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The VBP and a1/EBP Leucine Zipper Factors Bind Overlapping Subsets of Avian Retroviral Long Terminal Repeat CCAAT/Enhancer Elements

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Two long terminal repeat (LTR) enhancer-binding proteins which may regulate high rates of avian leukosis virus (ALV) LTR-enhanced c-*myc* transcription during bursal lymphomagenesis have been identified (A. Ruddell, M. Linial, and M. Groudine, Mol. Cell. Biol. 9:5660–5668, 1989). The genes encoding the a1/EBP and a3/EBP binding factors were cloned by expression screening of a λgt11 cDNA library from chicken bursal lymphoma cells. The a1/EBP cDNA encodes a novel leucine zipper transcription factor (W. Bowers and A. Ruddell, J. Virol. 66:6578–6586, 1992). The partial a3/EBP cDNA clone encodes amino acids 84 to 313 of vitellogenin gene-binding protein (VBP), a leucine zipper factor that binds the avian vitellogenin II gene promoter (S. Iyer, D. Davis, and J. Burch, Mol. Cell. Biol. 11:4863–4875, 1991). Multiple VBP mRNAs are expressed in B cells in a pattern identical to that previously observed for VBP in other cell types. The LTR-binding activities of VBP, a1/EBP, and B-cell nuclear extract protein were compared and mapped by gel shift, DNase I footprinting, and methylation interference assays. The purified VBP and a1/EBP bacterial fusion proteins bind overlapping but distinct subsets of CCAAT/enhancer elements in the closely related ALV and Rous sarcoma virus (RSV) LTR enhancers. Protein binding to these CCAAT/enhancer elements accounts for most of the labile LTR enhancer-binding activity observed in B-cell nuclear extracts. VBP and a1/EBP could mediate the high rates of ALV and RSV LTR-enhanced transcription in bursal lymphoma cells and many other cell types.

Avian leukosis virus (ALV) is a slowly transforming retrovirus that induces B-cell lymphoma in chickens, after proviral integration next to the c-*myc* proto-oncogene (reviewed in references 15 and 33). The oncogenic potential of ALV is regulated by the long terminal repeat (LTR) enhancer, which drives high levels of c-*myc* hyperexpression 50- to 100-fold over normal levels in bursal lymphoma cells. In contrast, the endogenous avian retroviruses generally lack the LTR enhancer sequences and are transcriptionally silent (10). These endogenous viruses fail to induce lymphoma (31, 39), suggesting that the LTR enhancer is essential for high levels of oncogene expression and tumor induction.

The ALV LTR enhancer drives high levels of viral expression in many tissues (42), while inducing tumors only in immature B cells. One feature of LTR-enhanced transcription which may be important for B-cell-specific tumor induction has been discovered. Labile factors appear to regulate LTR-enhanced transcription in B cells, as treatment with protein synthesis inhibitors specifically decreases LTR-driven transcription (29). This lability is specific for B cells, as inhibition of protein synthesis in T cells or fibroblasts does not affect LTR-enhanced transcription. Moreover, LTR-enhanced transcription is labile in hematopoietic tissues from ALV lymphoma-susceptible strains, while it is stable in lymphoma-resistant strains (42). This correlation of labile LTR-enhanced transcription with tumor susceptibility suggests that the labile factors are somehow important for B-cell tumor induction. Down-regulation of these labile factors during B-cell development could influence c-*myc* hyperexpression in a manner that is important for tumor induction. For example, this down-regulation could reduce the cytotoxic effects of high-level c-*myc* expression (14, 56, 57) or allow differentiation events required for bursal lymphomagenesis (8, 38).

The ALV LTR-binding factors of B cells were characterized to determine how they could regulate labile LTR-enhanced transcription. Five distinct LTR enhancer-binding proteins were identified (41). Three of these activities (a1, a3, and b*) are specifically labile in B cells, while they are stable in other cell types. The binding sites of the labile a1 and a3 proteins cover a large region of the 140-bp LTR enhancer. This region contains a number of CCAAT/enhancer elements, which may be important for binding by the labile a1 and a3 proteins (41). The Rous sarcoma virus (RSV) LTR sequence is nearly identical to the ALV LTR (5) and also contains several of these elements (44). These RSV CCAAT/enhancer elements bind C/EBP-α, which is the prototype of a family of leucine zipper transcription factors (25, 28). This family of about 10 proteins shares conserved carboxy-terminal basic regions specifying DNA binding activity and leucine zipper regions involved in factor dimerization. The amino-terminal regions of these proteins are variable and can mediate transcription-activating or -repressing activity (17, 37). Several members of this family in addition to C/EBP-α have been shown to bind the RSV LTR enhancer. These include NF/IL6, a rodent factor that also binds to interleukin 6 gene promoter elements (1), and Ig/EBP, a murine factor that binds to the immunoglobulin gene enhancer and promoter elements (40). C/EBP-α and NF/IL6 show tissue-restricted expression in liver and other tissues (1, 4), while Ig/EBP is preferentially expressed in

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lymphoid cell types (40). These factors could act in different combinations to mediate the widespread high-level expression of ALV and RSV (16, 42). Alternatively, ubiquitous factors could regulate LTR-enhanced transcription.

The genes encoding the a1 and a3 LTR-binding proteins were cloned from B-lymphoma cells by a phage library screening technique (48, 54). A λgt11 cDNA expression library was screened for fusion proteins binding to the a1 and a3 LTR binding site probes. The identification of an a1 LTR enhancer-binding protein designated a1/EBP was reported previously (6). The a1/EBP cDNA encodes 209 amino acids of a novel leucine zipper transcription factor, which is most closely related to Ig/EBP (40). The a1/EBP bacterial fusion protein binds two large LTR regions that include conserved CCAAT/enhancer elements. Gel shift analysis indicates that a1/EBP recognizes these CCAAT/enhancer elements in a manner similar to the a1 binding activity from bursal lymphoma cells.

In this report, we describe the identification of the gene encoding a second leucine zipper transcription factor. This factor is designated a3/EBP, as it was identified by screening the cDNA library with the a3 LTR oligonucleotide probe. The partial a3/EBP cDNA clone encodes amino acids 84 to 313 of the vitellogenin gene-binding protein (VBP), a leucine zipper factor previously identified by Iyer et al. (23). VBP belongs to the PAR subfamily of leucine zipper factors, which share a conserved proline- and acidic amino acid-rich region in the amino terminus that is not found in the C/EBP-related leucine zipper factors (12). This subfamily includes the DBP and HLF factors, which show tissue-specific expression patterns (22, 32). VBP was originally identified as a vitellogenin II gene promoter-binding protein that interacts with an estrogen-responsive promoter element important for vitellogenin II gene transcription in liver cells (23). However, VBP mRNA is widely expressed, suggesting that VBP could regulate viral and cellular gene expression in many cell types. We demonstrate that VBP binds multiple LTR CCAAT/enhancer elements in a manner similar to the labile a3 binding activity previously identified in B cells. These sites overlap but are distinct from the a1/EBP binding sites, suggesting that both proteins could contribute to the high rates of LTR-enhanced transcription in B cells and in other cell types.

MATERIALS AND METHODS

Cell culture and RNA analysis. The DT40 and S13 bursal lymphoma cell lines were grown as previously described (2, 42). Primary chicken embryo fibroblasts (CEF) were prepared from 10-day-old embryos and cultured as previously described (42).

RNA was isolated by guanidinium isothiocyanate-cesium chloride centrifugation (46), and poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography (Bethesda Research Laboratories). For Northern (RNA) blot analysis, RNA was resolved in a 2.2 M formaldehyde agarose gel (52) and transferred to nitrocellulose (Schleicher & Schuell). Total RNA electrophoresed on the same gel was stained with ethidium bromide to detect the migration of 18S and 28S rRNAs. The a3/EBP cDNA was ^{32}P labelled by random prime reaction (U.S. Biochemical) and hybridized to blots under standard high-stringency hybridization conditions (46).

Isolation of an a3/EBP cDNA clone. A λgt11 expression cDNA library was prepared from poly(A)$^+$ RNA of S13 bursal lymphoma cells, and 1.5×10^6 primary phage were screened by the technique of Singh et al. (48), as described previously (6). Duplicate filters were screened for proteins that bound a ^{32}P-labelled concatenated a3 oligonucleotide probe

5′-CATGCTTATGTAACGATGAGCTTCA-3′
3′-AATACATTGCTACTCGAAGTGTACG-5′

that was prepared by ligation and nick translation (54). The a3/EBP cDNA insert was purified from λgt11 phage DNA by *Not*I digestion and subcloned into Bluescript plasmid (Stratagene) for dideoxy-sequence analysis (U.S. Biochemical). The DNA sequence was analyzed by using GCG computer programs (11).

Protein purification. The a3/EBP cDNA was cloned into the glutathione *S*-transferase (GST) vector pGEX-2T (Pharmacia), expressed as a bacterial fusion protein, and purified by glutathione-agarose chromatography (49). The purified protein was adjusted to 8% glycerol, 50 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol; frozen in liquid nitrogen; and stored at −70°C. Nuclear extracts were prepared from S13 bursal lymphoma cells by 0.5 M NaCl extraction of purified nuclei, as previously described (42). Protein concentration was determined by the Bradford assay (7).

Gel shift assay. Complementary overlapping LTR oligonucleotides were synthesized and annealed. The sequences are given in Fig. 4D. The double-stranded probes were end labelled with Klenow polymerase, [^{32}P]dCTP, and [^{32}P]dTTP (3,000 Ci/mmol; NEN). Bacterial GST fusion protein or S13 B-cell nuclear extract was incubated with 5,000 cpm (about 0.1 ng) of ^{32}P-labelled oligonucleotide probes and 1 μg of poly(dI-dC)-poly(dI-dC) in a 15-μl reaction volume. Protein-DNA complexes were resolved on 4% polyacrylamide gels in TAE buffer as previously described (42).

DNase I footprinting assay. The 245-bp *Mst*II-*Eco*RI fragment of the ALV LTR enhancer from BK25 bursal lymphoma cells (42) was subcloned into pBluescribe (Stratagene), so that end-labelled probes for the coding and noncoding strands could be obtained by *Eco*RI or *Hin*dIII digestion. DNA was end labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP (6,000 Ci/mmol; New England Nuclear), followed by *Hin*dIII or *Eco*RI digestion and polyacrylamide gel purification (41). The 235-bp *Acc*I-*Hin*dIII fragment of the SR-A RSV LTR (19) was subcloned into pBluescribe (provided by K. Conklin) and end labelled after digestion with *Bam*HI or *Hin*dIII. Fusion proteins were incubated with 10,000 cpm of either probe and then subjected to brief DNase I digestion (18). DNA was purified and resolved on 8 M urea–8% polyacrylamide gels in parallel with A+G sequence reactions (30), followed by autoradiography.

Methylation interference assay. Coding and noncoding a1 oligonucleotides spanning the ALV LTR enhancer sequence from −264 to −232 were synthesized, annealed, and cloned into the *Xho*I and *Bam*HI sites of pBluescribe (pBSa1). The a2/a3 site probe was prepared by PCR (45) from the BK25 ALV LTR (42). The primers used (5′ primer, GCGGGATC CCAATACTCTTGTAGTCT; 3′ primer, GCGGAATTCCCT TATAAGGCATGTTG) created an a2/a3 site from position −231 to −169, which was subcloned into the pBluescribe *Bam*HI and *Eco*RI sites (pBSa2/a3). pBSa1 and pBSa2/a3 were digested with *Eag*I or *Eco* 0109 and end labelled with polynucleotide kinase and [γ-^{32}P]ATP. The probes were then restricted with *Eco* 0109 or *Eag*I, respectively, and purified from 5% polyacrylamide gels. The probes were made blunt ended by filling in with unlabelled deoxynucleotides and Klenow fragment.

The end-labelled LTR probes were partially methylated at guanine and adenine residues with dimethylsulfate (Aldrich) as described by Maxam and Gilbert (30). Methylated DNA was used in gel shift reactions scaled up 10-fold with either a1/EBP-GST or a3/EBP-GST fusion proteins. The shifted

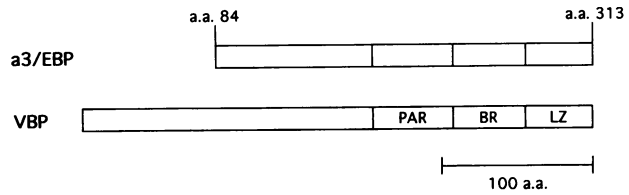

FIG. 1. The a3/EBP cDNA is identical to the VBP cDNA from nucleotides 390 to 1077. The a3/EBP cDNA encodes amino acids (a.a.) 84 to 313 of VBP, which includes the regions encoding the proline- and acidic amino acid-rich region (PAR), basic region (BR), and leucine zipper (LZ).

complexes were resolved in 4% gel shift gels, followed by wet-gel autoradiography. The bound and the free probes were purified from the polyacrylamide gel by electrophoresis onto DEAE NA45 paper (Schleicher & Schuell), as described by Baldwin (3). The DNA samples were precipitated with ethanol, and the guanine and adenine residues were cleaved in 0.1 M piperidine (Fisher Scientific) by heating at 90°C for 30 min. The piperidine was removed by lyophilization, and equal counts per minute of bound and free probe were electrophoresed on 8 or 10% denaturing polyacrylamide gels, followed by autoradiography (21).

RESULTS

The a3/EBP cDNA encodes VBP. The a1 and a3 LTR-binding activities of B cells interact with a 70-bp region of the LTR enhancer region which includes multiple CCAAT/enhancer elements. The genes encoding the proteins with a1 and a3 binding activities were identified by an expression library screening technique (48, 54). A λgt11 cDNA expression library was prepared with S13 bursal lymphoma cell poly(A)$^+$ RNA and was screened for expression of β-galactosidase–cDNA fusion proteins that bind the a1 or a3 LTR CCAAT/enhancer element probes (see Materials and Methods). An a1/EBP clone obtained by screening with the a1 oligonucleotide probe was found to encode a novel member of the leucine zipper family of transcription factors, as previously reported (6).

A cDNA encoding the a3 LTR-binding activity was obtained by screening the library with the a3 oligonucleotide probe. This a3/EBP cDNA contains a 687-bp insert which was analyzed by DNA sequencing, as described in Materials and Methods. The a3/EBP cDNA encodes a 229-amino-acid open reading frame that is identical to amino acids 84 to 313 of VBP (Fig. 1). VBP was previously identified as a vitellogenin II gene promoter-binding protein in an expression screen of an avian liver cDNA library (23). VBP belongs to a subfamily of leucine zipper factors which share a conserved PAR domain enriched for proline and acidic amino acid residues (12). The partial a3/EBP cDNA clone encodes part of the amino-terminal putative transcription-activating region and all of the PAR, basic, and leucine zipper domains of VBP (Fig. 1). The a3/EBP cDNA lacks an initiation codon at its 5′ terminus and hence is incomplete. This cDNA could represent a partial copy (nucleotides 390 to 1077) of the full-length VBP mRNA (23), or it potentially could encode a VBP transcript with alternatively spliced 5′ or 3′ termini.

The VBP mRNA is widely expressed in many tissues in addition to liver, suggesting that it could encode a general transcription factor (23). The a3/EBP cDNA was analyzed in high-stringency Northern blots with poly(A)$^+$ RNA from the DT40 bursal lymphoma cell line, to confirm that this gene is

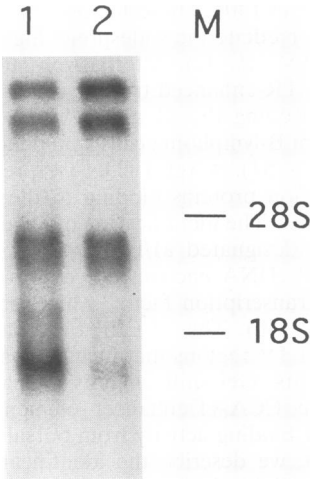

FIG. 2. Expression of multiple a3/EBP mRNAs similar to the pattern of VBP mRNAs. The a3/EBP cDNA clone was used in Northern blot hybridization of polyadenylated RNA (8 μg) from DT40 bursal lymphoma cells (lane 1) and CEF (lane 2). The migration of 18S and 28S rRNAs is indicated (M).

also expressed in B cells. The a3/EBP cDNA hybridizes multiple RNAs of roughly 1.5 to 4 kb, as well as two larger RNAs (Fig. 2). The mRNA size distribution is similar in CEF and in several other B-lymphoma cell lines (data not shown). This complex pattern is the same as the pattern of multiple VBP mRNA species previously described for CEF and other cell types by Iyer et al. (23), who used a slightly different VBP cDNA probe (nucleotides 459 to 1179). The protein coding potential of these multiple mRNAs is not yet known, but they are all large enough to encode the full-length 313-amino-acid VBP. These findings indicate that VBP could encode the labile a3 LTR-binding protein of B cells.

a3/EBP and a1/EBP bind LTR CCAAT/enhancer elements. The ALV LTR-binding activity of the a3/EBP-GST fusion protein was characterized by DNase I footprinting analysis of the ALV LTR enhancer region. The a3/EBP cDNA was expressed in a GST expression vector, so that fusion protein could be expressed and purified from bacterial lysates by glutathione-agarose chromatography (49). This purified a3/EBP-GST fusion protein binds two large ALV LTR sites designated a1 and a3 (Fig. 3A), which correspond to sites protected by the labile a1 and a3 activities purified from B-lymphoma cells (41). a3/EBP binds the two sites with similar affinity in vitro, as DNase I protection of both sites is maintained as protein concentration is decreased (data not shown).

The a3/EBP LTR-binding sites overlap those previously characterized as a1/EBP binding sites (6). The two proteins protect very similar regions of the a1 site, while their binding to downstream LTR sites is distinct (Fig. 3A). The DNA sequences protected by each protein are summarized in Fig. 3B (a3/EBP) and Fig. 3C (a1/EBP). a3/EBP binds the a3 site, while the a1/EBP-GST fusion protein preferentially binds the more 5′ a2 site. Moreover, a1/EBP binding induces a strong DNase I-hypersensitive site between the a1 and a2 sites (Fig. 3C), while a3/EBP binding to the a1 and a3 sites does not induce DNase I hypersensitivity (Fig. 3B). The LTR region bound by both proteins spans about 70 bp, covering the region protected by the labile B-cell proteins (41).

The a1, a2, and a3 LTR binding sites include a number of CCAAT/enhancer elements (Fig. 3D) related to the consensus

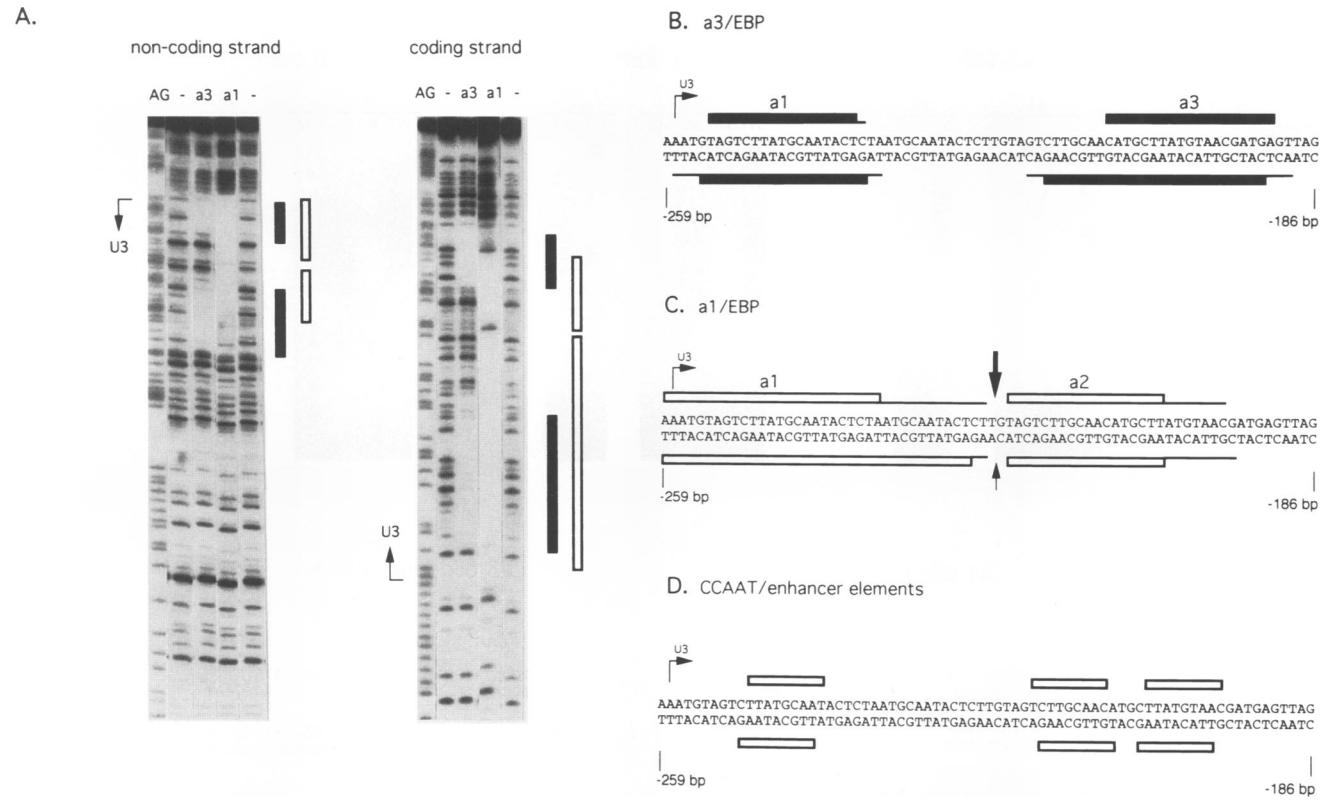

FIG. 3. a3/EBP and a1/EBP bind multiple ALV LTR enhancer sites. (A) The ALV LTR probe was ^{32}P labelled on the noncoding or coding strand and incubated in mock reactions (−) or with the a3/EBP-GST (a3) or a1/EBP-GST (a1) fusion proteins. Samples were treated with DNase I and resolved on denaturing polyacrylamide gels alongside Maxam-Gilbert A+G sequence reactions (AG). The solid bars represent sequences protected by a3/EBP, while the open bars show a1/EBP binding sequences. The 5′ end of the U3 LTR sequence is indicated. (B) a3/EBP LTR binding sites. The lines indicate partially protected sequences. (C) a1/EBP LTR binding sites. DNase I-hypersensitive sites are indicated by arrows. (D) Boxes represent CCAAT/enhancer elements.

sequence (T/G)TNNG(C/T)AA(T/G) (43). Gel shift assays with wild-type and mutated a1 and a3 oligonucleotide probes were used to determine if a3/EBP recognizes these elements. a3/EBP shows three protein-DNA complexes in gel shift assays with the wild-type a1 and a3 site probes (Fig. 4A), which could represent multimerization of purified a3/EBP. Binding of a3/EBP to each probe is sequence specific, as judged by gel shift competition assays with homologous or heterologous oligonucleotides (data not shown). Three-base-pair mutations (changed to different bases) in the a1 oligonucleotide CCAAT/enhancer element (M1 and M3) abolish a3/EBP binding, while the M2 mutation flanking the CCAAT/enhancer element does not affect a3/EBP binding (Fig. 4). A 3-bp mutation in the a3 oligonucleotide CCAAT/enhancer element (M4) also abolishes a3/EBP binding activity. These data indicate that a3/EBP recognizes CCAAT/enhancer elements in the a1 and a3 sites.

The gel shift binding activity of a3/EBP was compared with that of the a1/EBP-GST bacterial fusion protein and nuclear extract from bursal lymphoma cells. We previously determined that a1/EBP and B-cell nuclear extract proteins both bind the a1 CCAAT/enhancer element in gel shift assays with the wild-type a1 and M1 oligonucleotide probes (6). The purified a1/EBP-GST fusion protein gives a diffuse ladder of protein binding to the wild-type a1 oligonucleotide probe, while a single weaker gel shift complex is observed with the wild-type a3 probe (Fig. 4B). The CCAAT/enhancer element mutations M1, M3, and M4 abolish a1/EBP binding, while the flanking M2 mutation has no effect on binding. These data suggest that a1/EBP and a3/EBP can recognize the same CCAAT/enhancer elements. A similar pattern is observed with nuclear extracts from S13 bursal lymphoma cells. The S13 nuclear extract shows a diffuse ladder of binding activity with the wild-type a1 and a3 oligonucleotide probes (Fig. 4C). The M1, M3, and M4 CCAAT/enhancer element mutations greatly decrease gel shift binding to either the a1 or a3 oligonucleotide probe. In contrast, the flanking-region M2 mutation does not affect gel shift binding. These data suggest that the majority of the B-cell nuclear extract LTR-binding activity recognizes CCAAT/enhancer elements. The a1/EBP and a3/EBP factors could contribute significantly to the labile CCAAT/enhancer element binding activity of B cells. Additional minor proteins may recognize flanking LTR sequences included in the oligonucleotide probes. It is also possible that the mutations used here create novel binding sites for other factors present in the crude extract.

a3/EBP and a1/EBP bind different subsets of ALV LTR CCAAT/enhancer elements. The methylation interference technique (21) was used to further map and compare the specific nucleotides contacted by a3/EBP and a1/EBP. Initial experiments using a probe including the whole LTR showed a weak interference pattern, probably due to binding to multiple sites (data not shown). Smaller probes containing either the a1 or the a2 and a3 LTR binding sites were then prepared from plasmid constructs, as described in Materials and Methods.

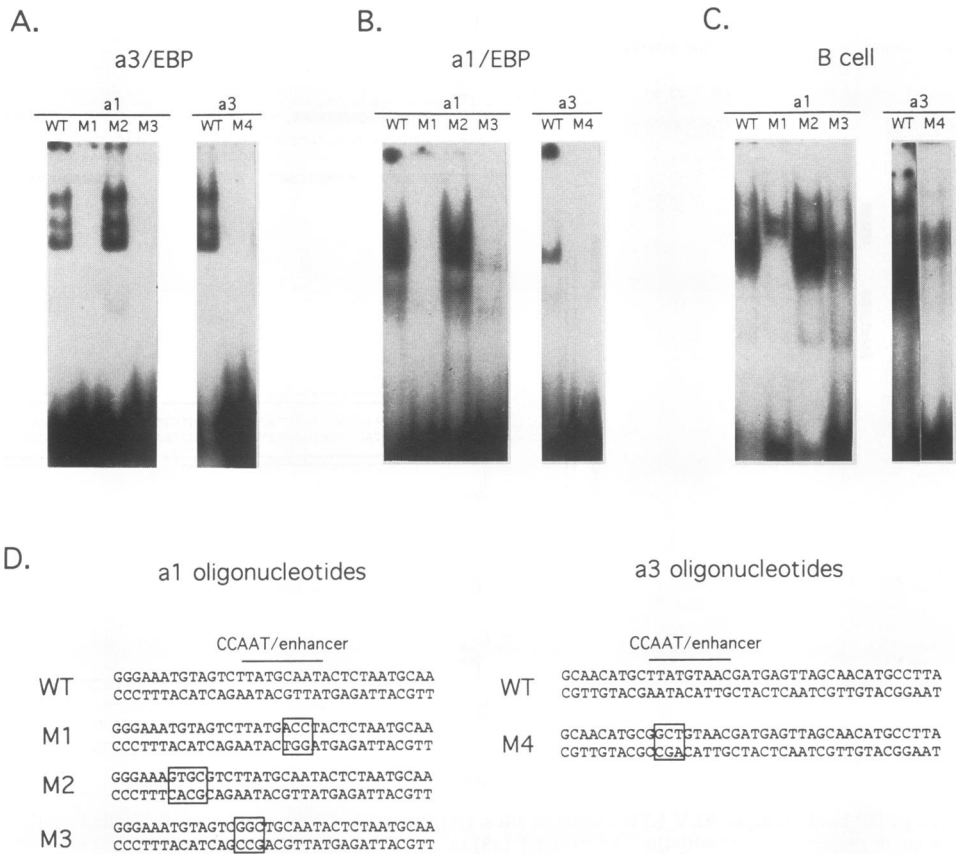

FIG. 4. Binding of a3/EBP, a1/EBP, and B-cell proteins to CCAAT/enhancer elements. The a3/EBP-GST protein (A), a1/EBP-GST protein (B), and B-cell nuclear extract (C) were incubated in gel shift assays with ^{32}P-labelled oligonucleotide probes of the a1 and a3 LTR-binding sites. (D) The probes tested for each site include the wild-type (WT) a1 oligonucleotide, the a1 CCAAT/enhancer element mutants M1 and M3, and the flanking mutant M2. The WT a3 oligonucleotide probe and the CCAAT/enhancer mutant M4 were also tested.

The end-labelled 45-bp a1 probe shows an overlapping pattern of close contact sites with a3/EBP and a1/EBP (Fig. 5). This region includes the a1 site CCAAT/enhancer element found to be important for a3/EBP and a1/EBP binding in gel shift assays (Fig. 4). The binding of both proteins is inhibited by methylation of G residues at positions −245 on the plus strand and −244 on the minus strand (Fig. 5). a3/EBP also shows interference at the G residue at positions −252, −243, and −242. This suggests that a3/EBP and a1/EBP recognize different DNA sequence motifs in this region. Interestingly, the plus-strand CCAAT/enhancer element sequence TTATGCAAT is slightly different from the overlapping and inverted minus-strand element TTGCATAAG (Fig. 5C), which could explain the distinct interference patterns observed for each protein.

a3/EBP and a1/EBP also showed differential binding to downstream sites in DNase I footprinting assays using the whole LTR enhancer as a probe (Fig. 3). a3/EBP preferentially binds the a3 site, while a1/EBP binds the a2 site. Both binding sites contain motifs that are closely related to the a1 CCAAT/enhancer element. A smaller probe containing only the a2 and a3 sites was prepared to further map the sequence elements recognized by a3/EBP and a1/EBP. DNase I footprinting experiments using this a2/a3 LTR probe demonstrate that a3/EBP preferentially binds the a3 site and a1/EBP binds the overlapping a2 site (Fig. 6). Methylation interference experiments confirm that these proteins bind different LTR sites. The a3/EBP binding shows interfering methylated G residues from positions −206 to −196, included within the a3 site protected in DNase I footprinting assays. In contrast, a1/EBP shows interference with G and A residues from −213 to −205, corresponding to the DNase I-protected a2 site. Both proteins share interference at the G residue at position −206. In summary, a3/EBP and a1/EBP bind different subsets of LTR CCAAT/enhancer elements, sharing the a1 binding site but differing in binding to the a2 and a3 LTR sites (Fig. 7).

Comparison of the CCAAT/enhancer elements in the a1, a2, and a3 sites shows that slightly different sequences are found on the plus and minus strands in each element (Fig. 7C). The a1 minus-strand and a3 minus-strand elements share a consensus sequence TT(G/A)CATAAG, which could mediate a3/EBP binding. The a1 plus-strand and a2 plus- or minus-strand elements share a consensus sequence, TN(A/T)TGCAAN, which could mediate a1/EBP binding. The sequence differences at positions 4, 5, and 6 of the CCAAT/enhancer element could mediate the preferred LTR binding pattern observed with each protein.

a3/EBP and a1/EBP bind RSV LTR CCAAT/enhancer elements. The closely related ALV and SR-A RSV LTR strains share identical LTR enhancer sequences except for two deletions in the region bound by a1/EBP and a3/EBP (5, 51). One deletion removes 13 bp between the a1 and a2 LTR sites. The other deletion removes 5 bp (CTTAT) from the a3 site CCAAT/enhancer element (Fig. 8). The effect of these deletions on the binding of a3/EBP and a1/EBP was examined by

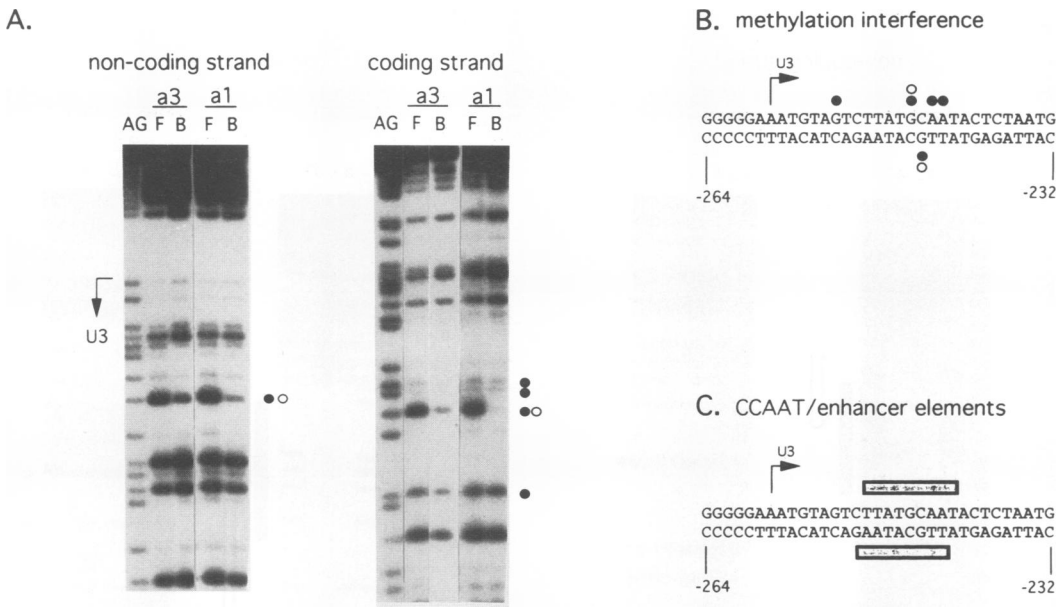

FIG. 5. Methylation interference analysis of a3/EBP and a1/EBP binding to the a1 LTR site. (A) The pBSa1 probe for the a1 region of the ALV LTR was ^{32}P end labelled on the noncoding or coding strand, partially methylated, and incubated in gel shift reactions. Free DNA (F) and DNA-protein complexes (B) were separated on polyacrylamide gels and purified for sequence gel analysis. The a3/EBP (a3) and a1/EBP (a1) interference patterns are shown. The guanine and adenine residues interfered with by a3/EBP are indicated by closed circles for a3/EBP and open circles for a1/EBP. The corresponding A+G sequence reactions (AG) are shown. (B) Sequence of the a1 site at positions −264 to −232 of the ALV LTR. The positions of the residues showing interference with a3/EBP and a1/EBP are indicated by closed and open circles, respectively. Boxes represent CCAAT/enhancer elements.

DNase I footprinting analysis. The pattern of a3/EBP binding to the SR-A RSV LTR (Fig. 8A) is very different from that observed with the ALV LTR (Fig. 3). Binding to the a1 site is maintained, but binding to the a3 site is abolished (Fig. 8B). This is probably due to the 5-bp deletion in the a3 site, which removes most of the CCAAT/enhancer element required for a3/EBP binding in gel shift assays (Fig. 4) and in methylation interference experiments (Fig. 7).

The SR-A RSV LTR deletions do not affect binding of a1/EBP to either site (Fig. 8C). The strong DNase I-hypersensitive site induced between the a1 and a2 regions is also preserved, although the sequences involved are different from those in the ALV LTR site. This suggests that the DNase I hypersensitivity induced by a1/EBP binding is not sequence specific. The 5-bp deletion in the a3 site also does not affect a1/EBP binding activity. These findings confirm that a3/EBP requires the a3 site CCAAT/enhancer element for ALV LTR binding, while a1/EBP preferentially binds the a2 element present in both the ALV and RSV LTRs. The binding of a3/EBP and a1/EBP to multiple LTR sites indicates that these proteins may be important for RSV LTR transcription enhancement.

DISCUSSION

A B-cell cDNA which encodes a protein identical to 229 amino acids of VBP has been identified. The a3/EBP cDNA encodes most of the amino-terminal region and all of the basic region and leucine zipper domains found in the VBP cDNA. We find that this truncated protein shows the same LTR-binding activity as the full-length VBP provided by John Burch (data not shown). The shared DNA sequence, mRNA hybridization pattern, and DNA-binding specificity of these factors indicate that a3/EBP is a partial cDNA copy of the VBP mRNA, and so a3/EBP will be referred to as VBP in this discussion. The 5′ and 3′ a3/EBP sequences have not been identified, although they could be identical to those of the VBP cDNA. It is also possible that the full-length a3/EBP cDNA encodes a VBP transcript with alternatively spliced 5′- or 3′-terminal sequences. The multiple VBP mRNAs observed in B cells and other cell types suggest that the VBP mRNA transcript could be differentially spliced or processed.

VBP binds two large regions extending over 70 bp of the ALV LTR enhancer. The recognition sequences in the a1 and a3 sites are CCAAT/enhancer elements, as shown by several approaches. Gel shift assays with mutated oligonucleotide probes indicate that the CCAAT/enhancer element in each site is required for VBP binding. These elements are also identified by DNase I protection and methylation interference assays. The SR-A strain of RSV shows a 5-bp deletion in the a3 site CCAAT/enhancer element, which abolishes VBP binding. Taken together, these data identify two LTR CCAAT/enhancer elements recognized by VBP. Comparison of the a1 and a3 binding sites indicates a shared consensus sequence, TT(G/A)CATAAG, on the minus strand of the a1 site and the minus strand of the a3 site. This inverted CCAAT/enhancer element sequence is somewhat related to the vitellogenin gene promoter element (TTTATGTAAAC) originally used to identify VBP, and to the rat albumin promoter element sequence (TTTTGTAAT) which VBP also binds (23).

a1/EBP was previously identified as a novel leucine zipper factor which binds multiple LTR CCAAT/enhancer elements (6). The LTR-binding pattern of a1/EBP is distinct from the pattern of VBP binding. Gel shift assays with mutated oligonucleotide probes indicate that a1/EBP and a3/EBP both can recognize the a1 and a3 CCAAT/enhancer elements. However, DNase I footprinting experiments with the whole LTR enhancer show that while VBP binds the a1 and a3 sites, a1/EBP

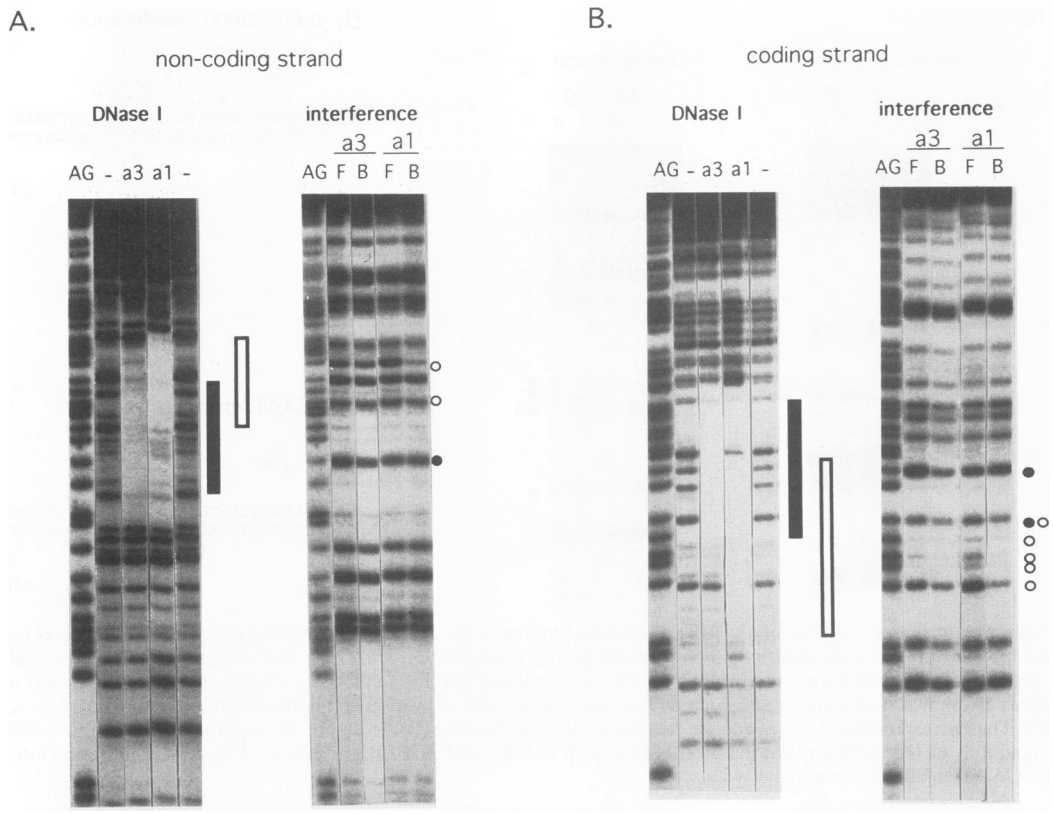

FIG. 6. DNase I footprinting and methylation interference analysis of a3/EBP and a1/EBP binding to the a2/a3 LTR region. The pBSa2/a3 clone for the a2/a3 ALV LTR region was ^{32}P end labelled on the noncoding (A) or coding (B) strand and used in DNase I footprinting reactions (DNase I) with a3/EBP or a1/EBP. The probe was partially methylated and used in methylation interference assays (interference). Gel lanes and symbols are as described in the legend to Fig. 5.

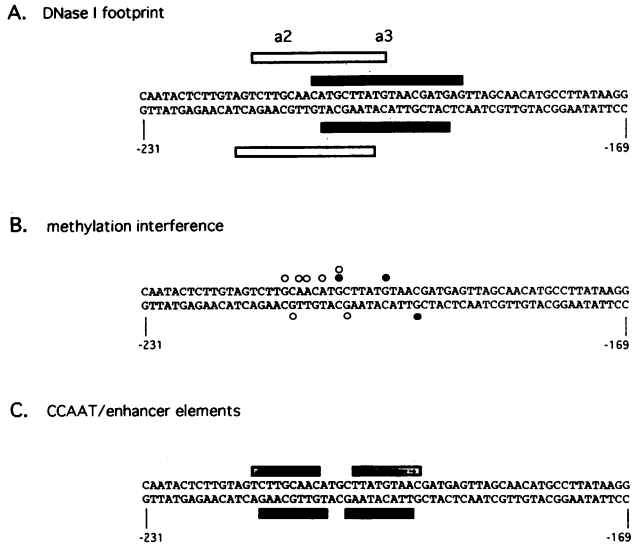

FIG. 7. Summary of a3/EBP and a1/EBP binding to the a2/a3 LTR region. (A) The a3 and a2 DNase I-protected sites of a3/EBP (closed bars) and a1/EBP (open bars). (B) Methylation interference pattern of a3/EBP (closed circles) and a1/EBP (open circles). (C) CCAAT/enhancer elements (boxes).

preferentially binds the a1 and a2 CCAAT/enhancer elements. Methylation interference experiments demonstrate that the DNA contacts of each protein also differ within each LTR CCAAT/enhancer element. Comparison of the CCAAT/enhancer element sequences in the a1, a2, and a3 sites indicates that the binding specificity of these proteins could be determined by small differences in the recognition sequence. VBP could bind the consensus sequence TT(G/A)CATAAG on the minus strand of the a1 and a3 sites, while a1/EBP could preferentially recognize the consensus TN(A/T)TGCAAN on the plus strand of the a1 site and either strand of the a2 site. The a1/EBP consensus matches the C/EBP consensus derived by using a number of avian retroviral LTR sequences (43). The major difference between the VBP and a1/EBP CCAAT/enhancer elements is in positions 4, 5, and 6 (CAT versus TGC). These proteins show additional differences in their DNase I protection patterns, as a1/EBP binding induces a strong DNase I-hypersensitive site while VBP does not. We do not yet know if a1/EBP and VBP could recognize these elements as heterodimers, although there appears to be very little heterodimer formation between VBP and other leucine zipper factors (23, 53).

Comparison of the VBP and a1/EBP basic regions identifies residues that could mediate CCAAT/enhancer element binding. The observation that VBP and a1/EBP bind similar LTR CCAAT/enhancer elements was surprising, as these are very different members of the leucine zipper family of transcription factors. Comparison of the basic DNA binding regions of these proteins indicates that their primary sequences are only about

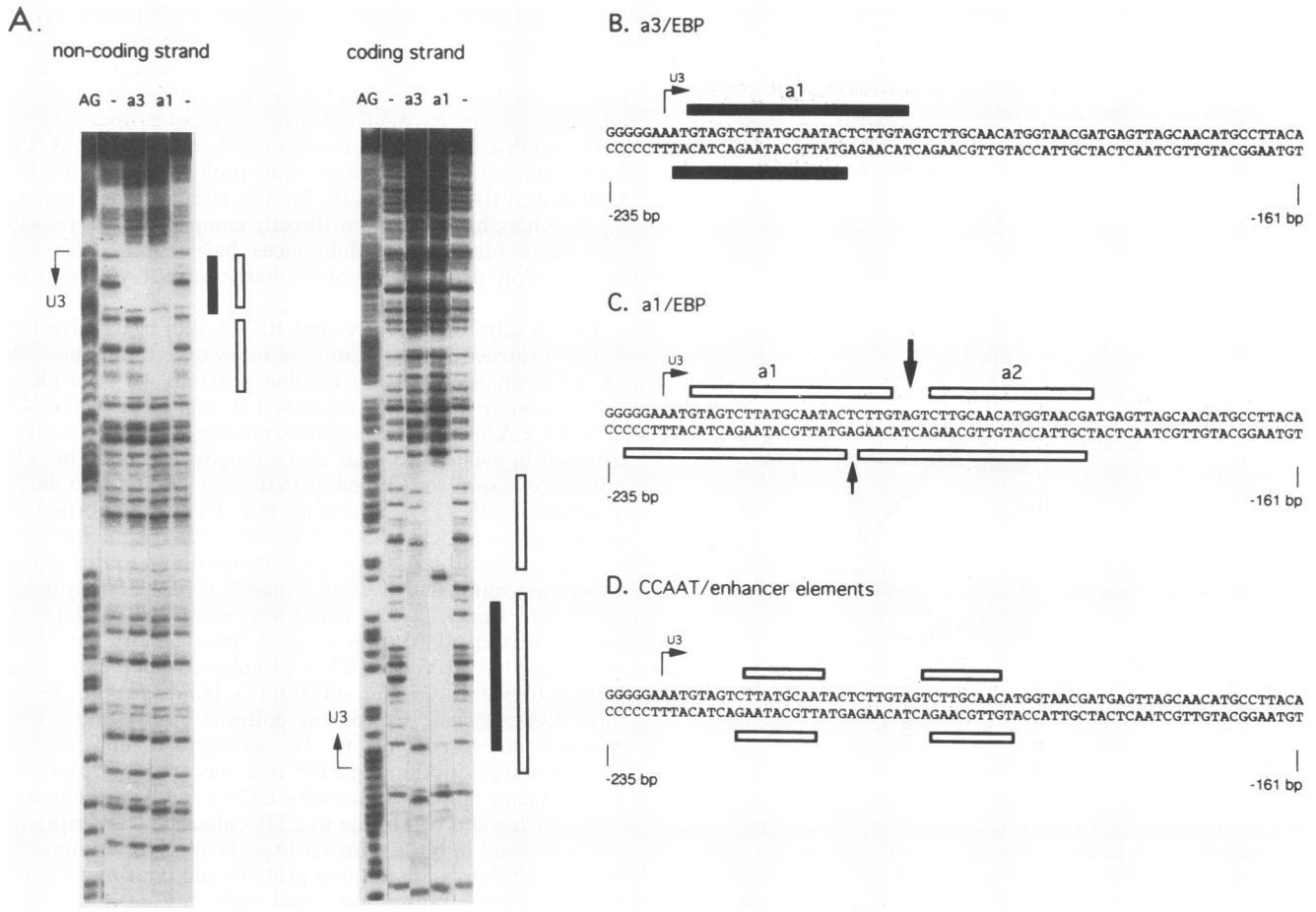

FIG. 8. DNase I footprinting analysis of a3/EBP and a1/EBP binding to the SR-A RSV LTR. (A) The RSV LTR probe was ^{32}P labelled on the coding or noncoding strand and incubated alone (−) or with the a3/EBP-GST (a3) or a1/EBP-GST (a1) fusion proteins. Bars indicate regions of DNase I protection. The Maxam-Gilbert sequence (AG) is shown. The 5′ end of the U3 LTR sequence is indicated. (B) a3/EBP binding sites. (C) a1/EBP binding sites. (D) CCAAT/enhancer elements (boxes).

30% identical (Fig. 9A). C/EBP-α also binds the RSV LTR CCAAT/enhancer elements (43), even though it is only 30% identical to VBP and 64% identical to a1/EBP (data not shown). The conserved amino acids in VBP and a1/EBP correspond to most of the basic-region residues conserved within the leucine zipper family of transcription factors (Fig. 9A). The basic regions of C/EBP-α and GCN4 have been most intensively characterized, and the residues important for DNA recognition have been identified by a variety of approaches. The basic region of these dimerized proteins binds each half of the CCAAT/enhancer element as an alpha helix that tracks along the major groove of the DNA (13, 35, 36, 55). A small number of residues aligned on one face of the alpha helix mediate recognition of specific nucleotides in the CCAAT/enhancer element (24, 50).

Projection of the VBP and a1/EBP basic regions onto an alpha-helical wheel model (assuming 3.5 residues per helical turn [24, 36]) gives insight into their recognition of similar but distinct LTR CCAAT/enhancer elements. Residues that are conserved in both proteins are clustered on the adjacent spokes numbered 1, 2, and 4 (Fig. 9B). These conserved residues could mediate the shared recognition of CCAAT/enhancer element sequences, as these spokes of the helix are directly involved in DNA recognition (13, 24). Four of the five residues found to be important for C/EBP-α recognition of CCAAT/enhancer element sequences are conserved in VBP and a1/EBP at positions 16, 19, 23, and 24 (Fig. 9B). Interestingly, VBP and a1/EBP differ at residue 20, showing alanine for VBP and valine for a1/EBP. This residue is especially important for distinguishing the DNA-binding specificity of GCN4 and C/EBP-α (24, 50). The presence of alanine at position 20 in GCN4 mediates recognition of a thymidine residue (AT GAGTCAT), while valine at position 20 in C/EBP-α determines recognition of a cytidine residue (ATTGCGCAAT). The alanine residue at position 20 could mediate VBP binding to the thymidine residue [TT(G/A)CATAAG] in the CCAAT/enhancer element, while the valine residue in a1/EBP could mediate binding to the cytidine residue in the TN(A/T)TG CAAN element. The VBP and a1/EBP basic-region sequences show a number of additional differences on this face of the helix (Fig. 9B) which could contribute to the differential recognition of LTR CCAAT/enhancer sequences by each protein.

VBP and a1/EBP may encode the labile LTR-binding proteins in B cells. The susceptibility of immature B cells to ALV lymphoma appears to be regulated by widely expressed LTR-binding factors that are specifically labile in pre-B target cells while stable in other cell types. The labile factors were partially

A.

```
VBP      PDEQKDEKYWTRRKKNNVAAKRSRDARRLKENQ
                |  ||  ||  |  |  ||      |
a1/EBP   FVDRNSDEYRQRRERNNMAVKKSRLKSKQKAQD

basic motif          BB-BN--AA-B-R-BB

         1                                33
```

B.

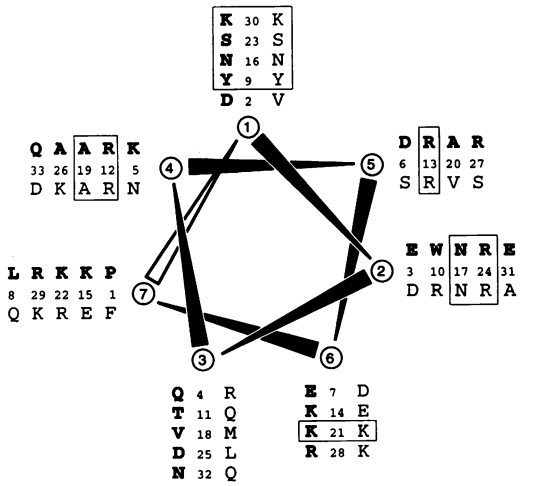

FIG. 9. VBP and a1/EBP share basic-region amino acid residues that could mediate CCAAT/enhancer binding. (A) Primary amino acid sequence alignment of the VBP basic region (amino acids [aa] 238 to 270) with the a1/EBP basic region (aa 116 to 148). The conserved basic motif residues (A, acidic; B, basic; N, neutral) are indicated. (B) Projection of the VBP (bold letters) and a1/EBP basic regions on an alpha-helical wheel model of 3.5 aa per turn. Boxed residues are shared between VBP and a1/EBP.

purified from B-lymphoma cells by several chromatography steps and separated into activities preferentially binding the a1, a2, and a3 LTR sites (6, 41). These labile protein-binding sites correspond to those recognized by VBP and a1/EBP. Gel shift comparison of the effects of the mutated a1 and a3 oligonucleotide probes on VBP, a1/EBP, and B-cell protein binding show that these proteins recognize similar LTR CCAAT/enhancer elements. Most but not all of the B-cell binding to the a1 and a3 oligonucleotide probes is abolished by mutations in the CCAAT/enhancer elements, indicating that the majority of the labile LTR-binding activity recognizes these elements. These data suggest that VBP and a1/EBP encode the labile LTR-binding proteins of B cells.

VBP and a1/EBP together bind a large region of the ALV and RSV LTR enhancers. Transfection studies using LTR enhancer deletion constructs have shown that this region is required for RSV LTR transcription enhancement in avian embryo fibroblasts (9, 27, 34). Moreover, the endogenous avian retroviruses show deletion of the a1, a2, and a3 CCAAT/enhancer elements and are transcriptionally inactive (20, 58). Ryden et al. (44) found that point mutations within the a1 CCAAT/enhancer element decrease RSV LTR-driven transcription roughly 40% in CEF transfection experiments. Mutation of the a2 and a3 CCAAT/enhancer elements also reduced LTR-driven transcription. Sears and Sealy (47) found that the concatemerized a1 CCAAT/enhancer element is a strong transcription enhancer in CEF transfection experiments. These data support the hypothesis that binding of VBP and a1/EBP to CCAAT/enhancer elements regulates the high-level transcription activity of the ALV and RSV LTR enhancers. The a3 site CCAAT/enhancer element is found in the ALV and Pr-C RSV LTRs, while it is deleted in the SR-A RSV LTR enhancer (5). All of these viral strains show high levels of LTR-driven transcription (10, 34, 42), although their enhancing activities have not been directly compared. It is possible that these binding site differences have subtle effects on transcription or in oncogenesis that have not yet been detected.

The closely related ALV and RSV LTRs both drive high levels of retroviral transcription in many different tissues. The CCAAT/enhancer element binding activities we have identified appear to be widely expressed in many cell types (42). Similar CCAAT/enhancer element binding activities have been detected in avian fibroblasts and embryos (26, 47). The VBP mRNA is expressed in many tissues in addition to bursal lymphoma cells (23), suggesting that VBP is important for mediating the widespread high levels of RSV LTR transcription. The a1/EBP mRNA could also contribute to this ubiquitous transcription activity. Other members of the family of 5 to 10 leucine zipper factors could also contribute to LTR-enhanced transcription, as several of these factors have been shown to bind RSV LTR CCAAT/enhancer elements. These include C/EBP-α (25) and NF/IL6 (1). However, both factors show tissue-specific expression patterns (1, 4) and so are probably not responsible for the widespread high levels of retroviral transcription. Ig/EBP is a more widely expressed murine factor that also binds RSV CCAAT/enhancer elements (40), which could contribute to LTR-enhanced transcription if it is expressed in birds. Further experiments will examine the lability and expression of these proteins and their role in labile LTR-enhanced transcription and induction of B-cell lymphoma.

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