JOURNAL OF VIROLOGY, Aug. 1997, p. 5972–5981 Vol. 71, No. 8
0022-538X/97/$04.00+0

VBP and RelA Regulate Avian Leukosis Virus Long Terminal Repeat-Enhanced Transcription in B Cells

SHEILA M. CURRISTIN, KELLY J. BIRD, ROBERT J. TUBBS, AND ALANNA RUDDELL*

Department of Microbiology and Immunology and Cancer Center, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

Received 13 February 1997/Accepted 6 May 1997

The avian leukosis virus (ALV) long terminal repeat (LTR) contains a compact transcription enhancer that is active in many cell types. A major feature of the enhancer is multiple CCAAT/enhancer element motifs that could be important for the strong transcriptional activity of this unit. The contributions of the three CCAAT/enhancer elements to LTR function were examined in B cells, as this cell type is targeted for ALV tumor induction following integration of LTR sequences next to the c-*myc* proto-oncogene. One CCAAT/enhancer element, termed a3, was found to be the most critical for LTR enhancement in transiently transfected B lymphoma cells, while in chicken embryo fibroblasts all three elements contributed equally to enhancement. Gel shift assays demonstrated that vitellogenin gene-binding protein (VBP), a member of the PAR subfamily of C/EBP factors, is a major component of the nuclear proteins binding to the a3 CCAAT/enhancer element. VBP activated transcription through the a3 CCAAT/enhancer element, supporting the idea that VBP is important for LTR enhancement in B cells. A member of the Rel family of proteins was also identified as a component of the a3 protein binding complex in B cells. Gel shift and immunoprecipitation assays indicated that this factor is RelA. Gel shift assays demonstrated that while RelA does not bind directly to the LTR CCAAT/enhancer elements, it does interact with VBP to potentiate VBP DNA binding activity. The synergistic interaction of VBP and RelA increased CCAAT/enhancer element-mediated transcription, indicating that both factors may be important for viral LTR regulation and also for expression of many cellular genes.

The avian leukosis virus (ALV) long terminal repeat (LTR) contains compact enhancer and promoter sequences which form a powerful transcription unit in many cell types (42, 53). The enhancer region is important for high rates of LTR-driven transcription in fibroblasts (19, 47) and is essential for hyperexpression of the c-*myc* oncogene during ALV induction of bursal lymphomas in chickens (52). ALV infects and produces a high level of viral expression in many tissues (56), but the tumors that develop are bursal in origin, suggesting that cell-specific host factors are important in tumor induction. Labile transcription factors appear to regulate c-*myc* hyperexpression in B lymphoma cells, as LTR-enhanced c-*myc* and viral transcription is specifically reduced after inhibition of protein synthesis (40), whereas LTR-enhanced transcription is stable in infected T cells or chicken embryo fibroblasts (CEF). Moreover, this lability is restricted to immature B cells of lymphoma-susceptible chicken strains, while LTR-enhanced transcription is stable in all tissues of lymphoma-resistant chicken strains (56). We have proposed that differential regulation of LTR-enhanced c-*myc* transcription by labile factors could be important in targeting immature B cells for ALV tumor induction (11).

A number of protein binding sequences have been identified in the ALV and closely related Rous sarcoma virus (RSV) LTR enhancers (reviewed in reference 55). The RSV strain Pr-RSV-C and SR-RSV-A sequences show a few small deletions and several point mutations relative to the ALV LTR sequence; however, all strains produce high levels of viral transcription, suggesting that these sequence differences do not appreciably influence LTR function. The LTR enhancer region consists of multiple repeats of closely related CCAAT/enhancer elements, T(T/G)TNNG(C/T)AA(T/G), with four in Pr-RSV-C, three in ALV, and two in SR-RSV-A LTRs (59, 65). Transfection studies in CEF have shown that the CCAAT/enhancer motifs contribute to RSV LTR enhancer function (59).

Protein activities that bind to the LTR CCAAT/enhancer motifs have been identified in several avian cell types, and these activities were named a1 and a3 (57), FI and FIII (25), E2BP (35), and EFII (61). The CCAAT/enhancer-binding protein (C/EBP) family of transcription factors includes several proteins that are characterized by a highly conserved C-terminal basic region and leucine zipper domain, which mediate DNA binding and dimerization, respectively (reviewed in references 2 and 48). The first member of the C/EBP family to be cloned was C/EBPα (34, 37), and it was later demonstrated that purified C/EBPα can bind to all four CCAAT/enhancer elements in the Pr-RSV-C LTR (58). C/EBPγ, also known as Ig/EBP, was also shown to bind the RSV LTR CCAAT/enhancer elements (54). C/EBPβ (14), also known as NF-IL6 (1), was identified as a component of the EFII complex of fibroblasts, which binds to the two CCAAT/enhancer elements in the SR-RSV-A LTR (62, 73).

We previously identified two C/EBP-related factors, VBP (vitellogenin gene-binding protein) and A1/EBP, by a B-cell cDNA expression library screen for proteins binding to the a1 and a3 LTR CCAAT/enhancer motifs (12, 65). A1/EBP is a 40-kDa ubiquitously expressed protein related to C/EBPγ, which interacts with a 70-kDa Rel-related protein in B cells (5, 10). The NF-κB/Rel transcription factors are a family of DNA-binding proteins that regulate many cellular genes, including those involved in immunoregulatory responses and cell growth control (reviewed in reference 4). VBP/TEF is a member of the PAR (proline- and acidic amino acid-rich) subfamily of

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Box 672, University of Rochester School of Medicine and Dentistry, 601 Elmwood Ave., Rochester, NY 14642. Phone: (716) 275-6916. Fax: (716) 473-9573. E-mail: aruddell@medinfo.rochester.edu.

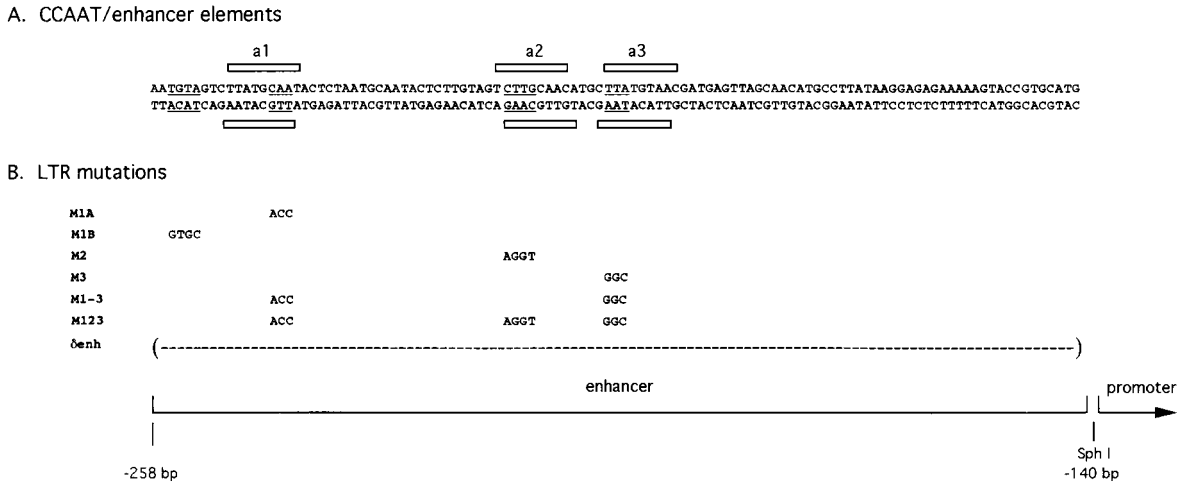

FIG. 1. Mutagenesis of LTR enhancer sequences. (A) Sequence of the ALV U3 LTR enhancer. The a1, a2, and a3 CCAAT/enhancer elements on each strand (boxes) and mutagenized sequences (underlined) are indicated. (B) Base change mutations introduced into the LTR enhancer by site-directed mutagenesis. The mutated sense strand is indicated. The enhancer sequences deleted in the Δenh-LUC construct (dashed line) and the distance from the transcription start site at the U3-R border (in base pairs) are shown.

C/EBP factors (22, 33, 64). The members of this family include rat albumin D-site-binding protein (43) and human hepatic leukemia factor (30, 32). All PAR proteins have high sequence similarity (65 to 73%) and share an auxiliary DNA-binding domain, the PAR region, located upstream of the basic region (22, 26).

Proteins binding to the ALV CCAAT/enhancer elements account for most of the labile LTR enhancer-binding activity of B cells (57). The a1 and a3 protein binding complexes exhibit B-cell-specific lability, as these binding activities disappear from extracts treated with protein synthesis inhibitors, whereas they are stable in fibroblasts and T cells. These findings suggest that the a1 and a3 CCAAT/enhancer element-binding proteins are important for labile regulation of transcription in B cells and stable transcription in other cell types. Mapping analysis determined that A1/EBP binds the a1 and a2 elements [TN(A/T)TGCAAN], while VBP binds the a1 and a3 motifs [TT(G/A)CATAAG], due to amino acid differences in the DNA-binding basic region of each protein (65).

In this study, we tested the contributions of the three CCAAT/enhancer elements to ALV LTR enhancer function in B cells and CEF. We find that the a3 CCAAT/enhancer element is the most critical for enhancer function in DT40 B cells, while all three elements contribute equally in CEF. The αβ isoform of VBP was identified as the a3-specific binding protein and was shown to transactivate CCAAT/enhancer element-mediated transcription. Interestingly, we found that RelA is a component of the a3 LTR binding complex and that RelA enhanced both VBP LTR binding activity and VBP transactivation of CCAAT/enhancer element-mediated transcription. These results suggest that VBP may be responsible for high levels of LTR-enhanced expression of the c-*myc* gene in B lymphoma cells and that Rel factors may contribute to the regulation of LTR-enhanced transcription via an interaction with VBP.

MATERIALS AND METHODS

Cell culture. DT40 (3) and S13 (28) bursal lymphoma cells and primary CEF were cultured as previously described (56).

Cell transfection and luciferase assay. The wild-type and mutated ALV-LTR reporter plasmids and the ALV promoter reporter plasmid were described previously (10). Briefly, the RAV-2 ALV LTR (60) was cloned upstream of the luciferase gene in the pXP1 expression vector (45). Site-directed mutagenesis of the CCAAT/enhancer elements was achieved by PCR amplification (38). The reporter plasmid $p(a3)_8$-LUC was generated by cloning eight concatemerized a3 sequence oligonucleotides (57) upstream of a minimal TATA-Inr sequence in the pGL2 luciferase vector, provided by M. Hannink (21). The VBP expression construct was generated by cloning the full-length VBP cDNA (provided by J. Burch) downstream of the cytomegalovirus (CMV) promoter in the pcDNA1 vector (Invitrogen). The RelA expression vector was generated by cloning the human cDNA from pBluescript-RelA (AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health; pBluescript-RelA [p65] from G. Nabel and N. Perkins) into pcDNA1. In some experiments, 1.5×10^7 DT40 cells were transfected by the electroporation method (16) and assayed 18 h later for luciferase activity (20), or 8×10^5 DT40 cells were transfected by a DEAE-dextran method (29) and assayed 48 h later for luciferase activity. A cotransfected CMV–β-galactosidase reporter plasmid (provided by A. Geballe) was assayed as described previously (46).

Preparation of VBP antiserum. Glutathione *S*-transferase (GST)-VBP was produced in bacteria from a partial cDNA clone (amino acids [aa] 84 to 313) as described previously (65). Glutathione agarose-purified protein was used for immunization of rabbits (East Acre Biologicals, Inc.). The resulting polyclonal antiserum was ammonium sulfate precipitated (27), precleared through a CNBr-activated Sepharose 4B (Pharmacia) column of GST protein and *Escherichia coli* lysates, and concentrated by Centricon-30 filter centrifugation (Amicon, Inc.).

Protein purification and supershift assays. Nuclear extracts were prepared from S13 cells by 0.5 M NaCl extraction of purified nuclei (56). GST-VBP and GST-RelA (expression vector was provided by M. Hannink) were purified by glutathione agarose chromatography (66). GST-E1 was provided by D. McCance (71). The R2 antiserum (provided by M. Hannink) was raised against the Rel homology domain (aa 92 to 456) of v-Rel (21). The anti-RelA serum (provided by H. Bose) was raised against chicken RelA (aa 506 to 521). The sequences of the a3, a1, and EF1 oligonucleotide probes and the gel shift assay protocol were described previously (10, 65).

Reverse transcription-PCR analysis. $Poly(A)^+$ RNA was isolated from DT40 bursal lymphoma cells by the guanidinium thiocyanate method (15) followed by oligo(dT)-cellulose chromatography (Bethesda Research Laboratories). cDNA was synthesized by reverse transcription of 1 μg of $poly(A)^+$ RNA, 50 pmol of random hexamers, and 200 U of Superscriptase II (Bethesda Research Laboratories) for 60 min at 42°C. cDNA synthesis was confirmed by PCR analysis using a 2-μl aliquot and 50 pmol of internal-control PCR primers that were made against VBP exon 2-exon 3 (13). The conditions used to PCR amplify the four specific VBP transcripts were as follows: 30 cycles of 1 min at 95°C, 1 min at 62°C, and 1 min at 72°C, followed by 10 min at 72°C. The sense primers for this analysis were specific for either alpha or beta first exons (13). The antisense primers were specific for alpha exon 4 (TCTGAGAGTCTCTACAGAAGGC) or beta exon 5 (TCACTCGGAATCAGATAAGTCA). Aliquots (10 μl) were resolved on 1% Tris-acetate-EDTA (TAE) agarose gels.

Immunoblot and immunoprecipitation analysis. For Western blot (immunoblot) analysis, a 50-μl reaction mixture of in vitro-transcribed and -translated VBP was acetone precipitated and resuspended in 50 μl of sodium dodecyl sulfate (SDS) sample buffer. One-tenth of the reaction mixture and 10 μg of S13 bursal lymphoma cell lysates or nuclear extracts were separated on an SDS–10%

polyacrylamide gel. The gel was electroblotted and analyzed with anti-VBP (4 mg/ml) and alkaline phosphatase-conjugated secondary goat anti-rabbit antibody (Bio-Rad).

For immunoprecipitation analysis, 10^7 S13 cells were metabolically labelled in Dulbecco modified Eagle medium minus methionine and cysteine and with 340 μCi of [^{35}S]methionine-[^{35}S]cysteine (DuPont NEN). The cells were lysed in Triton X-100 buffer (1% Triton X-100, 25 mM Tris [pH 7.5], and 150 mM NaCl) on ice for 20 min. After lysis, the lysates were precleared with protein A-Sepharose beads at 4°C for 20 min. Samples (250 μl) were then incubated with 25 μg of appropriate antiserum and incubated at 4°C for 1 h. Complexes were precipitated with protein A beads at 4°C for 20 min. The beads were washed in radioimmunoprecipitation assay buffer and resuspended in SDS sample buffer. Proteins were resolved on SDS–10% polyacrylamide gels, fluorographed, and autoradiographed.

For coimmunoprecipitation experiments 2×10^7 cells were lysed in low-stringency buffer (phosphate-buffered saline and 1% Nonidet P-40) at 4°C for 2 h. After preclearing, the 250-μl lysates were incubated overnight at 4°C with the appropriate antisera. Complexes were precipitated with protein A beads at 4°C for 20 min. The beads were washed in Triton X-100 buffer and resuspended in SDS sample buffer. Proteins were resolved on SDS–10% polyacrylamide gels. After electroblotting, the samples were analyzed with anti-RelA and horseradish peroxidase-conjugated secondary goat anti-rabbit antibody, and the proteins were detected by using the enhanced chemiluminescence (ECL) Western blotting system (Amersham).

RESULTS

Previous studies of RSV LTR enhancer function demonstrated that all of the LTR CCAAT/enhancer motifs contribute to LTR-enhanced transcription in transiently transfected fibroblasts (59). We were interested in analyzing the contribution of the CCAAT/enhancer motifs to ALV LTR-enhanced transcription in B cells, as we have proposed that labile proteins binding these sites are important for regulation of labile LTR-enhanced transcription and ALV tumor induction in immature bursal cells (57). The ALV LTR enhancer region and the a1, a2, and a3 CCAAT/enhancer elements are depicted in Fig. 1A. The transcription-enhancing activity of these CCAAT/enhancer elements was analyzed by transient-transfection assays using wild-type or mutated LTR-luciferase reporter gene constructs. Three or four base pair changes were introduced into each of the motifs by amplification of the left and right halves of the LTR with primers carrying the mutated sequences shown in Fig. 1B, followed by amplification of the whole LTR with flanking primers. The amplified RAV-2 enhancer and promoter sequences (U3, R, and U5; bp −258 to +99) were cloned in the sense orientation upstream of the luciferase gene in pXP1 luciferase reporter plasmid. The M1A and M3 CCAAT/enhancer element mutations were chosen because they greatly decrease the binding activity of B-cell nuclear proteins in oligonucleotide gel shift assays, while the M1B mutation flanking the 5′ a1 CCAAT/enhancer element does not affect protein binding (65).

The a3 CCAAT/enhancer element mediates LTR-enhanced transcription in B cells. The wild-type or mutated LTR-luciferase constructs were transfected into the avian DT40 ALV bursal lymphoma cell line, and luciferase activity was assayed 18 h later. In some experiments, an internal control CMV promoter β-galactosidase expression plasmid was cotransfected and assayed by using a colorimetric assay (46). Similar results were obtained whether or not luciferase activity was expressed relative to β-galactosidase activity (data not shown). The M3 mutation in the a3 CCAAT/enhancer element produced the strongest effect on luciferase expression in DT40 B cells, showing a statistically significant 57% decrease in activity relative to that of the wild-type LTR construct, which was arbitrarily set at 100% (Fig. 2A). The M2 mutation in the a2 site reduced luciferase activity by 36%, although this decrease is not statistically significant. The M1A mutation showed only a slight decrease in luciferase activity, even though this mutation greatly decreased the binding of cellular factors to the a1 CCAAT/enhancer element in vitro (65). Similar results were obtained with a construct mutated in the 5′ half of the a1 CCAAT/enhancer element (data not shown). The M1B mutation flanking the a1 CCAAT/enhancer element also did not influence luciferase expression (Fig. 2A).

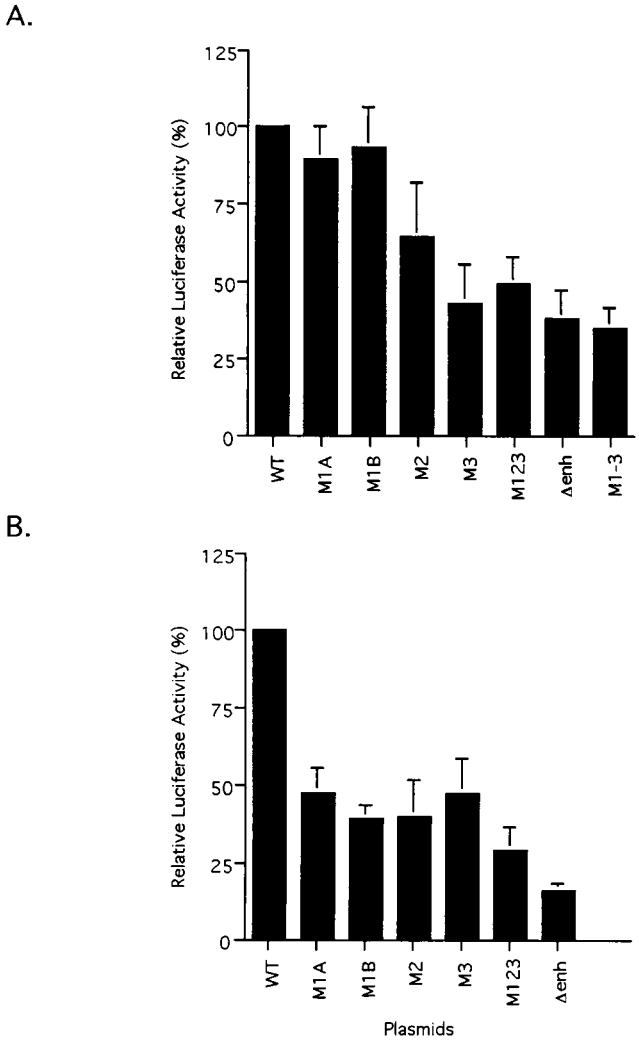

FIG. 2. Activity of LTR-luciferase constructs in B cells and CEF. (A) DT40 bursal lymphoma cells (1.5×10^7) were transfected by electroporation and assayed 18 h later. Luciferase activities of mutated LTR-luciferase constructs (5 μg) are expressed as percentages relative to that of the wild-type (WT) LTR-luciferase constructs (5 μg). The data are averaged from five to eight experiments, and standard errors are indicated. (B) Luciferase activity of LTR-luciferase constructs in CEF.

The LTR a1, a2, and a3 CCAAT/enhancer motifs could cooperate to drive high rates of LTR-enhanced transcription. This possibility was tested by using luciferase constructs with combinations of mutations in these sites (Fig. 1B). The M1-3 and the M123 constructs decreased luciferase activity by 65 and 51%, respectively, to a level similar to that observed with the single M3 mutation (Fig. 2A). These data indicate that the three CCAAT/enhancer motifs do not work cooperatively in B lymphoma cells. The finding that mutation of all three LTR CCAAT/enhancer elements decreases luciferase activity by only 60% suggests that other enhancer elements could contribute to LTR activity. The potential involvement of other enhancer sequences was analyzed by using an LTR-luciferase

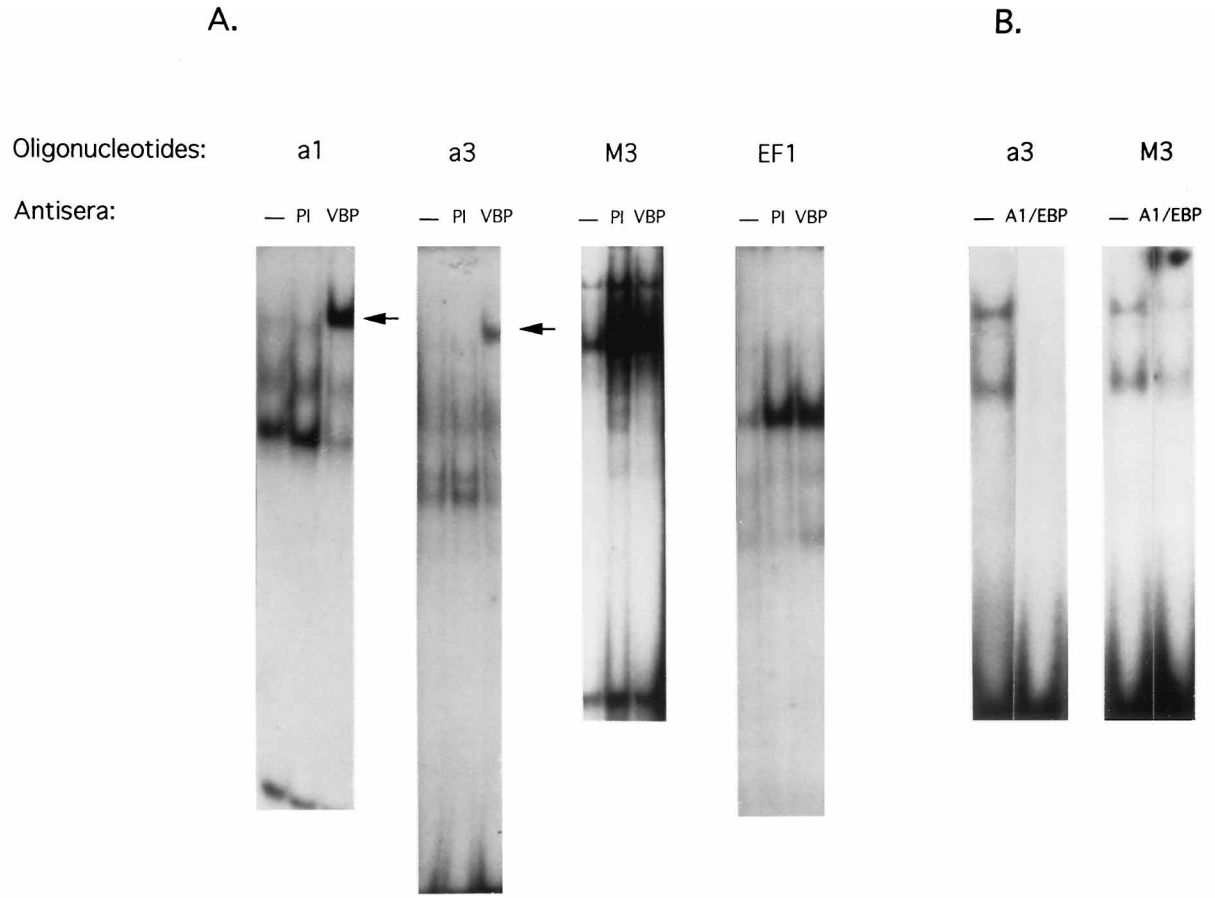

FIG. 3. VBP is a component of the a1 and a3 LTR CCAAT/enhancer element binding complexes. (A) Nuclear extracts from B lymphoma cells were incubated in a gel shift assay with ^{32}P-labelled a1, a3, M3, or EF1 LTR binding site probe alone (—), with preimmune serum (PI), or with VBP antiserum (VBP). Samples were resolved on a native Tris-borate-EDTA–5% polyacrylamide gel. Supershift complexes (arrows) are indicated. (B) Nuclear extracts from B lymphoma cells were incubated in a gel shift assay with ^{32}P-labelled a3 or M3 LTR binding site probe alone (—) or with A1/EBP antiserum (A1/EBP). Samples were resolved on a native TAE–5% polyacrylamide gel.

construct with the enhancer region deleted but retaining the promoter, R, and U5 sequences (Fig. 1B). This Δenh-LUC construct showed a 62% decrease in luciferase activity, similar to the reduction observed with the M3 construct (Fig. 2A). These data demonstrate that the a3 CCAAT/enhancer element is the most critical element for LTR enhancer function in B lymphoma cells, while the a1 and a2 elements make minor contributions. The promoter region also contributes to LTR function, as the Δenh-LUC construct showed considerable activity in the absence of the enhancer (Fig. 2A). The promoter sequences involved are located in the region from bp −140 to −50 relative to the transcription start site, as a luciferase construct with further deletion of these sequences is inactive (data not shown).

These observations were surprising, as similar mutations of the corresponding Pr-RSV-C LTR CCAAT/enhancer elements all decreased LTR-driven transcription in CEF (59). Moreover, previous studies have shown that the RSV LTR enhancer supplies approximately 90% of the LTR transcription activity in avian or mammalian fibroblasts (18, 47). This could reflect differences in the cell types analyzed or sequence differences between the closely related ALV and RSV LTRs (9). These possibilities were analyzed by comparing the activities of the wild-type and mutated ALV LTR constructs in CEF. The single M1A, M2, and M3 mutations each decreased luciferase activity by more than 50% (Fig. 2B), confirming that all of these sites, like the RSV elements, contribute equally to ALV LTR enhancer function in fibroblasts. The M123-LUC and the Δenh-LUC constructs showed 71 and 84% decreases in activity, respectively, indicating that the ALV LTR-CCAAT/enhancer elements are essential for enhancer function in CEF. Surprisingly, the M1B-LUC construct showed a 61% reduction in luciferase activity in CEF (Fig. 2B), while this construct showed wild-type activity in DT40 B cells (Fig. 2A). These findings indicate that different combinations of LTR sequence elements contribute to the high rate of LTR-enhanced transcription in different cell types, with the a3 CCAAT/enhancer element being the most critical element in lymphoma B cells.

VBP is a major component of the a3 LTR CCAAT/enhancer element binding activity. The in vitro binding of the A1/EBP and VBP C/EBP-related transcription factors to the ALV LTR was previously characterized by using GST fusion proteins isolated from bacteria. A GST-A1/EBP fusion protein binds to the a1 and a2 CCAAT/enhancer elements (12), while GST-VBP binds to the a1 and a3 CCAAT/enhancer elements (65). These findings suggest that VBP, but not A1/EBP, is responsible for binding to the a3 CCAAT/enhancer element that is critical for LTR enhancer function in B cells. An antiserum was raised against a GST-VBP (aa 84 to 313) fusion protein to

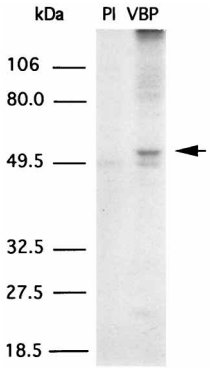

FIG. 4. A 50-kDa VBP is expressed in B cells. S13 cells were metabolically labelled, lysed, precleared, and incubated with either preimmune serum (PI) or with VBP antiserum (VBP) and protein A beads. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The migration of the 50-kDa VBP (arrow) is indicated.

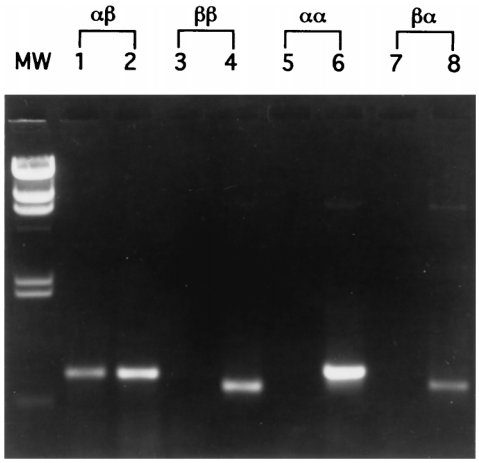

FIG. 5. VBPαβ is the single isoform expressed in B cells. (A) VBP cDNA synthesized from DT40 poly(A)$^+$ RNA by reverse transcription was PCR amplified by using the indicated VBP isoform-specific primers (lanes 1, 3, 5, and 7). VBP cDNA plasmids were used as positive controls for the PCR analysis (lanes 2, 4, 6, and 8). The PCR products were resolved on a 1% agarose gel and stained with ethidium bromide. Lane MW, molecular weight markers.

determine if VBP does contribute to the a3 CCAAT/enhancer element binding activity of B cells.

Multiple DNA-protein complexes binding to the a1 and a3 CCAAT/enhancer elements are resolved in gel shift assays of B lymphoma cell nuclear extracts (65). To test whether VBP is a component of these DNA-protein complexes, the VBP antiserum was incubated with B-cell nuclear extracts prior to the addition of ^{32}P-labelled oligonucleotide. The addition of the VBP antiserum decreased the mobility of both the a1 and the a3 LTR protein binding complexes (Fig. 3A). The M3 oligonucleotide probe, which is mutated in the a3 CCAAT/enhancer element, was tested since this mutated probe is unable to bind GST-VBP (65). The VBP antiserum did not affect the mobility of the complexes binding to the M3 probe (Fig. 3A), indicating that mutation of this sequence abolished the binding of VBP to the a3 CCAAT/enhancer element. The addition of preimmune serum had no effect on the migration pattern of the a1 or a3 complexes (Fig. 3A). As a further control, the VBP antiserum did not affect the mobility of proteins binding the EF1 LTR promoter element (Fig. 3A). The contribution of A1/EBP to the a3 LTR binding complex was also tested since previous findings had indicated that A1/EBP binds to the adjacent a2 CCAAT/enhancer element (65). The addition of A1/EBP antiserum to both the wild-type (a3) and the mutated (M3) oligonucleotide probes caused a depletion of the binding complexes (Fig. 3B). This result is consistent with A1/EBP binding to the flanking a2 CCAAT/enhancer motif and is in agreement with the in vitro binding data for GST-A1/EBP (65). These data indicate that VBP binds to the a3 CCAAT/enhancer element in nuclear extracts from B cells, confirming the finding that GST-VBP preferentially binds to this site in vitro (65). Taken together with the mutational analysis, these findings suggest that VBP is the major regulator of ALV LTR-enhanced transcription in B cells.

A 50-kDa αβ protein is the only VBP isoform expressed in B cells. We used the VBP antiserum to analyze VBP protein expression in B cells. This antiserum immunoprecipitated a 50-kDa protein from metabolically labelled B lymphoma cells (Fig. 4, lane VBP). This observed size of VBP is much greater than the 38-kDa size predicted from the 313-aa open reading frame (33). This apparent size increase could be due at least in part to the high proline content of VBP, as all of the PAR proteins migrate about 10 kDa larger than expected for their predicted molecular masses (23, 32).

The VBP gene produces four mRNA transcripts by alternative splicing and processing (13). The internal three exons are common to all types and encode the DNA-binding and dimerization domains. The αα isoform consists of these three exons and an additional 5′ exon. The ββ isoform has a novel 5′ and 3′ exon compared to αα. The remaining two isoforms (αβ and βα) are splicing variants of the first two proteins. Reverse transcription-PCR analysis was performed to determine which of these isoforms encodes the VBP expressed in B cells. Using primers specific for each of the 5′ and 3′ exons and cDNA synthesized from B lymphoma poly(A)$^+$ RNA, we determined that only the VBPαβ isoform mRNA is expressed in B cells (Fig. 5, lane 1). The PCR conditions amplified all four isoforms, as determined by use of plasmid control reaction mixtures containing VBP cDNAs (Fig. 5, lanes 2, 4, 6, and 8). The finding that the VBP αβ isoform is the only VBP mRNA expressed in B cells was confirmed by protein analysis. The four isoforms produce proteins ranging in size from 45 to 50 kDa, and the 50-kDa αβ isoform comigrates with the 50-kDa protein from B cells (Fig. 6). Taken together, these data indicate that the αβ protein is the only VBP isoform expressed in B cells. These findings are in agreement with previous studies

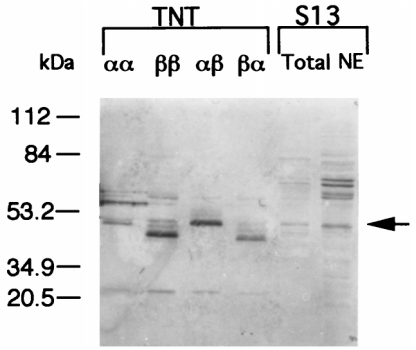

FIG. 6. The VBP from B cells comigrates with the VBP αβ isoform. VBP isoforms prepared by in vitro transcription and translation (TNT) were resolved on an SDS-polyacrylamide gel alongside total protein and nuclear extract (NE) samples from S13 B lymphoma cells. The migration of VBP αβ as determined by Western blot analysis with the anti-VBP serum is indicated (arrow).

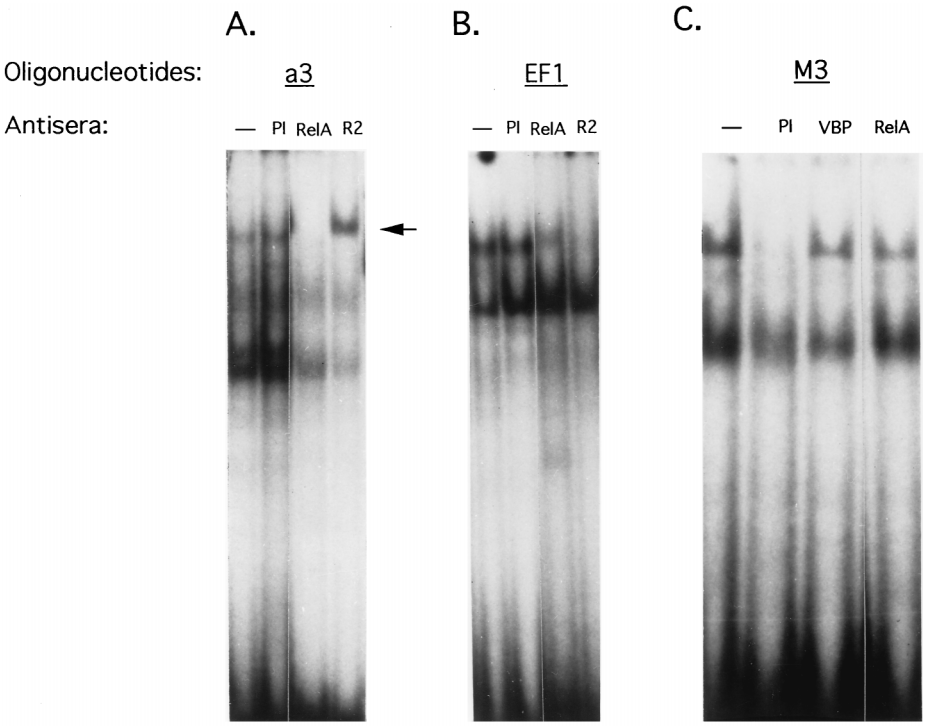

FIG. 7. A Rel-related factor(s) is a component of the a3 LTR CCAAT/enhancer element binding complex. Nuclear extract from S13 B lymphoma cells was incubated in a gel shift assay with ^{32}P-labelled a3 (A), EF1 (B), or M3 (C) LTR binding site probe alone (—) or with preimmune serum (PI), RelA-specific antiserum (RelA), v-Rel antiserum (R2), or VBP antiserum (VBP). Samples were resolved on a native Tris-borate-EDTA–5% polyacrylamide gel. Supershift complexes (arrow) are indicated.

by Burch and Davis (13) which demonstrated that the αβ isoform is the only ubiquitously expressed VBP mRNA, while the other isoforms show more tissue-restricted expression.

A Rel factor contributes to the a3 CCAAT/enhancer element binding activity. A 70-kDa Rel-related factor that interacts with A1/EBP in the a1 LTR binding complex was previously identified (10). This indirect interaction with the ALV LTR modestly increased a1 CCAAT/enhancer element-mediated transcription in B cells. However, we have now determined that the a3 CCAAT/enhancer element is the major control element for ALV LTR transcription enhancement in B cells. We were interested in determining if Rel factors also interact with VBP on the a3 CCAAT/enhancer element. To test whether a Rel-related factor is a component of the multiple a3 LTR DNA-protein complexes, we used R2, an antiserum to the Rel homology domain (RHD) of avian v-Rel (21). The R2 antiserum is made against a highly conserved region of Rel proteins and is cross-reactive with several Rel proteins, including RelA (6). We also used an antiserum raised against the unique sequence of the C terminus of chicken RelA. The incubation of R2 serum with the a3 LTR protein binding complexes resulted in a strong mobility supershift (Fig. 7A, lane R2). Interestingly, the addition of RelA-specific antiserum decreased the a3 binding activity (Fig. 7A, lane RelA). These effects are specific, since the addition of preimmune serum did not affect the mobility of the complexes (Fig. 7A, lane PI). These antisera had no effect on proteins binding to the heterologous oligonucleotide EF1 (Fig. 7B), illustrating the specificity for the CCAAT/enhancer element binding complexes. As a further control, the M3 oligonucleotide probe, which is mutated in the a3 CCAAT/enhancer element, was tested. The VBP and RelA antisera did not alter the mobility of the residual gel shift binding activity (Fig. 7C), suggesting that VBP binding is required for the interaction with Rel proteins in the a3 CCAAT/enhancer element binding complex. Although A1/EBP binding to the flanking sequence on the a3 oligonucleotide may account for the residual protein binding activity (Fig. 3B), this binding is not sufficient for binding of RelA (Fig. 7C). These results indicate that RelA is a component of the a3 CCAAT/enhancer element protein binding complex.

RelA interacts with VBP in B cells. The finding that a Rel protein is a component of the a3 CCAAT/enhancer DNA binding complex is interesting, as the a3 oligonucleotide sequence does not include a Rel binding site. In addition, data presented below indicate that RelA does not directly bind to the a3 CCAAT/enhancer element. These findings suggest that Rel factors indirectly bind to the a3 site by interacting with VBP. This possibility was examined by immunoprecipitation and Western blot analysis. Low-stringency precipitation with the VBP antiserum followed by Western blot analysis of coprecipitating proteins using the RelA-specific antiserum demonstrated that VBP and RelA interact in B cells (Fig. 8). A 70-kDa Rel protein immunoprecipitated with VBP- and RelA-specific antisera, but not with preimmune serum. The similar migrations of the immunoglobulin chains and VBP prevented the experiment from being done in the reverse fashion.

VBP and RelA activate ALV LTR CCAAT/enhancer element-driven transcription in B cells. We performed transient-transfection assays to examine the functional significance of the VBP and RelA interaction with the a3 CCAAT/enhancer element. First, the transactivation activity of the αβ VBP isoform was determined by using a reporter gene construct driven by multiple copies of the a3 LTR CCAAT/enhancer element. The luciferase reporter plasmid p$(a3)_8$-LUC was constructed

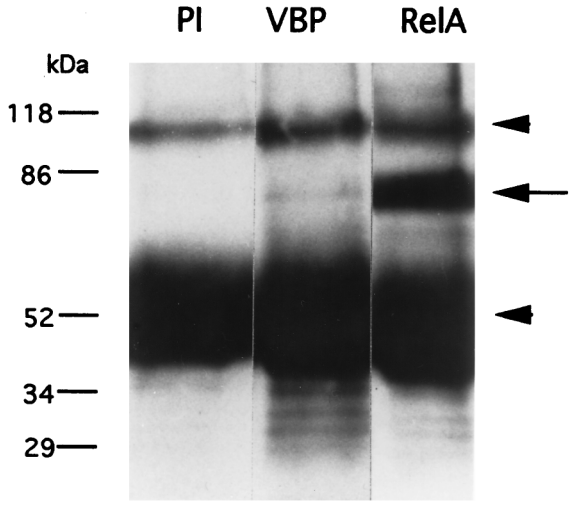

FIG. 8. VBP and RelA interact in B cells. Lysates from S13 B lymphoma cells were precipitated with preimmune (PI), anti-VBP (VBP), or anti-RelA (RelA) serum. Proteins were resolved on an SDS–10% polyacrylamide gel and electroblotted. Coprecipitating RelA was detected by immunoblotting with anti-RelA serum and chemiluminescence detection. The RelA protein (arrow) and the immunoglobulin chains (arrowheads) are indicated.

by cloning eight copies of the oligonucleotide containing the a3 CCAAT/enhancer element upstream of a minimal promoter-driven luciferase gene. DT40 bursal lymphoma cells were cotransfected with a CMV promoter-driven VBP expression vector, the $p(a3)_8$-LUC construct, and an internal control CMV–β-galactosidase plasmid. The parental plasmid pcDNA1 was added to each sample to equalize the total amount of CMV promoter sequence in each reaction mixture. The VBPαβ expression construct increased $p(a3)_8$-LUC expression 25-fold (Fig. 9A), while it did not influence luciferase expression from the minimal promoter construct lacking the CCAAT/enhancer elements. These data indicate that the αβ VBP isoform activates a3 CCAAT/enhancer element-mediated transcription in B cells.

We then examined the contribution of the RelA interaction with VBP to a3 CCAAT/enhancer element-mediated transcription. For these experiments, we used the available human RelA construct which shares 71% similarity with the avian protein (31). Cotransfection of the VBPαβ and RelA expression vectors increased $p(a3)_8$-LUC expression 12-fold compared to the level of activity obtained with VBP alone (Fig. 9B). The concentration of the VBP plasmid was lowered in the cotransfection experiments, which allowed the effect of RelA to be observed over the strong transactivating effect of VBP. Transfection of the RelA expression vector in the absence of VBP did not affect CCAAT/enhancer element-mediated luciferase expression, illustrating the requirement of VBP for RelA activity on the ALV LTR (Fig. 9B). These results suggest that the interaction of RelA with VBP has a functional role in activating ALV LTR-enhanced transcription.

RelA increases VBP LTR CCAAT/enhancer element binding activity. Our experiments indicate that RelA can interact with VBP within the a3 CCAAT/enhancer element binding complex to augment VBP-mediated transactivation from this element. One possible mechanism for this indirect effect is that interaction with VBP increases the efficiency of its binding to a3 CCAAT/enhancer elements. To examine this hypothesis, the effect of RelA on VBP DNA binding activity was examined in a gel shift assay. The addition of GST-RelA protein to suboptimal amounts of GST-VBP greatly increased the binding of VBP to the a3 CCAAT/enhancer element motif (Fig. 10A). In contrast, the addition of either control GST protein or human papillomavirus GST-E1 protein had no effect on VBP binding activity (Fig. 10A). Similar results were obtained with Rel proteins generated by in vitro transcription and translation (data not shown). This effect does not involve direct binding of RelA to the a3 CCAAT/enhancer element, as GST-RelA alone does not bind to the a3 CCAAT/enhancer element, although it does bind to an NF-κB oligonucleotide as expected (Fig. 10B). These data indicate that RelA interaction with VBP increases VBP DNA binding activity, which may be the mech-

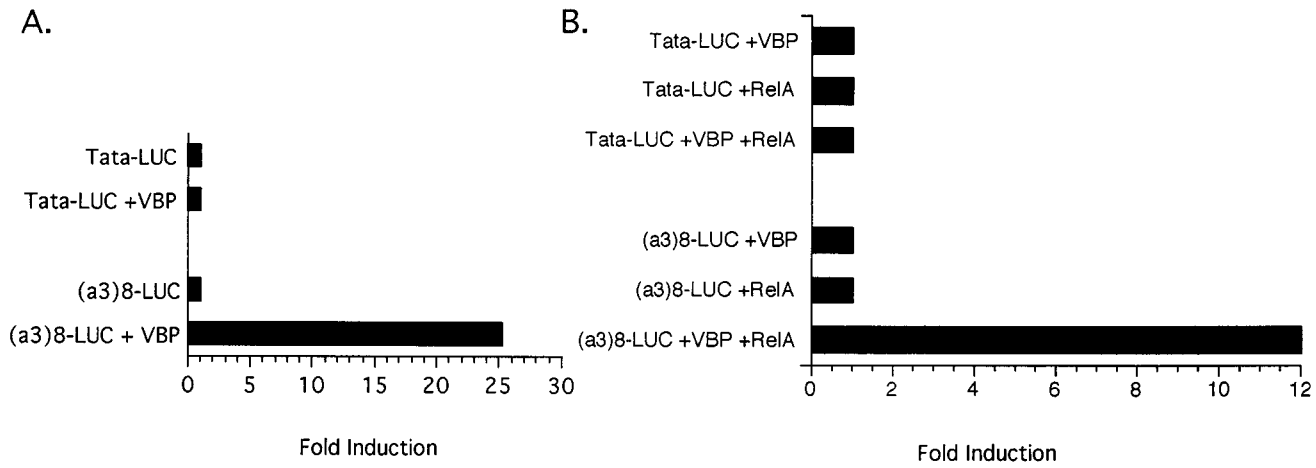

FIG. 9. VBP and RelA activate LTR CCAAT/enhancer element-mediated transcription. (A) DT40 bursal lymphoma cells (1.5×10^7) were transfected by electroporation and assayed 18 h later. Luciferase activity of the VBPαβ expression construct (0.5 μg) and the TATA-LUC or $p(a3)_8$-LUC constructs (5 μg) is presented as fold induction relative to the activity obtained with transfected pcDNA alone. The samples with pcDNA1 alone were arbitrarily set at 1. The standard errors were 20% of the means ($n = 3$). (B) RelA enhances VBP transactivation of LTR CCAAT/enhancer element-mediated transcription. A total of 8×10^5 cells were transfected by a DEAE-dextran method and assayed 48 h later. The results of a representative transfection experiment with the expression constructs VBP αβ (5.33 ng), RelA (5.33 ng), and the TATA-LUC or $p(a3)_8$-LUC reporter constructs (300 ng) are presented as fold induction of luciferase activity relative to activity obtained with transfected VBP alone. The samples with VBP alone were arbitrarily set at 1. The standard errors were less than 25% of the means ($n = 3$).

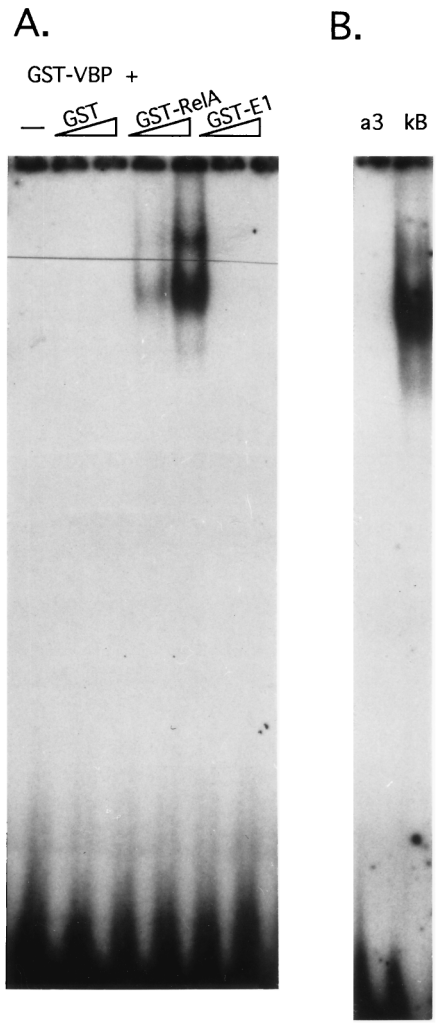

FIG. 10. RelA increases VBP binding to the a3 CCAAT/enhancer element. (A) Suboptimal amounts of GST-VBP (1 ng) incubated with either 10 or 25 ng of purified GST, GST-RelA, or GST-E1 and ^{32}P-labelled a3 oligonucleotide probe. (B) A 15-ng amount of GST-RelA incubated with ^{32}P-labelled a3 oligonucleotide or ^{32}P-labelled κB oligonucleotide. The DNA binding complexes were resolved on a 5% native polyacrylamide gel.

anism by which Rel transactivates VBP-mediated CCAAT/enhancer element-driven transcription.

DISCUSSION

The ALV LTR enhancer and promoter constitute a strong transcription unit which is essential for c-*myc* hyperexpression and induction of B-cell lymphomas (52). In this study, we directly tested the contributions of the three CCAAT/enhancer elements to ALV LTR enhancer function in B cells and in CEF. The a3 CCAAT/enhancer element is the major determinant of LTR enhancement in DT40 B cells, while the a1 and a2 elements show minor contributions. In contrast, all three CCAAT/enhancer elements contribute to LTR-driven transcription in CEF, a result that is similar to that observed with the closely related Pr-RSV-C strain LTR (59). Neither the ALV nor the RSV LTR shows a dominant contribution by the a3 site to LTR-driven transcription in CEF. These findings indicate that different combinations of CCAAT/enhancer elements mediate enhancer function in B lymphoma cells and fibroblasts. It is not yet clear why the a3 site is especially important for LTR enhancer function in B cells, while all three CCAAT/enhancer elements contribute in fibroblasts. It is interesting that the SR-RSV-A strain LTR contains a 5-bp deletion within the a3 site CCAAT/enhancer element that abolishes binding of B-cell proteins to this site in vitro (65). While the SR-RSV-A LTR shows high transcription activity in avian fibroblasts, this LTR could potentially be less active in B cells. The M1B site 5′ of the a1 CCAAT/enhancer element also shows cell-type-specific activity in that this site contributes to LTR enhancement in fibroblasts but not in B cells. Proteins binding to the M1B site have not yet been identified. These findings indicate that while the ALV and RSV LTR enhancers are transcriptionally active in many cell types, different combinations of enhancer elements may contribute to their regulation in each cell type. Similar results were obtained in studies of the Moloney murine leukemia virus LTR (67). Mutational analysis of the key protein binding sites in the Moloney murine leukemia virus enhancer found that some sites contributed to the ubiquitous transcription of the enhancer, while others affected transcription specifically in lymphoid cells or fibroblasts.

Our finding that the a3 CCAAT/enhancer element is the major determinant of LTR-enhanced transcription in B lymphoma cells suggests that VBP, which was previously identified as binding the a3 CCAAT/enhancer element (65), is especially important for LTR enhancer activity. Burch and Davis demonstrated that the VBP mRNA transcript is alternatively spliced into four variants, which exhibit different expression patterns and variable levels of transcriptional transactivation (13). The αβ isoform of VBP is the only isoform ubiquitously expressed at the RNA level in embryonic and adult tissues and activates transcription from a vitellogenin gene promoter element in fibroblasts and hepatoma cells. We have demonstrated that the αβ isoform is the only VBP variant expressed in bursal lymphoma cells. VBP could make a major contribution to both RSV and ALV LTR CCAAT/enhancer element-mediated transcription in many cell types, as it binds to both the a1 and the a3 CCAAT/enhancer elements. The VBP αβ isoform activated transcription from the a3 CCAAT/enhancer element in B cells, suggesting that VBPαβ could have a general role in CCAAT/enhancer element-mediated transcription in many cell types. A1/EBP is also a ubiquitously expressed protein that could contribute to transcription mediated by CCAAT/enhancer elements having the a1 type of sequence motif (10). Other members of the C/EBP family show more-restricted expression and could contribute to tissue-specific transcription involving CCAAT/enhancer elements (8). The C/EBPβ and C/EBPγ proteins repress LTR-driven transcription (17, 62), suggesting that the activity of LTR or cellular gene CCAAT/enhancer motifs can be modulated in a positive or negative manner by different C/EBP family members. Our findings suggest that the VBP αβ isoform is a good candidate to regulate general activation of viral and cellular gene CCAAT/enhancer elements in many cell types, and in particular the widespread activity of the ALV and RSV LTR enhancers.

The findings that VBP interacts with RelA and that both are components of the major regulatory ALV LTR binding complex of B cells suggest that this interaction is important for the regulation of ALV CCAAT/enhancer element-mediated transcription. The cross-coupling of transcription factors is increasingly being recognized as a mechanism for regulation of gene expression (reviewed in reference 44). A number of studies have demonstrated C/EBP-Rel interactions on cellular and viral promoter elements. Bowers et al. showed that a Rel-related protein interacted with A1/EBP in the ALV LTR a1 CCAAT/enhancer element binding complex in avian B cells

(10), and Diehl and Hannink demonstrated a C/EBP-Rel complex binding to the interleukin-6 gene in avian T cells (21). Regulation of the interleukin 8 gene and the serum amyloid A gene has also been shown to involve interactions between C/EBP and NF-κB (51, 68, 70). Stein et al. determined that RelA, p50, or Rel could functionally synergize with C/EBPα, C/EBPβ, and C/EBPδ to activate CCAAT/enhancer element-mediated transcription (69). Interestingly, cross-coupling of these factors resulted in inhibition of promoters containing κB motifs.

In this study, we determined that RelA and VBP synergistically activate transcription through the major B-cell ALV LTR a3 CCAAT/enhancer element, indicating that PAR-type C/EBP factors also interact with Rel proteins. We also demonstrated that RelA increases VBP binding to the CCAAT/enhancer element. RelA does not directly bind to the a3 LTR CCAAT/enhancer element, and therefore it is likely that the transactivating effect is mediated via RelA interaction with VBP. Stein et al. also demonstrated that RelA stimulates the binding activity of C/EBPα, C/EBPβ, and C/EBPδ (69). The molecular mechanism for this binding augmentation of VBP and other C/EBP factors by RelA has not yet been determined, although it is known to require the Rel RHD and C/EBP bZIP domains of each protein (69). Recent studies of the human T-cell leukemia virus type 1 Tax transactivator protein demonstrated that Tax enhances the DNA binding activity of C/EBP and Rel proteins (50, 72, 74). This interaction has been mapped to the basic region of C/EBP factors and the RHD of Rel proteins (7, 49). In both cases, Tax increased the dimerization of these proteins by shifting the monomer-dimer equilibrium to favor dimerization, thereby increasing the rate of association of the factors with their cognate DNA binding sequences (7, 49, 72). VBP also binds DNA as a dimer (22), and it seems likely that interaction with RelA could promote VBP dimerization and stabilization of the VBP-DNA complex.

It has been proposed that differential regulation of LTR-enhanced c-*myc* transcription by labile factors could be important in targeting immature B cells for ALV tumor induction (11). Rel proteins interacting with VBP and A1/EBP could be important for the B-cell-specific lability of these factors. The NF-κB family of proteins is multigenic and includes p50, p52, RelA (p65), c-Rel, and RelB (reviewed in reference 4). The 300-aa RHD is shared by all members and mediates dimerization and DNA binding. The RHD also mediates interactions with the bZIP domains of C/EBP factors (69). NF-κB (p50/RelA) was first identified as a B-cell-specific activity that regulates the enhancer of the immunoglobulin κ light-chain gene (63). However, NF-κB activity can be induced in pre-B cells and other cell types by a variety of agents. Different members of the Rel family are either inducibly or constitutively active during murine B-cell development (41). The most abundant dimer of inducible NF-κB is a p50-RelA (p65) heterodimer, although RelA homodimers which activate transcription in T cells have been identified (24, 36). Constitutively active forms consist of dimers of p50 and p52 with RelB and c-Rel (39, 41). Although we have demonstrated that RelA is involved in the regulation of the ALV enhancer, it is possible that other Rel factors may also interact with VBP. The developmental expression and activity of these Rel proteins may provide insight into the B-cell-specific labile LTR binding activity of VBP and the susceptibility of immature B cells to ALV tumor induction.

ACKNOWLEDGMENTS

We thank Henry Bose, Bill Bowers, John Burch, and Mark Hannink for generously sharing antisera, reagents, and advice.

This work was supported by NIH grants CA54768 to A. Ruddell and T32 DEO7202 to S. Curristin.

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