G   T   A   G   S   A

GAA GGC TGC AAG AGG AAG CGG CCC CGT GTG GCT GGG GGC GGC GGC   458
68  E   G   C   K   R   K   R   P   R   V   A   G   G   G   G

GCA GGT GGT AGC GCG GGC GGT GGT GGC AAG AAG CCC CTC CCG GCC   503
83  A   G   G   S   A   G   G   G   G   K   K   P   L   P   A

AAG GGC TCA GCC GCA GAG TGC AAG CAG TCG CAG CGG AAC GCG GCC   548
98  K   G   S   A   A   E   C   K   Q   S   Q   R   N   A   A

AAC GCC CGT GAG CGT GCC CGG ATG CGC GTG CTG AGC AAA GCC TTC   593
113 N   A   R   E   R   A   R   M   R   V   L   S   K   A   F

TCC AGG CTC AAG ACC AGC CTG CCC TGG GTG CCC CCC GAC ACT AAG   638
128 S   R  [L]  K   T   S   L   P   W   V   P   P   D   T   K

CTC TCC AAG CTG GAC ACG CTC CGG CTG GCT TCC AGT TAC ATC GCT   683
143 L   S   K   L   D   T   L   R   L   A   S   S   Y   I   A

CAC CTG CGG CAG CTG TTG CAG GAG GAC CGC TAT GAG AAC GGC TAC   728
158 H   L   R   Q   L   L   Q   E   D   R   Y   E   N   G   Y

GTG CAC CCA GTG AAC CTG ACA TGG CCA TTC GTG GTC TCG GGA AGA   773
173 V   H   P   V   N   L   T   W   P   F   V   V   S   G   R

CCG GCC TCT GCA CCA AAG AAG TTT CCG CAG CCA ACA GAC TAT GTG   818
188 P   A   S   A   P   K   K   F   P   Q   P   T   D   Y   V

GAA CCA CCG CTT AAA TCG GAC TGG AAC TCA CTT GAT GGG ATT ATT   863
203 E   P   P   L   K   S   D   W   N   S   L   D   G   I   I

CGT TAA ATGCGAGTGTTTGGGGGCCACGGAGAGAAGGGAGAGCTCGTGAGATGGGAAG   921
218 R   *
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AAGTTTCCGCTGGATTCTCCTTGACCCTTCCCCTTGCCCTGGAACTGTGATCGTGACAGG   981
TGGCGGGTGTGGCTGTCACTGCACAGCGCCCACGGCTACAGCTGCGCCGGATCTGGGCGA  1041
CCACGTTTTGCCTCTCCAAAAAGAGCTTCCTTTCGTGACGAGACGCGGACGCAGGTCCAC  1101
CCTCGGGCCCTAGCTCTGTAGACTAACTCTCGGCTGCTGCCCCAGCCCGCGCCAGACAGC  1161
CCACGGATCCGTTCTCAGCGGAGCCAGATTCATCGCACACGTGCGGGACGGTTCCACACA  1221
GCCCCGGCCTTTCGCGGTGACACAATGGTTAGGGAACGGTTAGACGCGCTCTACATCCGC  1281
TTTAAAGACAGAGGTCTAGACGTGAGATCCGCGTCGGGACAGGGTTTTAAGTGACAAAGA  1341
AGGGCGAGTGGCTTCTCTGGGCCGGGTTCGTACTCCAGCACAGCGCCCTTCTAACGGGCG  1401
GGAGGAAGGCCGCTGCTCGCAGGGCTAGGTGGAGACACACTTCCCAGATCACCGCAGGCG  1461
GGTTTTACCCGGAGAGCTCTGGCCGTTCGGCCTCCCTGCCGGGTGGCTTCTTCAATCCCG  1521
TCTCCTTCCCAAGCTCCCGGCTTTTTCTAATCAGGCAGGCGTCTGTCAACCCTCTCCACT  1581
TCTGGGCTGAAGCCTCCCCAAGCCCCGTGCGCCAACCTGTGTGGGGTCTTCTGTGCCGCT  1641
CCCTCTCCGCCCCGCTCCTGCTCCTACCTGCAGCACCCCCAGCTCCGACTCCAGACTCTC  1701
TGCATCAGGTCTCCCCACTCCACGCTCCGGGCGCCCAACTCCAACACCACGTCCTGCCG   1761
CGCAGGTTCTTCCCCGCGCGGAGGAGCGCGCAGGGTGGGCGGCTTACCATGCAAGTGAT   1821
CCTGCGATAGGGAACGCGCCCTTGCCCCGAGGCTGCACTACCACAGGAAATAACGGCCTT  1881
CGTGGCCTCGAG                                                 1893
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FIG. 2. Nucleotide sequence of the human *ABF-1* cDNA. The longest open reading frame encodes a protein 218 amino acids in length. Although not full length (see Fig. 4), the ~1.9-kb cDNA contains two potential start codons, both with good Kozak sequences (58). The conceptual translation product predicts a 23.6-kDa protein with an estimated pI of 9.5. In addition to the bHLH motif (bold underline), there is a putative nuclear localization signal (dashed underline), a glycine-rich region (boldface), and a stretch of acidic residues (double underlined). The boxed leucine residue (position 130) represents the fusion point of clone 95 with the GAL4 AD. The asterisk denotes the stop codon.

was constructed by subcloning an *Xho*I (blunted)/*Eco*RV-digested ABF-1 cDNA into pGEX2TK linearized with *Sma*I. The GAL4 DNA binding domain–ABF-1 fusions used in the repression assays were constructed as follows. The full coding region of ABF-1 was amplified by PCR with the primers ABF-GF1 (5′-ATA GGA ATT CAT GTT CAC GGG CTC GGT GAG T-3′) and ABF FLAG R1. The *Eco*RI-digested PCR fragment was ligated into the *Eco*RI site of pBXG1 (51) to create pGAL4-ABF-1(FL). The GAL4–ABF-1 deletion was created with
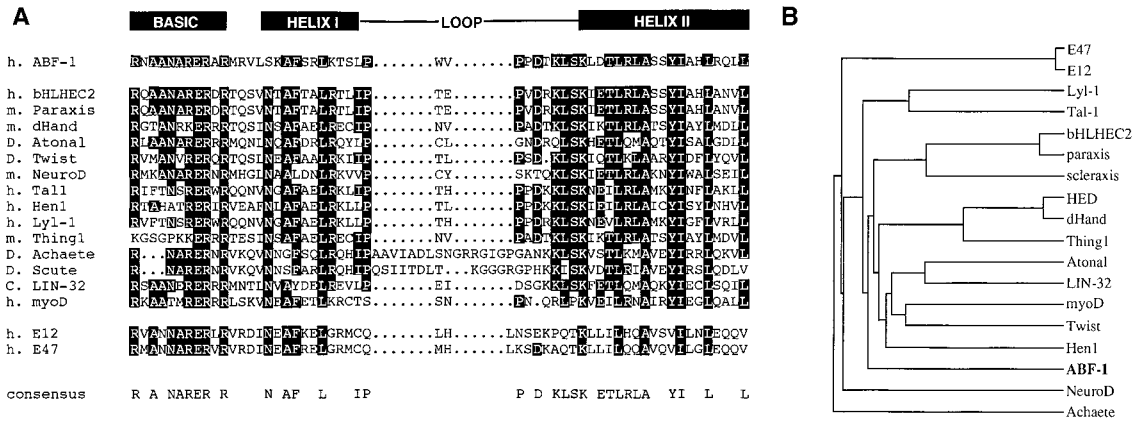
FIG. 3. Sequence alignment of ABF-1 with the class II family of bHLH proteins. (A) Multiple sequence alignment of the bHLH region of human (h.) ABF-1 with the most related class II bHLH proteins was created by using the PileUp and Pretty algorithms (Genetics Computer Group sequence analysis software package). Amino acids identical in at least half of the sequences are shown as blackened boxes. For reference, the bHLH regions of the class I proteins E12 and E47 are shown. m., mouse; D., *Drosophila*; C., *Caenorhabditis elegans*. (B) Dendrogram displaying a graphical output of the pairwise alignments of ABF-1 and related bHLH family members generated by PileUp.

the primers ABF-GF1 and ABF-GR1 (5′-ATA GGG ATT CTT ACC GCT GCG ACT GCT TGC ACT C-3′). The PCR product was subsequently cloned into pBXG1 at the *Eco*RI and *Bam*HI sites to create pGAL4-ABF-1(ΔC). All constructs were sequenced to confirm the correct reading frame. pFLAG-ABF-1 was digested with *Eco*RI and *Hin*dIII to release the FLAG–ABF-1 cDNA. This fragment was made blunt with Klenow enzyme and ligated into pHβAneo (23) at the *Bam*HI site (blunted). pHβA-E47 was kindly provided by Gretchen Bain.

Northern blot analysis. Total RNA was isolated from cells by using Trizol reagent as instructed by the manufacturer (Gibco-BRL). RNA samples were separated on formaldehyde gels, transferred, and hybridized as described previously (58). All Northern blots were hybridized with a ^{32}P-labeled 0.8-kb 3′ untranslated region fragment derived from *Bam*HI digestion of pBSK-ABF-1.

Protein purification and generation of ABF-1-specific antiserum. Host strain *Escherichia coli* BL21(DE3), harboring the pGST-ABF-1 expression vector, was grown to an optical density at 600 nm of 0.6 and induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 1.5 h at 37°C. Cells were subsequently harvested and lysed as described previously (38). Glutathione *S*-transferase (GST)–ABF-1 protein was solubilized from the pellet fraction in 6 M urea and subjected to dialysis in phosphate-buffered saline. GST–ABF-1, which precipitated out of solution during dialysis, was pelleted and resuspended in 1× sodium dodecyl sulfate (SDS) sample buffer. The sample (approximately 70% pure) was fractionated on an SDS–10% polyacrylamide gel and Coomassie blue stained to reveal the GST–ABF-1 fusion protein. The protein band was excised from the gel and prepared for injection into rabbits as described previously (24). Animals were immunized with 250 to 300 μg of protein, and serum was collected and purified by using standard techniques (24).

Western blotting. Western blot analyses were performed as described previously (38). Briefly, 50 μg of nuclear extract was fractionated on SDS–12% polyacrylamide gel, transferred to an Immobilon-P membrane (Millipore), and incubated with affinity-purified polyclonal ABF-1 antiserum used at a 1:500 dilution. Bands were visualized by chemiluminescence with a horseradish peroxidase-conjugated goat anti-rabbit antibody.

Isolation and in vitro activation of human B cells. Human B cells were purified from peripheral blood obtained from healthy donors. T cells were eliminated by complement-mediated lysis using an anti-CD3 antibody. Fluorescence-activated cell sorting analysis indicated that greater than 85% of the purified cells were CD19$^+$ and less than 5% stained positive for OKT3. B cells were activated by culturing in a 1:10,000 dilution of *Staphylococcus aureus* Cowan 1 (SAC) (Calbiochem). Interleukin-2 (IL-2) was used at a final concentration of 50 U/ml. Cells were cultured in RPMI 1640 supplemented with 2.5% human serum, penicillin-streptomycin, and L-glutamine.

Cell culture and transfections. HeLa S3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, L-glutamine, and antibiotics. For transient transfections, 3×10^5 HeLa S3 cells were plated onto 6-cm-diameter dishes and transfected with Superfect reagent the next day as instructed by the manufacturer (Qiagen). Cells were harvested 48 h posttransfection and assayed for either chloramphenicol acetyltransferase (CAT) or luciferase activity as described previously (58).

RESULTS

Identification of a novel bHLH gene. To identify potential B-cell-specific class II bHLH proteins, we performed a yeast

two-hybrid screen. Using the bHLH region of E2-2 as bait, we screened a human B-cell GAL4 activation domain (AD)-cDNA fusion library for interacting proteins (Fig. 1) (19). From approximately 1.9×10^6 yeast transformants plated, we isolated a total of 249 clones that were capable of growth on 25 mM 3-aminotriazole (3AT), which indicates activation of the *HIS3* reporter gene (19). Of these, 131 also activated a *lacZ* reporter gene and thus turned blue when assayed for β-galactosidase activity. Clones that could activate both reporter genes, which is indicative of a potential interaction between the E2-2 bait and a library-encoded GAL4 AD fusion protein, were analyzed further. Approximately 89% of the 131 clones failed to interact with the unrelated bait SNF2, lamin, p53, or CDK2, suggesting that these clones were highly specific for the E2-2 bait (data not shown). Subsequent characterization of the cDNAs isolated from the positive clones revealed that the vast majority were identical to human Id2 and Id3 (data not shown). One cDNA, clone 95, encoded a protein with significant homology to the tissue-specific class of bHLH transcription factors. Because of the expression pattern of clone 95 (discussed below), it will be hereafter referred to as ABF-1, for activated B-cell factor 1.

Because we were unable to isolate ABF-1 by using other E-protein baits (38a), we further used the two-hybrid system to assess whether the gene product is capable of interacting with different E-protein family members. The original isolate, clone 95, was able to interact with the E2-2, HEB, and E12 bait proteins, as indicated by growth on selective medium containing 25 mM 3AT (Fig. 1). However, when the entire bHLH domain of ABF-1 was fused to the GAL4 AD, an interaction with all E proteins, including E47, could be detected (Fig. 1). These interactions were strong enough to support growth on 50 mM 3AT (data not shown). As a positive control, the E-protein baits were shown to interact with a GAL4 AD-Id2 fusion protein (Fig. 1).

ABF-1 encodes a novel HLH protein. The data described above suggest that *ABF-1* encodes a protein which has the ability to interact with class I HLH proteins. To examine whether *ABF-1* encodes an HLH protein, a longer cDNA clone was isolated from a B-cell library and completely sequenced (Fig. 2). A computer database search using the *ABF-1* nucleotide sequence and the BLAST algorithm revealed that *ABF-1* indeed encodes a bHLH protein. Sequence alignment of *ABF-1*
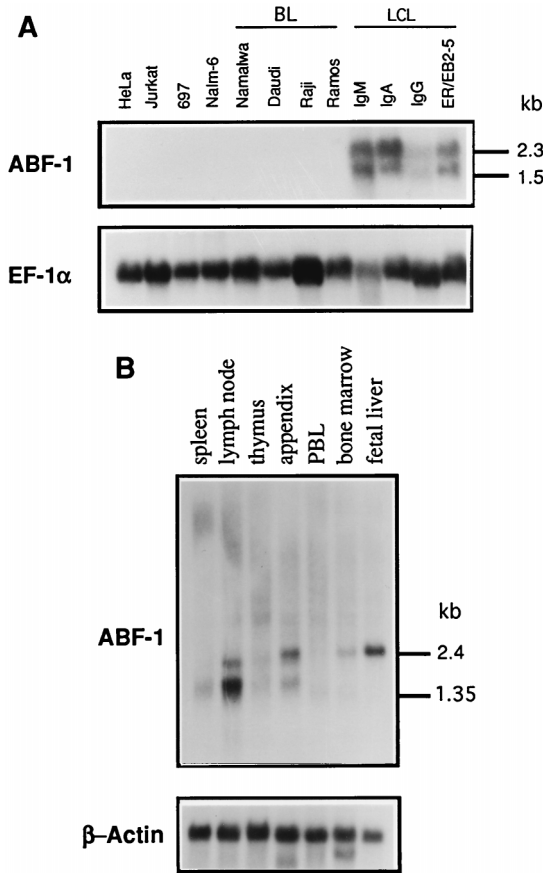
FIG. 4. Northern blot analysis of *ABF-1* expression. (A) Expression pattern of *ABF-1* in human cell lines. Ten micrograms of total RNA was isolated from each cell line and analyzed by Northern blotting. The blot was probed with the *ABF-1* cDNA (top), stripped, and subsequently reprobed with the human elongation factor 1 alpha (EF-1α) cDNA (6) as a loading control (bottom). HeLa, carcinoma; Jurkat, T-cell leukemia; 697, pre-B ALL harboring a t(1;19) translocation; Nalm-6, pre-B; BL, Burkitt lymphoma; LCL, lymphoblastoid cell line of the indicated Ig isotype. The ER/EB2-5 cell line was grown in the presence of 1 μM β-estradiol (27). (B) A human tissue Northern blot (Clontech) containing 2 μg of poly(A)$^+$ RNA per lane was sequentially hybridized with *ABF-1* (top) and β-actin (bottom). PBL, peripheral blood lymphocyte.

FIG. 5. *ABF-1* mRNA is highly abundant in activated human B cells. Northern blot analysis of 10 μg of total RNA isolated from purified peripheral blood B lymphocytes activated in vitro. B cells were treated with SAC for 3 days or with SAC and IL-2 for 6 days or were left untreated. The Northern blot was probed with the *ABF-1* cDNA (top), stripped, and then reprobed with elongation factor 1 alpha (EF-1α; bottom) as a control.

with various class II family members revealed a remarkable conservation within the HLH region (Fig. 3A). ABF-1 is 60% identical to bHLHEC2 and paraxis and 53% identical to dHAND and Atonal within this domain. In addition, ABF-1 also shows significant identity to tal-1, NeuroD, and members of the achaete-scute family (Fig. 3A). Outside the bHLH region, however, ABF-1 shows no significant homology to any known proteins. To examine the evolutionary relationship of ABF-1 to other HLH proteins, a dendrogram was constructed (Fig. 3B). Interestingly, the ABF-1 protein is more closely related to class II HLH proteins, including the muscle- and pancreatic-specific HLH proteins, than to the class I HLH proteins E12 and E47 (Fig. 3B). These data indicate that a novel HLH protein closely related to the class II HLH proteins is expressed in hematopoietic lineage cells.

ABF-1 is highly expressed in activated human B lymphocytes. To determine the expression pattern of *ABF-1*, RNA derived from various cell lines and lymphoid tissues was isolated and analyzed by Northern blotting. A panel of human cell lines derived from both lymphoid and nonlymphoid origin was examined for the presence of *ABF-1* transcripts (Fig. 4A). No
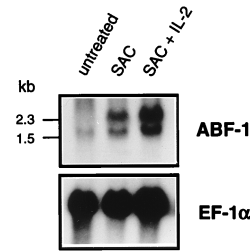
expression of *ABF-1* was detected in HeLa, Jurkat, and a number of Burkitt lymphoma B-cell lines, including Namalwa, Daudi, and Raji (Fig. 4A). Since *ABF-1* was isolated from an Epstein-Barr virus (EBV)-transformed human B-cell cDNA library, we next analyzed RNA derived from a number of EBV-immortalized LCLs for the presence of *ABF-1*. *ABF-1* mRNA was detected in all LCLs analyzed (Fig. 4A). Most LCLs expressed ABF-1 at relatively high levels. The mRNA consisted of a doublet running at 1.5 and 2.3 kb, which likely represents alternative splicing of *ABF-1* transcripts (Fig. 4A). *ABF-1* message is also highly abundant in ER/EB2-5, an EBV-transformed B-cell line expressing an estrogen receptor (ER)-EBNA2 fusion protein (Fig. 4A) (32).

To examine for the presence of *ABF-1* in primary cells, a Northern blot containing poly(A)$^+$ RNA isolated from human lymphoid tissues was probed with a radiolabeled *ABF-1* cDNA (Fig. 4B). Interestingly, ABF-1 transcripts were detected in the lymph node, appendix, fetal liver, and to a lesser degree bone marrow (Fig. 4B). However, no expression was detected in peripheral blood lymphocytes (Fig. 4B). Taken together, these data indicate that *ABF-1* is expressed both in a subset of human B-cell lines and in primary cells present in lymphoid tissues.

Since EBV-transformed B-cell lines resemble activated human B lymphocytes (8, 15, 53, 69, 73), we wished to address whether *ABF-1* expression could be induced in mitogen-treated peripheral blood B lymphocytes. Human B cells were purified from peripheral blood and activated in vitro with SAC or SAC plus IL-2. B cells left untreated showed little, if any, expression of *ABF-1* (Fig. 5), consistent with the lack of ABF-1 mRNA in the peripheral blood lymphocyte sample (Fig. 4B). However, treatment with SAC alone caused a dramatic upregulation of *ABF-1* which could be further induced by addition of IL-2 (Fig. 5). These data indicate that *ABF-1* expression is induced upon B-cell activation. Although *ABF-1* transcripts were not detected in the thymus or the T-cell line Jurkat, we wished to determine whether *ABF-1* is expressed in activated human T cells. Northern blot analysis revealed that mitogen-activated T cells do not express detectable levels of *ABF-1* mRNA (38a). Thus, in lymphoid cells, activation of *ABF-1* expression is restricted to B-lineage-derived cells.

ABF-1 has the ability to bind either as a homodimer or as a heterodimer to DNA. To assess whether ABF-1 has the ability to bind the E2-box site, we performed an EMSA. E12, E47, and FLAG-tagged ABF-1 were translated in vitro and analyzed by SDS-gel electrophoresis. Each of the gene products was translated efficiently (data not shown). Subsequently, the in vitro-translated proteins were incubated with a ^{32}P-labeled
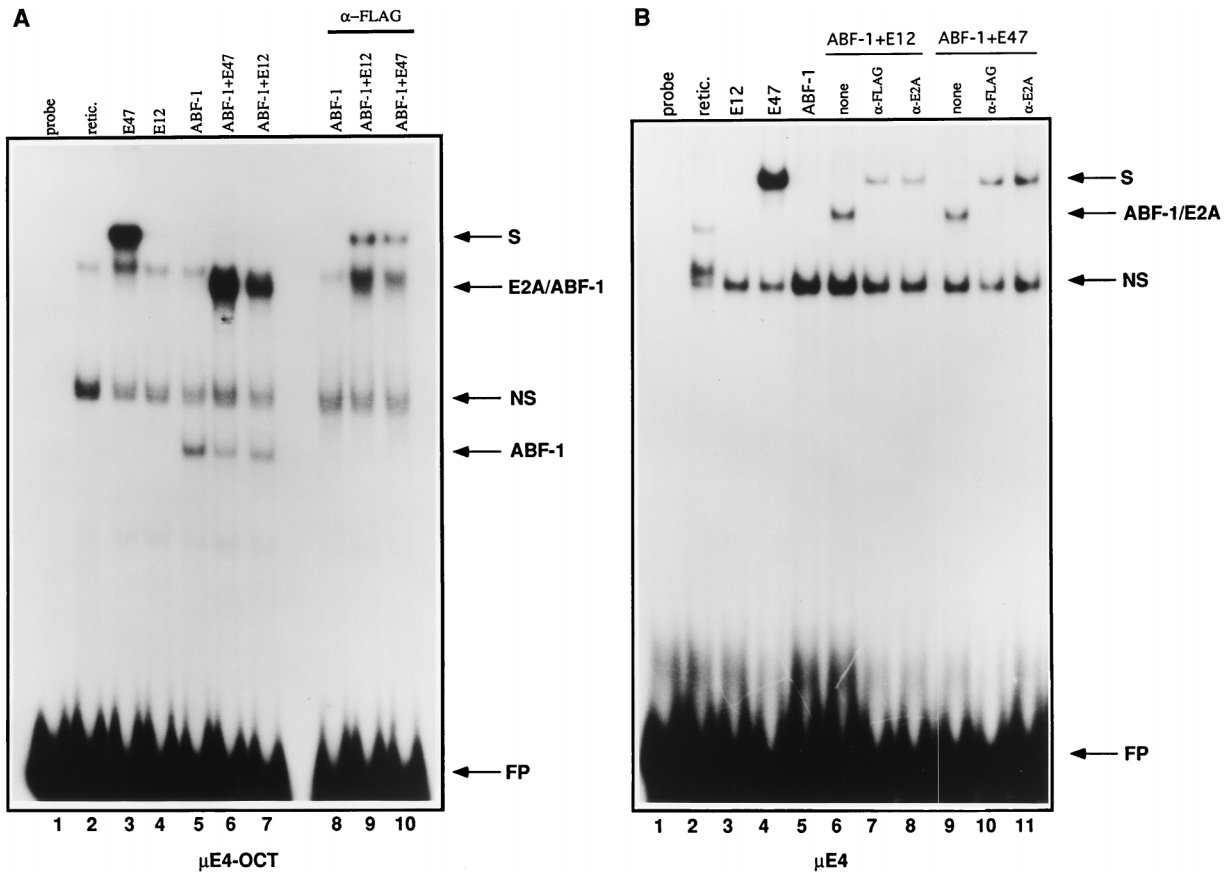
FIG. 6. ABF-1 binds to an E box in vitro. (A) EMSA analysis of FLAG ABF-1 homo- and heterodimeric complexes formed on the μE4-OCT probe. Arrows indicate the position of ABF-1 homodimers and ABF-1–E2A heterodimers. retic., reticulocyte lysate; FP, free probe; NS, nonspecific complex; S, supershifted complex. (B) ABF-1 binds as a heterodimer to the μE4 probe. Addition of a nonspecific control antibody had no effect on complex formation (not shown).

μE4-OCT-containing oligonucleotide probe derived from the IgH intronic enhancer and analyzed by EMSA. The μE4 site is closely related to the μE5 and κE2 sites and binds with high affinity to E47 (5) and (38a). As predicted, E47 was capable of binding to the μE4 site as a homodimer (Fig. 6A, lane 3). As expected, E12 was not able to bind efficiently to the site, as it has been shown to contain an inhibitory domain which interferes with homodimerization (Fig. 6A, lane 4) (67). In contrast to E12, ABF-1 has the ability to bind the μE4-OCT site alone (Fig. 6A, lane 5). This complex was eliminated by the addition of an anti-FLAG antibody and thus likely represents a homodimer of ABF-1 (Fig. 6A, lane 8). When ABF-1 was cotranslated with either E12 or E47, a complex of intermediate mobility was formed (Fig. 7A, lanes 6 and 7). This complex, which could be supershifted with an anti-FLAG antibody, most likely represents a heterodimer composed of ABF-1 and E2A (Fig. 6A; compare lanes 6 and 7 with lanes 9 and 10). Alternatively, incubation with the anti-FLAG antibody may inhibit DNA binding of ABF-1–E2A heterodimers, thus allowing E47 homodimers to form on the μE4 probe (Fig. 6).

To confirm that ABF-1 bound to the E-box site, we performed an EMSA with a minimal oligonucleotide containing only the μE4 site. In the presence of either E12 or E47, ABF-1 could bind the E-box site as a heterodimer (Fig. 6B, lanes 6 and 9). However, ABF-1 could not bind as a homodimer to the E-box site (Fig. 6B, lane 5). As expected, the heterodimeric complexes could be supershifted with both an anti-FLAG and an anti-E2A antibody, confirming that ABF-1 with either E12

or E47 has the ability to bind as heterodimers to DNA (Fig. 6B, lanes 7, 8, 10, and 11). E2-2 and HEB are also able to interact with ABF-1 in the two-hybrid system as demonstrated above (Fig. 1). However, gel shift analysis failed to detect any binding of HEB- or E2-2–ABF-1 heterodimers to the μE4 site (not shown).

Since the μE4 and μE4-OCT oligonucleotide probes contain the same E-box element, we postulated that the octamer site may be involved in facilitating the binding of ABF-1 homodimers. To test if the octamer site is required, we generated
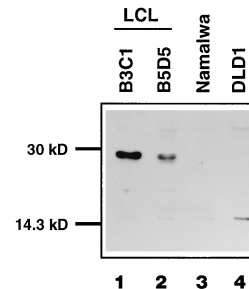

FIG. 7. Western blot analysis of ABF-1 in nuclear extracts isolated from several human cell lines. Lane 1, EBV-immortalized LCL B3C1; lane 2, B5D5 (LCL); lane 3, Namalwa (Burkitt lymphoma); lane 4, DLD1 (colon carcinoma). ABF-1 was detected by using a polyclonal antibody as described in Materials and Methods.
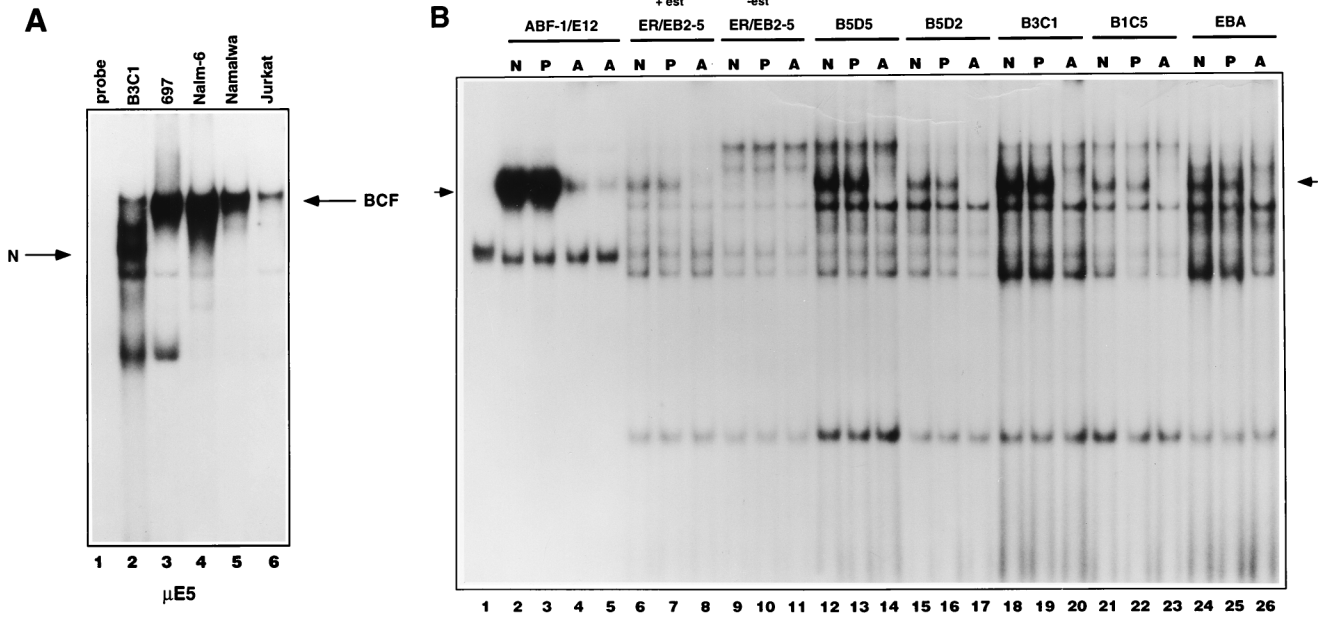
FIG. 8. ABF-1 is part of an E-box binding complex present in EBV-immortalized LCLs. (A) Gel shift analysis with a μE5 probe reveals a novel complex (N) whose mobility differs from that of BCF present in the EBV-immortalized line B3C1 (lane 2). (B) The ABF-1-containing nucleoprotein complex, indicated by arrows, was detected in all EBV-immortalized LCLs (seven cell lines in total were analyzed). Lane 1, unprogrammed reticulocyte lysate; lanes 2 to 5, in vitro-cotranslated ABF-1 and E12 proteins; lanes 6 to 11, nuclear extract derived from the conditional cell line ER/EB2-5 (32) grown in the presence of 1 μM β-estradiol (+est) or estrogen starved for 48 h (−est); lanes 12 to 26, nuclear extract isolated from independent LCLs. N, no antibody; P, preimmune serum; A, ABF-1-specific antiserum. The free probe was run off the gel.

a μE4-OCT mutant probe in which the octamer site has been destroyed. ABF-1 homodimers were equally capable of forming on both the wild-type and mutant μE4-OCT oligonucleotide probes, demonstrating that the octamer site is not required (38a).

ABF-1 forms heterodimers with E2A proteins in vivo. To determine whether ABF-1 protein is present in nuclear extracts derived from B-cell lines, we generated a polyclonal antibody specific for ABF-1. Nuclear extracts derived from a human B-cell line, Namalwa, and two EBV-transformed LCLs were analyzed by immunoblotting. ABF-1 protein was detected in nuclear extracts derived from EBV-immortalized B-cell lines but not from the Burkitt lymphoma line Namalwa or the colon carcinoma line DLD1 (Fig. 7). Both in vitro-translated and endogenous ABF-1 proteins have an apparent molecular mass of 29 kDa, as revealed by Western blot analysis using an ABF-1-specific polyclonal antibody (Fig. 7 and data not shown). Therefore, as suggested by the Northern blot analysis described above, ABF-1 protein is expressed in EBV-transformed LCLs.

Previously, we have shown that two B-cell-specific μE5 binding complexes are present in nuclear extracts from a wide variety of pre-B- and mature B-cell lines, designated BCF1 and BCF2. The BCF1 and BCF2 complexes are composed mainly of homodimers of E2-2 and E47 in pre-B cells, whereas in mature B cells the binding complex consists predominantly of E47 homodimers (2, 44, 63). To determine whether ABF-1 DNA binding activity could be detected in vivo, we examined a number of nuclear extracts derived from various B-cell lines by EMSA using the μE5 site as a probe. B-cell-specific complexes BCF1 and BCF2 were detected in the pre-B line Nalm-6, the pre-B acute lymphoblastic leukemia (ALL) line 697, the mature B line Namalwa, and to a lesser degree the T-cell ALL line Jurkat as described previously (Fig. 8A) (2, 44). Interest-

ingly, the LCL B3C1 extract contained a nucleoprotein complex of faster mobility not seen in the other extracts (Fig. 8A, lane 2). To determine if the novel complex was a common feature of EBV-immortalized lines, we analyzed a number of nuclear extracts that were derived from various EBV-transformed B-cell lines. Of the seven lines examined, all contained the unique complex, albeit at various levels (Fig. 8B and data not shown). This group also included the EBNA2 conditional cell line ER/EB2-5, an estrogen-dependent LCL (32). Complex formation was clearly dependent on the presence of β-estradiol in the culture medium and thus required the presence of EBNA2 (Fig. 8B; compare lanes 6 and 9).

Interestingly, this complex comigrated with an in vitro-translated heterodimer of ABF-1 and E12 (Fig. 8B; compare lane 2 with lanes 6, 12, 15, 18, 21, and 24). To assess whether this complex contained ABF-1, preimmune serum or ABF-1-specific antiserum was added to the reactions. The preimmune sera had no effect on the complex, but addition of anti-ABF-1 antibody completely eliminated complex formation (Fig. 8B). Furthermore, addition of an E2A-specific monoclonal antibody supershifted the complex, indicating that the complex is composed of an ABF-1–E2A heterodimer (38a). Taken together, these data indicate that ABF-1 forms heterodimers with the E2A gene products in EBV-transformed lymphoblastoid B-cell lines.

ABF-1 is a transcriptional repressor. As shown above, ABF-1 has the ability to form heterodimers with E2A in vivo. This finding suggests that ABF-1 may be involved in modulating the transcriptional activity of E2A. We therefore checked whether coexpression of ABF-1 and E47 alters the transactivation properties of E47 in mammalian cells. An E47 expression vector was introduced into HeLa cells along with a CAT reporter gene driven by six copies of the μE5-μE2 E-box elements derived from the IgH gene enhancer (Fig. 9A) (27).
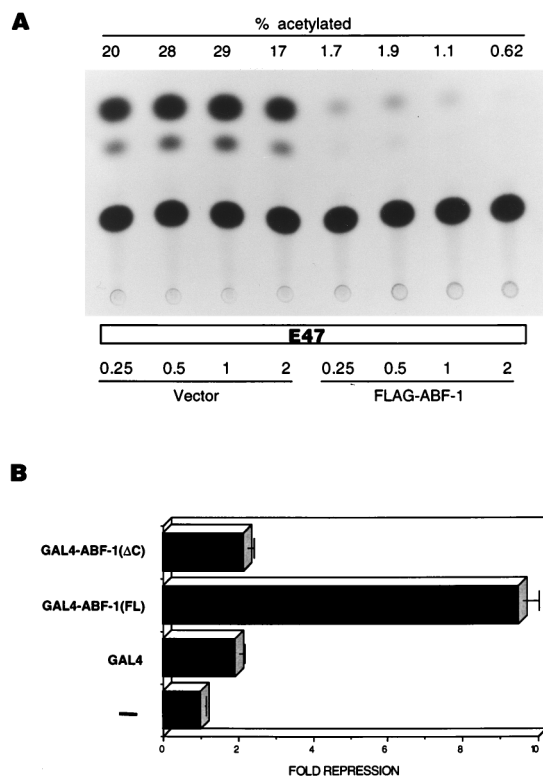
A

B

FIG. 9. ABF-1 functions as a transcriptional repressor in mammalian cells. (A) ABF-1 interferes with the ability of E47 to activate transcription in mammalian cells. HeLa S3 cells were transiently transfected with 1 μg of (μE5-μE2)$_6$CAT reporter plasmid, 0.25 μg of E47 expression vector (pHβA-E47), and increasing amounts (shown in micrograms) of empty expression vector (pHβAneo) or FLAG–ABF-1 expression vector (pHβA-FLAG-ABF-1) as indicated. The total amount of DNA used in each transfection experiment was adjusted to 5 μg by addition of pBSK; 30 μl of Superfect reagent was used for each transfection. Equal amounts of protein extracts were assayed for CAT activity. CAT activity is indicated by % acetylated, which represents the conversion of ^{14}C-chloramphenicol to acetylated forms. (B) ABF-1 contains a transcriptional repression domain. HeLa S3 cells were transiently transfected as described above with 1 μg of 3XUAS$_{GAL}$TK LUC reporter plasmid, 0.025 μg of pCMVβGAL, and 1 μg of the indicated GAL4 expression constructs. In the absence of effector plasmid, the 3XUAS$_{GAL}$TK LUC reporter gene activity was 16,546 relative light units. Luciferase activity was normalized to β-galactosidase levels as a control for transfection efficiency.

Cotransfection of increasing amounts of a FLAG–ABF-1 expression plasmid resulted in a dramatic inhibition of E47-mediated transactivation (Fig. 9A). In fact, cotransfection of equal amounts of E47 and FLAG–ABF-1 expression vectors resulted in a 12-fold reduction of reporter gene activation (Fig. 9A). As expected, cotransfection of increasing amounts of empty expression vector had no effect on E47 transcriptional activity (Fig. 9A).

To address the issue of how ABF-1 might inhibit E47-mediated transcriptional activation, we tested whether ABF-1 can function as a transcriptional repressor. The full-length ABF-1 protein [ABF1(FL)] was fused in-frame with the GAL4 DNA binding domain and introduced into HeLa cells with a luciferase reporter gene containing three GAL4 binding sites upstream of the thymidine kinase promoter (Fig. 9B) (26). This reporter, which is moderately active in HeLa cells, can be used to monitor transcriptional repression (26). The GAL4–ABF-1(FL) fusion was capable of repressing transcription ninefold (Fig. 9B). The deletion derivative GAL4–ABF-1(ΔC), which lacks the bHLH domain and C-terminal sequence, failed to

repress the activity of the reporter gene (Fig. 9B). Therefore, ABF-1 contains a domain(s) present in the carboxy-terminal half of the protein which can function as a transcriptional repressor when fused to a heterologous DNA binding domain.

DISCUSSION

Transcription factors belonging to the bHLH family are known to regulate a variety of developmental programs, including neurogenesis, myogenesis, sex determination, and hematopoiesis (1a, 3, 40, 41, 45, 49, 57, 79). Many of these processes are controlled by a heterodimer composed of a tissue-restricted bHLH factor and an E protein. It appears, however, that B-lymphocyte development requires the activity of E2A homodimers (2, 3, 63, 79). Although formally possible, there has been a dearth of evidence supporting the notion of a B-cell-specific class II bHLH factor. In undertaking this study, we reasoned that B-lymphocyte development may parallel many other developmental processes in their requirement for a cell-type-specific bHLH protein.

We have named this gene *ABF-1* because of its high expression levels in activated human B cells. *ABF-1* expression was induced upon treatment of purified human B cells with the polyclonal activator SAC. SAC activates human B cells through cross-linking of the surface Ig (21, 54, 55, 60, 64). This finding suggests that *ABF-1* may be a downstream target of the B-cell receptor signal transduction pathway and thus may be induced upon physiological encounter with antigen. The expression pattern in lymphoid tissues is consistent with a role in B-cell development. Significant levels of *ABF-1* mRNA were found in the appendix and lymph node, sites where activated B cells are known to be localized. Since *ABF-1* mRNA was also seen in fetal liver and bone marrow, however, we cannot rule out the possibility that ABF-1 also plays a role early in B-cell development. More extensive Northern blot analysis of poly(A)$^+$ RNA derived from 50 different human tissues showed that low-level expression of *ABF-1* was restricted to the lymph node and aorta (38a).

Intriguingly, the *ABF-1* gene was highly expressed in a number of EBV-immortalized LCLs. These lines have many features in common with activated B lymphocytes, including increased cell size, proliferation, and expression of surface markers (8, 15, 53, 69, 73). Indeed, resting B cells stimulated by physiological agents or by EBV infection exhibit identical patterns of expression of several cell cycle control genes (28). Taken together, these data suggest that EBV infection of resting B cells may induce the normal activation program, thus bypassing the need for physiological signals (28). This may explain why *ABF-1* mRNA is expressed in both LCLs and activated human B cells. Furthermore, it suggests that the ABF-1–E2A complex detected in nuclear extracts isolated from LCLs may also be present in physiologically activated B cells. In fact, a complex of similar mobility can be detected in extracts derived from human B cells activated with SAC and IL-2 (1). Surprisingly, the presence of EBV proteins is not sufficient to allow expression of ABF-1; all Burkitt lymphoma lines tested did not express *ABF-1* mRNA.

A conditional LCL, ER/EB2-5, also contains high levels of *ABF-1* transcripts. An ER-EBNA2 fusion protein renders this cell line dependent on estrogen for growth (32). Withdrawal of estrogen causes the cells to leave the cell cycle and arrest in G$_0$ (32). Interestingly, expression of ABF-1 mRNA is also estrogen responsive. When ER/EB2-5 is estrogen starved, *ABF-1* message becomes undetectable within 24 h (Fig. 4A and data not shown). This finding correlates well with the absence of *ABF-1* transcripts in noncycling, resting human B cells. Upon

addition of estrogen, *ABF-1* mRNA becomes apparent within 10 h and is maintained at a steady state thereafter (38a). As expected from these observations, formation of an active ABF-1–E2A DNA binding complex in ER/EB2-5 also appears to be critically dependent on the presence of estrogen. Perhaps, then, further study of ABF-1 function in LCLs may lead to insights as to its role in physiologically activated B lymphocytes.

The E-box hexanucleotide sequence CANNTG has been identified in a number of cell-type-specific promoter and enhancer elements (40, 41). E boxes have long been implicated in the regulation of a number of B-lineage genes. For example, the IgH intronic enhancer, the 3′ IgH enhancer, and the kappa enhancer all contain E-box elements (47, 65). Mutational analysis has shown that the integrity of these elements is crucial for full transcriptional activity (65). We have shown here that ABF-1–E2A heterodimers are capable of binding at least two types of E-box sequences, the μE4 element (CACCTG) and μE5 (CAGGTG), thus raising the possibility that ABF-1 contributes to the regulation of Ig gene transcription.

It is not entirely clear why ABF-1 is capable of forming homodimers on the μE4-OCT probe but not on the μE4 probe. We conclude, however, that sequences flanking the core E-box element, CACCTG, present in the μE4-OCT oligonucleotide are important for ABF-1 homodimer formation. Clearly, binding site selection studies are necessary to address this issue further. Nevertheless, the ability of ABF-1 to bind DNA as a homodimer will have important implications for understanding its role in B-cell development.

After antigenic stimulation and in the presence of T-cell help, activated B lymphocytes can undergo the process of Ig isotype switching. Recently, it was shown by targeted deletion in mice that the 3′ IgH enhancer is required for the process of class switch recombination (CSR) (14). Interestingly, the 3′ IgH enhancer core contains an E-box element which has been shown to be important for enhancer activity (39). The switch regions present in the IgH gene consist of repetitive DNA elements in which recombination breakpoints lie (33, 76). It has been postulated that DNA binding factors may contribute to the generation of loop structures formed during the CSR process (33, 76, 77). The S_γ switch regions have been shown to contain E2-box-like elements to which a binding activity known as SNAP recognizes (37). It appears that E47 or a related factor is part of the SNAP hetero-oligomeric complex (37). Although it has yet to be demonstrated that these E-box elements are important for CSR, it is interesting to speculate that ABF-1 plays a role in this process. ABF-1 could participate in higher order nucleoprotein complexes postulated to regulate recombination within the switch regions.

Studies of IgE synthesis in human B cells have shown that two signals are required to mediate the process: an activation signal and the presence of a cytokine (13, 31, 70). For example, switching to IgE requires that the B cell be exposed to both mitogen and IL-4 (31, 70). Although IL-4 treatment alone induces germ line epsilon transcripts, S_μ/S_ϵ deletional switch recombination does not occur and mature C_ϵ mRNA is not produced (30, 61, 62). Interestingly, infection with EBV could provide the activation stimulus required for the switching event to take place (31, 61, 70). Perhaps ABF-1, which is known to be induced upon such a mitogenic signal, is involved in this development event.

We have demonstrated here that ABF-1 is capable of inhibiting E47-directed transcriptional activation from an E-box-containing reporter gene. Furthermore, we have shown that when tethered to DNA, ABF-1 strongly represses transcription. The ability of ABF-1 to repress transcription may be

dependent on sequence context. The transcription factor Dorsal, for example, can function as either a transcriptional repressor or an activator in *Drosophila* (29, 68). Dorsal can activate transcription of the *twist* gene yet function as a repressor of the *zen* gene (29, 68). Dorsal can be converted into a transcriptional repressor by the protein DSP1, a member of the high-mobility-group protein family (36). Repression is dependent on a sequence element known as the negative regulatory element which is found adjacent to Dorsal binding sites in the *zen* promoter (18, 36). It is conceivable that the repressor activity of ABF-1 helps maintain the proliferative state of EBV-transformed LCLs and activated B cells. Recently, it has been shown that the cyclin-dependent kinase inhibitor, p21, contains E2A binding sites within its promoter element (50). E47 was shown to be capable of activating expression of a reporter gene driven by the p21 promoter element as well as activation of the endogenous p21 gene (50). This observation provides a possible model for growth inhibition conferred by overexpression of E2A, whereby increased expression of p21 interferes with cell cycle progression (50). The ability of ABF-1 to antagonize E47 transactivation suggests that ABF-1 may help promote proliferation of activated B cells and EBV-transformed LCLs by inhibiting E2A-directed p21 gene transcription. It will now be essential to generate mice lacking *ABF-1* to determine its role in B-cell activation.

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