JOURNAL OF VIROLOGY, May 1996, p. 3051–3059 Vol. 70, No. 5
0022-538X/96/$04.00+0

Regulation of Avian Leukosis Virus Long Terminal Repeat-Enhanced Transcription by C/EBP-Rel Interactions

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Received 29 December 1995/Accepted 13 February 1996

The avian leukosis and sarcoma virus long terminal repeat (LTR) enhancers feature directly repeated CCAAT/enhancer element sequences which are also found in many viral and cellular gene enhancers. While most members of the CCAAT/enhancer element-binding protein (C/EBP) transcription factor family exhibit tissue-restricted expression, there may be ubiquitously expressed C/EBP-like factors that regulate widespread CCAAT/enhancer element-driven transcription. An avian C/EBP-related factor designated A1/EBP was previously shown to bind CCAAT/enhancer elements within the avian leukosis virus (ALV) and Rous sarcoma virus (RSV) LTR enhancers in a pattern identical to that of a B-cell LTR-binding factor (W. J. Bowers and A. Ruddell, J. Virol. 66:6578–6586, 1992). An A1/EBP-specific antiserum recognizes a 40-kDa LTR CCAAT/enhancer element-binding protein purified from avian B lymphoma cells. A1/EBP is widely expressed at the mRNA and protein levels, suggesting that this protein could be important not only in regulating widespread expression of the ALV and RSV retroviruses but also in controlling the expression of other viral and cellular gene enhancers that possess CCAAT/enhancer motifs. We also found that an NF-κB/Rel-related protein is a component of the LTR CCAAT/enhancer element binding complex through its interaction with A1/EBP. At least one of the NF-κB family members, p65 (RelA), is capable of activating LTR CCAAT/enhancer element-driven transcription. These findings suggest a role for Rel-related factors in the regulation of ALV or RSV LTR-driven transcription via an interaction with A1/EBP.

Avian leukosis virus (ALV) provides a useful system for studying the mechanism involved in the retrovirus-mediated deregulation of c-*myc* expression and induction of lymphoma. ALV is a slowly transforming retrovirus that induces B-cell lymphomas in chickens after a rare integration of proviral long terminal repeat (LTR) enhancer sequences next to the c-*myc* proto-oncogene (reviewed in reference 37). Tissue-specific host factors appear to play an important role in tumorigenesis, in that ALV infects many tissue and cell types, but the majority of tumors that arise are of bursal origin. A correlation between the regulation of B cell-specific LTR transcription and immature B-cell tumor susceptibility which may play an important role in ALV lymphomagenesis has been observed (35, 46). ALV LTR-enhanced c-*myc* and viral gene transcription is specifically decreased 10- to 15-fold after the inhibition of protein synthesis in bursal lymphoma cells, while LTR-enhanced transcription is unaffected by protein synthesis inhibition in infected T cells or embryo fibroblasts. These findings suggest that labile or short-lived proteins regulate LTR enhancement in B cells. This lability is restricted to immature hematopoietic cells of lymphoma-susceptible chicken strains, while LTR-enhanced transcription is stable in all tissues of lymphoma-resistant chicken strains (46). These findings suggest that labile LTR-enhanced c-*myc* transcription may be an important determinant of ALV tumor susceptibility.

Multiple CCAAT/enhancer element motifs are found in the ALV LTR enhancer (8, 54), suggesting that these sites may be important not only for widespread retroviral gene expression but also for the labile characteristic of LTR-driven transcription in immature B cells. For example, different strains of ALV and Rous sarcoma virus (RSV) contain two to four CCAAT/enhancer motifs that cover most of the LTR enhancer region (47, 54). Mutation of these CCAAT/enhancer motifs reduces LTR-driven transcription and viral titers in fibroblasts (48), indicating that these sites are important for LTR enhancer function. Interestingly, the CCAAT/enhancer motifs are absent in endogenous avian retroviral LTRs, a finding which correlates with the very low transcriptional activity of these viruses (13, 20). Nuclear proteins that bind CCAAT/enhancer elements in the closely related ALV and RSV LTR enhancers have been identified in several avian cell types (reviewed in reference 44). The binding activities have been designated a1 and a3 (45), EFII (51), and FI and FIII (18) in different studies. The a1 and a3 binding activities are specifically labile in B cells, as these gel shift binding complexes are missing from extracts of B cells treated for 3 h with protein synthesis inhibitors while they are stable in T cells or fibroblasts (45). These findings correlate with the labile or stable LTR-enhanced transcription observed in each cell type (46), supporting the idea that these CCAAT/enhancer binding factors are important for LTR enhancer regulation.

The CCAAT/enhancer element-binding proteins (C/EBPs) that have been characterized thus far mainly exhibit tissue-restricted expression. For example, C/EBPα is expressed primarily in liver and adipose tissue (6). C/EBPβ is an inducible factor, as its expression is observed in cells only following the addition of lipopolysaccharide (LPS), serum stimulation, or cytokine stimulation (1, 9). Avian NF-M is expressed in myelomonocytic cells but not in other hematopoietic cell lineages (29). Candidate C/EBP-related factors which may regulate labile LTR-driven transcription in B cells and the high levels of viral expression in other cell types were identified. Two genes from an avian λgt11 cDNA expression library were cloned because of their ability to specifically bind the a1 and a3

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CCAAT/enhancer elements of the ALV LTR enhancer (8, 55). A1/EBP encodes an a1 LTR binding factor that is closely related to murine immunoglobulin gene enhancer binding protein (Ig/EBP) (8, 43), and A3/EBP (54) was identified as the avian vitellogenin II gene promoter-binding protein (VBP), which belongs to the PAR subfamily of C/EBP-related factors (17, 27). A1/EBP and VBP bind overlapping but distinct subsets of CCAAT/enhancer elements in the ALV and RSV LTR enhancers. The consensus binding site for A1/EBP is TN(A/T)TGCAAN, while VBP recognizes the sequence TT(G/A)CATAAG (54). These motifs are closely related to the binding sites determined for other C/EBP-related factors (24, 48). The binding specificities of the A1/EBP and VBP proteins are similar to those of the purified a1 and a3 proteins of B cells, respectively, suggesting that A1/EBP encodes the a1 LTR-binding protein and VBP encodes the a3 protein in vivo (54).

In this report, we characterize the expression pattern of A1/EBP and demonstrate that A1/EBP is ubiquitously expressed at both the mRNA and protein levels. A1/EBP is a major component of the a1 LTR activity in B and T cells, as judged by gel shift assays using A1/EBP antiserum. In addition, A1/EBP interacts with a 70-kDa NF-κB/Rel-related factor in S13 bursal lymphoma cells. p65 (RelA) binds to A1/EBP in a DNA-independent manner and is also capable of activating CCAAT/enhancer-driven transcription in B cells. These observations suggest a role for NF-κB/Rel family members in regulating LTR-driven expression and the labile character of LTR transcription enhancement in ALV target B cells.

MATERIALS AND METHODS

Cell culture. S13 bursal lymphoma cells (22), DT40 bursal lymphoma cells (4), MSB thymocytes (2), KBMC (pre-B, pre-T) cells (34), and primary chicken embryo fibroblasts were grown as described previously (46). The E3 embryonic splenic B-cell line (provided by H. Bose) was cultured as previously described (63).

Preparation of A1/EBP antiserum. The 72-amino-acid amino-terminal segment of the A1/EBP cDNA (8) was obtained by PCR amplification and was cloned in frame into the glutathione-*S*-transferase (GST) vector (55) to obtain glutathione agarose-purified A1/EBP-TA-GST fusion protein for the immunization of rabbits (East Acres Biologicals, Inc.). The resulting polyclonal antiserum was enriched for IgG by ammonium sulfate precipitation (21) and further purified for A1/EBP-specific immunoglobulins by passing the IgG fraction through a column composed of *Escherichia coli* lysate and GST protein conjugated to CNBr-activated Sepharose 4B (Pharmacia). The resulting flowthrough fractions were concentrated to >1 mg/ml by Centricon-30 filter centrifugation (Amicon, Inc.).

Immunoprecipitation and Western (immunoblot) analysis. For metabolic labeling, 10^7 tissue culture cells were incubated for 3 h in Dulbecco modified Eagle medium without methionine and cysteine but with 250 μCi of [^{35}S]methionine and [^{35}S]cysteine (DuPont NEN). Cells were lysed on ice for 20 min in Triton X-100 buffer (1% Triton X-100, 25 mM Tris [pH 7.5], and 150 mM NaCl) or RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], and 50 mM Tris [pH 8]). Following centrifugation, the supernatant was precleared with protein A Sepharose (Pharmacia) at 4°C for 2 h. Precleared samples were then incubated at 4°C for 4 h with protein A Sepharose that had been precoated with the appropriate antisera. In some experiments, 5 μg of glutathione agarose-purified GST protein was added as a competitor. For sequential immunoprecipitation experiments, cell samples were lysed at 4°C for 2 h in low-stringency immunoprecipitation buffer (phosphate-buffered saline, 1% Nonidet P-40) precleared with protein A Sepharose, incubated with primary antibody-conjugated protein A Sepharose beads, and washed with low-stringency buffer (59). The beads were resuspended in 300 μl of RIPA buffer and heated at 95°C to denature the proteins. Supernatants were transferred to new microcentrifuge tubes, and secondary antibody-conjugated protein A Sepharose beads were added. Samples were incubated overnight at 4°C, washed with RIPA buffer, and resuspended in 30 μl of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. All immunoprecipitated samples were analyzed by SDS-PAGE and fluorography. The R2 antiserum was raised against the Rel homology domain (amino acids 92 to 456) of v-Rel (generously provided by M. Hannink).

For Western blot analysis, protein samples (50 μg) were precipitated with 5 μg of bovine lactoglobulin (Sigma) and 8 μg of linear polyacrylamide (BDH) in 4 volumes of cold acetone by chilling on dry ice for 30 min. Precipitates were centrifuged at 12,000 × *g* for 30 min at 4°C, rinsed with cold 80% acetone, recentrifuged, dried, and resuspended in SDS sample buffer (31). Samples were separated on SDS–12% PAGE gels, electroblotted (60), and analyzed by alkaline phosphatase staining (36) with purified α-A1/EBP antiserum and alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Bio-Rad).

Protein purification and gel shift assay. S13 and MSB T-cell nuclear extracts were prepared by 0.5 M NaCl treatment of purified nuclei as previously described (46). C/EBPs were purified by S-300 Sepharose chromatography (Pharmacia) and then by heat treatment at 85°C for 10 min, followed by centrifugation for 10 min at 12,000 × *g* to remove heat-denatured protein. Proteins were then purified by binding to an a1 oligonucleotide agarose column as previously described (8). For gel shift assays, nuclear extracts (5 μg) were incubated in gel shift buffer (10 mM Tris-HCl, [pH 8.0], 50 mM NaCl, 10% glycerol, and 1 mM EDTA) with 1 μg of poly(dI-dC) · poly(dI-dC) and appropriate antisera (10 μg) for 1 h on ice prior to the addition of 5,000 cpm (approximately 0.1 ng) of ^{32}P-labeled a1 oligonucleotide probe (46). Following a 20-min room temperature incubation, the 15-μl reaction mixtures were electrophoresed on a native 5% polyacrylamide gel in TAE buffer (46) or in TBE buffer (22.5 mM Tris-borate [pH 8] and 0.5 mM EDTA). The double-stranded a1 oligonucleotide probe sequences are 5′-GGGAAATGTAGTCTTATGCAATACTCTAA-3′ and 5′-TTAGAGTATTGCATAAGACTACATTTCCC-3′ (9),and the double-stranded EFI oligonucleotide probe sequences are 5′-GCATGCCGATTGGTGGGAGTAAGGTGGT-3′ and 5′-ACCACCTTACTCCCACCAATCGGCATGC-3′ (41).

RNA isolation and Northern (RNA) analysis. Poly(A)$^+$ RNA was purified from tissue culture cells or from tissues isolated from 5-week-old White Leghorn chickens (generously provided by R. Rosier) by the guanidinium isothiocyanate-cesium chloride method (10) followed by oligo(dT) cellulose chromatography (Bethesda Research Laboratories). Eight micrograms of each RNA sample was separated by formaldehyde-agarose gel electrophoresis (58) and transferred to nitrocellulose (Schleicher & Schuell) for Northern blot hybridization. A 350-bp fragment from the 5′ terminus of the A1/EBP cDNA and a chicken glyceraldehyde-phosphate dehydrogenase (GAPDH) cDNA (57) were ^{32}P labeled (U.S. Biochemicals), used to sequentially probe Northern blots, and analyzed by autoradiography. For transcript size determination, the total RNA electrophoresed on the same gel was stained with ethidium bromide to detect the migration of 18S and 28S rRNAs with estimated sizes of 2.1 and 4.8 kb, respectively (14, 62).

GST retention assays. The T7 expression construct for p50 used in coupled in vitro transcription-translation reactions was generated by cloning the p50 insert from pRP50 (49) into a *Hin*dIII-*Eco*RV-restricted pcDNA1 eukaryotic expression vector. A T7 expression construct for p65 was described previously (53). Fifteen microliters of glutathione-agarose beads bound by GST fusion protein (55) were incubated for 2 h at 4°C with 3 μl of ^{35}S-labeled, in vitro-translated (TNT coupled reticulocyte lysate system; Promega), no-template control, p50, or p65 protein in 300 μl of binding buffer (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40) containing 0.1% bovine serum albumin (28) and either 0 or 400 μg of ethidium bromide per ml (32). The beads were then washed five times in binding buffer and resuspended in SDS-PAGE sample buffer. The proteins were resolved on SDS–10% PAGE gels and visualized by fluorography.

Cell transfection and luciferase assay. The expression construct for p65 (RelA) was described previously (49), and the pcDNAI plasmid was obtained from Invitrogen. An ALV LTR-luciferase reporter plasmid (pALV-LUC) was constructed as follows. The entire ALV LTR (bases −295 to +99 relative to the transcription start site) was amplified by PCR from the BK25 plasmid (50) that contains RAV-2 ALV proviral enhancer and promoter sequences, with a 5′ primer containing a *Kpn*I site and a 3′ primer possessing a *Hin*dIII restriction site being used. The restricted PCR product was ligated into the pXP1 luciferase reporter vector (39). To create pALVM123-LUC, the a1, a2, and a3 CCAAT/enhancer elements of pALV-LUC were sequentially mutated by site-directed PCR mutagenesis (33). The promoter-LUC construct was made by PCR amplification of the promoter sequence (−140 to +99) of pALV-LUC and cloning upstream of the luciferase gene in pXP1. The reporter plasmid, p(a1)$_8$-LUC, was constructed by concatemerization of an a1 LTR oligonucleotide, *Bam*HI linker addition, and cloning upstream of the ALV LTR promoter in pXP1. The a1 oligonucleotide sequences are 5′-GAAATGTAGTCTTATGCAATACTCTAATGCAATACT-3′ and 5′-ATTTCAGTATTGCATTAGAGTATTGCATAAGACTAC-3′. Five micrograms of each plasmid construct was transfected into DT40 cells by electroporation (11), and luciferase activity was measured 20 h later (15). A control cytomegalovirus promoter-driven β-galactosidase reporter plasmid (provided by A. Geballe) was assayed as previously described (40).

RESULTS

A1/EBP was previously shown to be a candidate for regulating labile LTR enhancement of c-*myc* transcription, as this protein binds to the same LTR enhancer sequences as the labile a1 LTR-binding protein of B cells (8). Analysis of A1/EBP involvement in the labile character of LTR-driven transcription in immature B cells would be aided by the development of an A1/EBP-specific antiserum. A rabbit polyclonal antiserum specific to the N-terminal 72 amino acids encoded

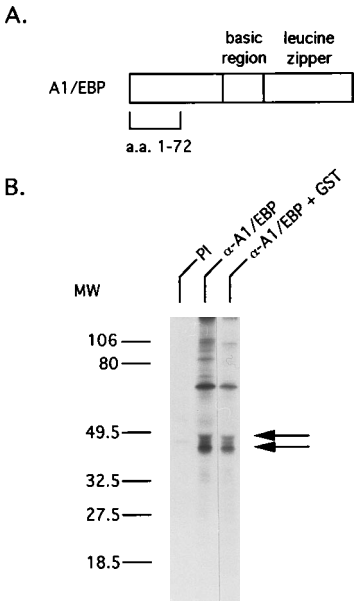

FIG. 1. α-A1/EBP recognizes B-cell proteins. (A) Schematic showing the 72-amino-acid (a.a.) A1/EBP region expressed as a GST fusion protein that was used for generation of A1/EBP-specific antiserum. (B) S13 cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine, lysed, incubated with rabbit preimmune serum (PI) or with A1/EBP antiserum (α-A1/EBP) and protein A beads, and analyzed by SDS-PAGE and fluorography. The immunoprecipitated 40- and 49-kDa proteins are indicated by arrows. Five micrograms of purified GST protein was also added as a competitor to the α-A1/EBP + GST immunoprecipitation sample. The migration of molecular weight markers (MW) is indicated.

by the partial A1/EBP cDNA was produced with an A1/EBP-TA-GST fusion protein antigen (Fig. 1A). This N-terminal peptide contains sequences that are specific to A1/EBP and does not include C-terminal regions which are related to other C/EBP factors (8). The antiserum detects A1/EBP in the context of a heterologous histidine-tagged bacterial fusion protein (23) by Western blot analysis, indicating that the antiserum specifically recognizes A1/EBP epitopes (data not shown). The A1/EBP antiserum was used to determine the size of the encoded protein in vivo. S13 bursal lymphoma cells were metabolically labeled, and the lysates were analyzed by immunoprecipitation and SDS-PAGE (Fig. 1B). The A1/EBP antibody recognizes three proteins with sizes of approximately 40, 49, and 70 kDa. These immunoprecipitated complexes were not eliminated by the addition of 10 μg of GST protein. A 45-kDa protein which coimmunoprecipitates with the A1/EBP antiserum also is immunoprecipitated with preimmune serum (Fig. 1B), indicating that this protein is nonspecific. Several proteins in the 80- to 110-kDa region are eliminated by GST addition, indicating that the purified antiserum still contains GST-reactive antibodies.

Several a1 LTR-binding proteins were previously purified from bursal lymphoma cells (8). Nuclear extracts were fractionated by S-300 Sepharose chromatography, heat treated, and centrifuged to remove denatured proteins (45). The heat-stable supernatant was further purified by binding to an a1 oligonucleotide-agarose affinity column and elution with 0.6 M KCl. SDS-PAGE analysis of the roughly 4,000-fold-purified protein fractions showed three enriched proteins with sizes of 35, 40, and 42 kDa (8). With Western blots being used, samples from steps in the protein purification process were analyzed for the presence of A1/EBP. The A1/EBP antiserum recognizes the 40-kDa protein in crude cell lysates, 0.5 M NaCl nuclear extracts from S13 B lymphoma cells, heat-treated nuclear extracts, and oligonucleotide affinity-purified fractions (Fig. 2). This result indicates that A1/EBP encodes the previously purified 40-kDa a1 LTR-binding protein of B cells. The affinity-purified fraction also shows several 60- to 70-kDa proteins that are not observed in crude fractions (Fig. 2). These bands sometimes appear in affinity-purified fractions after freezer storage and therefore do not appear to be A1/EBP-related proteins. The identities of the 35- and 42-kDa species also purified by a1 oligonucleotide affinity chromatography (8) are not yet known, although they could represent other C/EBP-related factors expressed in B cells. The 49-kDa protein recognized by the A1/EBP antiserum could represent a modified form of the 40-kDa A1/EBP species, which is denatured by heat treatment and therefore lost during purification. *Staphylococcus aureus* V8 protease and chymotrypsin were used in peptide mapping experiments with [^{35}S]methionine-labeled S13 bursal lymphoma cell lysates to determine if these two proteins are related (12). The peptide maps for these proteins are different (data not shown), indicating that the 49-kDa protein is not related to the 40-kDa a1 LTR-binding protein.

A1/EBP contributes to LTR CCAAT/enhancer element binding activity. The A1/EBP antiserum was used to determine if A1/EBP contributes to LTR CCAAT/enhancer element binding activity. Diffuse DNA-protein complexes are observed in gel shift assays with the a1 LTR CCAAT/enhancer element oligonucleotide probe with B- or T-cell nuclear extracts (45). These binding activities are labile or greatly decreased in nuclear extracts from B cells treated with protein synthesis inhibitors while they are stable in T cells, suggesting that they are important for labile LTR-enhanced transcription in B cells and stable transcription in other cell types. To determine if A1/EBP encodes the protein responsible for these DNA-protein complexes, the A1/EBP antiserum was used to deplete nuclear extracts of A1/EBP before they were used in gel shift assays. The antiserum specifically depletes most of the a1 oligonucleotide binding complexes in B- and T-cell nuclear extracts, as the addition of A1/EBP antiserum leads to a decrease in the intensity of the shifted complexes while the addition of preimmune serum has no effect (Fig. 3A). The use of a control oligonucleotide probe corresponding to the EFI site in the ALV and RSV LTR promoters (41) demonstrates that the A1/EBP depletion seen with the A1/EBP antiserum is specific for the a1 binding activity, as EFI binding activity is not decreased by the addition of A1/EBP antisera (Fig. 3B). The incomplete inhibition of binding by the A1/EBP antiserum

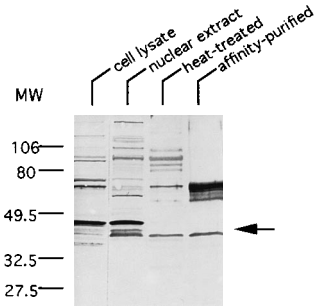

FIG. 2. A1/EBP encodes an a1 LTR-binding protein in B cells. Proteins from S13 cell lysates, nuclear extracts, heat-treated fractions, and a1 oligonucleotide affinity column-purified samples were analyzed by Western blots with the A1/EBP antiserum. The arrow indicates the 40-kDa protein. The migration of molecular weight markers (MW) is indicated.

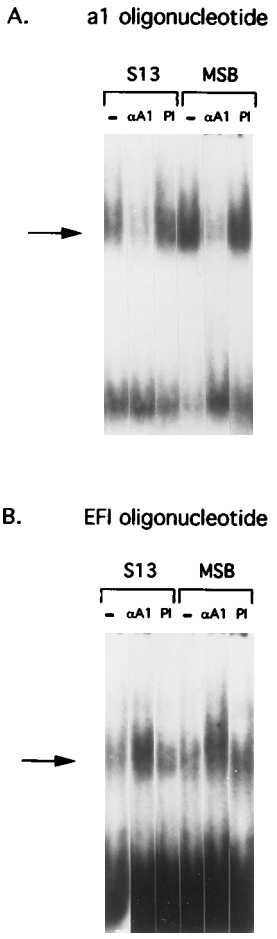

FIG. 3. A1/EBP contributes to LTR CCAAT/enhancer element binding activity. (A) Nuclear extracts from S13 or MSB cells were incubated in gel shift reactions with a ^{32}P-labeled a1 LTR binding site oligonucleotide alone (−), with A1/EBP antiserum (αA1), or with preimmune serum (PI) prior to incubation with the ^{32}P-labeled a1 oligonucleotide probe. Samples were separated on native 5% polyacrylamide–TAE gels and visualized by autoradiography. The arrow indicates the migration of shifted complexes. (B) Nuclear extracts were incubated in gel shift reactions with a ^{32}P-labeled EFI LTR binding site oligonucleotide probe alone (−), with A1/EBP antiserum (αA1), or with rabbit preimmune serum (PI) prior to incubation with labeled EFI oligonucleotide probe. The arrow indicates the migration of shifted complexes.

could reflect the inability of the antiserum to completely deplete A1/EBP from the nuclear extract. It is also possible that additional C/EBP-related factors contribute to LTR CCAAT/enhancer element binding activity. These observations suggest that A1/EBP is a major component of the labile a1 binding complexes in B cells. This binding activity is greatly decreased in extracts from cells treated for 3 h with protein synthesis inhibitors (45). A1/EBP also appears to be a major component of the stable LTR binding activity from T cells (Fig. 3B).

A1/EBP is ubiquitously expressed. The ALV and RSV proviruses are expressed at high levels in many cell types and tissues (42, 61), and this strong expression appears to be dependent upon the integrity of the LTR CCAAT/enhancer elements, as determined by transient transfection or retroviral infection assays in fibroblasts (13, 48). The a1 LTR CCAAT/enhancer binding activity is detected in B cells, T cells, fibroblasts, and erythroblasts from chickens (18, 45) and in mouse and rat cell lines (30, 52). This outcome suggests that the factors binding LTR CCAAT/enhancer elements are widely expressed. The possibility that A1/EBP encodes a ubiquitous factor was examined by Northern blot hybridization analysis. Poly(A)$^+$ RNA was purified from various cell lines and from tissues isolated from 5-week-old chickens. A 350-bp probe prepared from the 5′ end of the A1/EBP cDNA detects a 2.9-kb transcript in all the cell types and tissues analyzed. These cells and tissues include chick embryo fibroblasts, B cells (DT40 B lymphoma), embryo splenic B cells (E3), pre-B, pre-T cells (KBMC), T cells (MSB), brain, bursa, heart, kidney, liver, skeletal muscle, and spleen (Fig. 4, top panel). The same 2.9-kb transcript was observed with a probe corresponding to the A1/EBP basic region-leucine zipper domains (data not shown). The GAPDH probe was used as an internal control for the assessment of RNA sample loading (Fig. 4, bottom panel). Equal levels of GAPDH mRNA are observed in the tissue culture cell samples, while GAPDH levels fluctuate in tissues. The highest expression of GAPDH is seen in skeletal muscle, as previously described (57). The ubiquitous expression of A1/EBP mRNA was, however, reproducibly observed in independent experiments. These data indicate that A1/EBP is widely expressed at the mRNA level.

The A1/EBP antiserum was used to determine A1/EBP expression at the protein level. The 40-kDa A1/EBP protein is detected in DT40 and S13 B lymphoma cells, MSB T cells, and chicken embryo fibroblasts analyzed by radioimmunoprecipitation (Fig. 5). A1/EBP expression was also examined in lysates from various tissues of 5-week-old chickens by Western blot assays. The antiserum recognized a 40-kDa protein in all of the tissues examined (data not shown). These results indicate that A1/EBP is expressed in many cell types and therefore is a candidate for the widespread LTR CCAAT/enhancer element binding activity.

A Rel-related factor is a component of the a1 LTR binding complex. The A1/EBP antiserum immunoprecipitates the 40-kDa A1/EBP factor and an unidentified protein with a size of

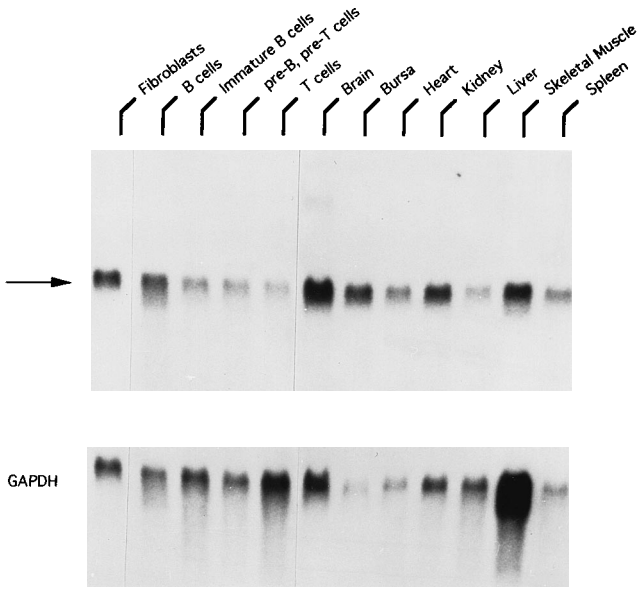

FIG. 4. A1/EBP is expressed ubiquitously at the mRNA level. Poly(A)$^+$ RNA was purified from chick embryo fibroblasts (fibroblasts), DT40 B cells, E3 immature B cells, KBMC pre-B, pre-T cells, and MSB T cells and from various tissues from 5-week-old chickens. Northern blot analysis was performed with a ^{32}P-labeled A1/EBP probe (top panel) and then with a ^{32}P-labeled GAPDH probe as a control (bottom panel). The 2.9-kb A1/EBP mRNA is indicated by the arrow.

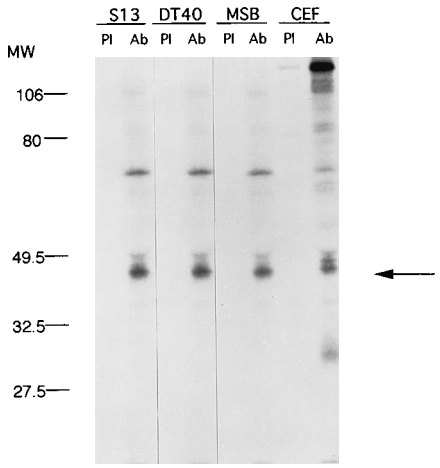

FIG. 5. A1/EBP is widely expressed at the protein level. Cell lines were metabolically labeled and lysates were analyzed by immunoprecipitation with preimmune serum (PI) or the A1/EBP antiserum (Ab) followed by SDS-PAGE and fluorography. The 40-kDa A1/EBP protein is indicated by the arrow. Molecular weight is expressed in thousands.

approximately 70 kDa (Fig. 5). The 70-kDa species could represent a protein that physically associates with A1/EBP. Other investigators have found that C/EBP-related factors complex with members of the NF-κB/Rel family of transcription factors (56). NF-κB proteins are also ubiquitously expressed transcription factors whose activities have been linked to immunological responsiveness, cell proliferation, and lymphoid cell differentiation (reviewed in references 5 and 19). NF-κB family members p50, p65, and c-Rel, along with C/EBPα, -β, and -δ, show a physical interaction that is dependent upon their respective Rel and bZIP (basic region-leucine zipper) domains (56). A 40-kDa C/EBP-related factor was previously found in a CCAAT/enhancer binding complex with a 70-kDa RelA-related factor in avian MSB T cells (16). The C/EBP-related factor involved could be A1/EBP, as we have shown that the 40-kDa A1/EBP protein is expressed in MSB T cells (Fig. 5).

We used a Rel-specific antiserum in gel supershift assays to determine if a Rel family member is a component of the a1 LTR binding complexes of B and T cells. This R2 antiserum was made against the Rel homology domain of avian v-Rel and recognizes multiple members of the NF-κB/Rel family (16). The Rel antiserum specifically shifts a portion of the a1 LTR binding complexes in B- and T-cell nuclear extracts, while the addition of preimmune serum has no effect (Fig. 6A). As a control, the Rel antiserum does not affect gel shift binding to the heterologous EFI promoter oligonucleotide probe (Fig. 6B). These observations suggest that a Rel-related factor is a component of the a1 LTR binding complexes in B and T cells.

A 70-kDa Rel-related factor interacts with A1/EBP. The RelA-related factor in avian MSB T-cell nuclear extracts is approximately 70 kDa in size (16). Immunoprecipitation experiments with the A1/EBP antiserum consistently demonstrate the presence of a 70-kDa protein in addition to the 40-kDa A1/EBP factor in all cell types (Fig. 5). Immunoprecipitation assays were performed to determine if this 70-kDa protein could be the Rel-related factor that interacts with A1/EBP to form the a1 LTR binding complex. The R2 and A1/EBP antisera both precipitate a 70-kDa protein from B cells under high-stringency conditions (Fig. 7A).

For sequential immunoprecipitations, low-stringency precipitation with A1/EBP antiserum was first performed (59). The precipitates were collected and washed, and the complexes were dissociated by heating in RIPA buffer. Rel proteins were then precipitated under high-stringency conditions with the R2 antiserum. The sequential immunoprecipitation experiments showed that α-A1/EBP coimmunoprecipitates the 70-kDa Rel-related factor, as the R2 antiserum is able to precipitate this factor from dissociated complexes initially immunoprecipitated by α-A1/EBP (Fig. 7B). The converse, however, does not appear to be the case, as α-A1/EBP is unable to bring down A1/EBP from dissociated complexes initially immunoprecipitated by the R2 antiserum. This outcome is not surprising, because under low-stringency conditions, R2 is unable to coimmunoprecipitate the 40-kDa protein (Fig. 7B). The R2 antiserum was made to the Rel homology domain of v-Rel, the region that appears to mediate the interaction with C/EBP (56). The R2 antiserum could dissociate the Rel-A1/EBP complex by binding to the Rel homology domain, thereby blocking A1/EBP binding. The A1/EBP antiserum, however, was made to a region N terminal to the interacting basic region domain, and as a result, the Rel-A1/EBP interaction is not interrupted. The coimmunoprecipitation of the p70 Rel factor by the A1/EBP antiserum is due to a specific interaction of these proteins rather than cross-reactivity of the A1/EBP antiserum with Rel

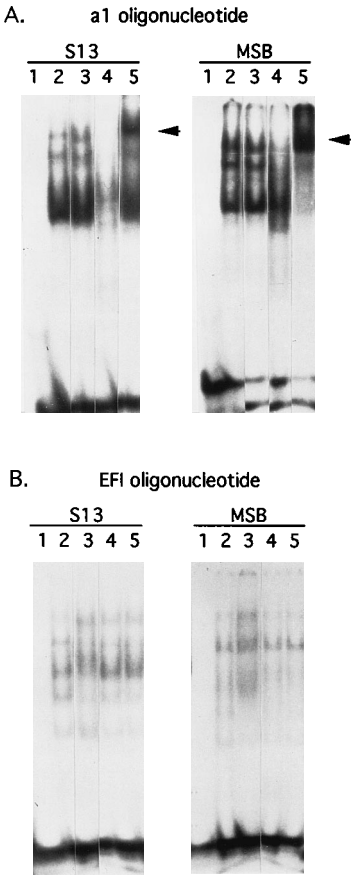

FIG. 6. A Rel-related factor(s) is a component of the a1 LTR binding complex. Nuclear extracts from S13 and MSB cells were incubated in gel shift reactions with various ^{32}P-labeled oligonucleotide probes alone (lanes 2), with rabbit preimmune serum (lanes 3), with A1/EBP antiserum (lanes 4), or with the R2 Rel-specific antiserum (lanes 5) prior to incubation with ^{32}P-labeled a1 LTR binding site oligonucleotide (A) or the EFI binding site oligonucleotide (B). Lanes 1 show the migration of probe alone. Samples were separated on native 5% polyacrylamide–TBE gels and visualized by autoradiography. The arrow indicates supershifted complexes.

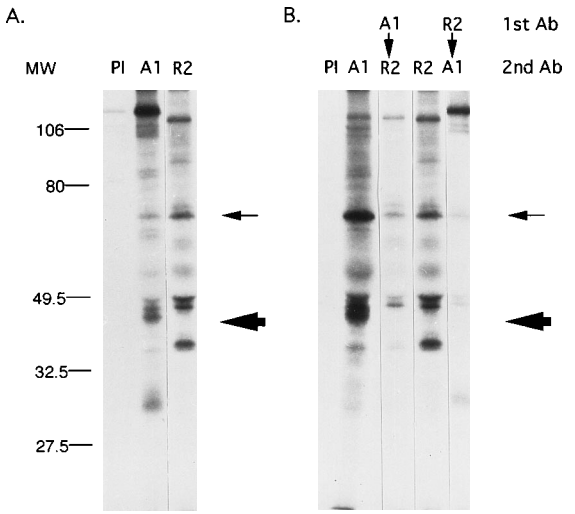

FIG. 7. A1/EBP interacts with a Rel-related factor in B cells. (A) S13 bursal lymphoma cells were metabolically labeled with [^{35}S]Met and [^{35}S]Cys, lysed in RIPA buffer, precleared, and incubated with rabbit preimmune serum (PI), A1/EBP antiserum (A1), or R2 Rel-specific antiserum (R2) and protein A beads. Samples were analyzed by SDS-PAGE and fluorography. The thin and thick arrows indicate the migrations of the 70-kDa Rel-related factor and the 40-kDa A1/EBP, respectively. Molecular weight (MW) is expressed in thousands. (B) Metabolically labeled S13 cells were analyzed by sequential immunoprecipitation assays (A1→R2 and R2→A1) with A1/EBP and R2 antisera (Ab). Other samples were immunoprecipitated only under low-stringency conditions (A1 and R2). Nonspecific proteins immunoprecipitated with preimmune serum (PI) are shown. The thin and thick arrows indicate the migrations of the 70-kDa Rel-related factor and the 40-kDa A1/EBP, respectively.

proteins. Figure 7B illustrates that the A1/EBP antiserum is unable to immunoprecipitate the 70-kDa Rel factor in the absence of A1/EBP (R2→A1 lane). These findings indicate that the Rel factor coimmunoprecipitates with the A1/EBP antiserum via its interaction with A1/EBP.

p65 (RelA) and p50 interact with A1/EBP in vitro. An in vivo complex containing A1/EBP and the 70-kDa Rel-related factor suggests that such an interaction plays a role in the regulation of LTR-enhanced transcription. To more directly analyze the A1/EBP-Rel interaction with known NF-κB/Rel proteins, two available murine Rel family members, p65 (RelA) and p50, were tested in in vitro GST retention assays for possible association with A1/EBP. The avian homologs of these proteins are closely related to those of mammals, sharing 71 and 86% identities, respectively, in the Rel homology domain region (25, 26). Murine p65 (RelA) migrates on SDS-PAGE gels close to 70 kDa, and previous investigators showed that the 70-kDa factor interacting with C/EBP factors in MSB T cells is antigenically related to p65 RelA (16). Two A1/EBP bacterial fusion constructs were used in this assay. The first consisted of the 209-amino-acid open reading frame of A1/EBP containing the basic region and leucine zipper domains fused to the GST protein (A1/EBP-GST), while the second construct consisted of the first 72 amino acids of A1/EBP fused to the GST protein (A1/EBP-TA-GST) (Fig. 1A). Both bacterial fusion proteins were coupled to glutathione-agarose beads for the purposes of the assay (56). T7 promoter-driven murine p50 and p65 constructs were used to program coupled in vitro transcription and translation reactions in the presence of [^{35}S]methionine and [^{35}S]cysteine (Fig. 8A). Both p65 and p50 bound to A1/EBP-GST beads but were unable to bind those coupled with A1/EBP-TA-GST (Fig. 8B), which encodes a segment of the N terminus but not the basic region and leucine zipper motifs. This result is in agreement with previous findings that the interaction between C/EBP-related factors and NF-κB/Rel members is mediated via the bZIP and Rel homology domains, respectively (56). The interactions of p50 and p65 with A1/EBP were not mediated by contaminating DNA (Fig. 8B), as similar results were obtained when experiments were performed in the presence of ethidium bromide (32). Only a small portion of the input p50 or p65 was retained on the GST columns, which could be due in part to the use of murine rather than avian Rel proteins. Posttranslational modification of the Rel protein in vivo could also be required for strong interaction with A1/EBP. ^{35}S-labeled S13 bursal lymphoma cell lysates were also incubated with fusion protein-conjugated agarose beads. Multiple proteins were retained by both bacterially expressed A1/EBP-TA and A1/EBP beads, but a 70-kDa protein appears to specifically bind to only the A1/EBP-GST beads (Fig. 8B).

p65 RelA increases ALV LTR CCAAT/enhancer element-driven transcription. To determine if the Rel-C/EBP interaction has functional significance for LTR CCAAT/enhancer element-driven transcription, a murine p65-containing eukaryotic expression construct (pRP65) was cotransfected into DT40 bursal lymphoma cells with a series of LTR-luciferase reporter constructs (Fig. 9A). An ALV LTR luciferase reporter plasmid, pALV-LUC (Fig. 9A), was constructed by cloning the ALV LTR enhancer and promoter sequences (bases −295 to +99) from the BK25 plasmid (50) into a luciferase reporter plasmid. Cotransfection of the RP65 expression construct with pALV-LUC increases LTR-driven luciferase expression by 30% in DT40 bursal lymphoma cells (Fig. 9B). However, cotransfection of pRP65 with pALVM123-LUC, a reporter construct which possesses mutations of the a1, a2, and a3 CCAAT/enhancer binding sites that abolish A1/EBP binding (54), does not affect luciferase expression, indicating that the LTR CCAAT/enhancer elements are required for p65 transactivation. The involvement of CCAAT/enhancer ele-

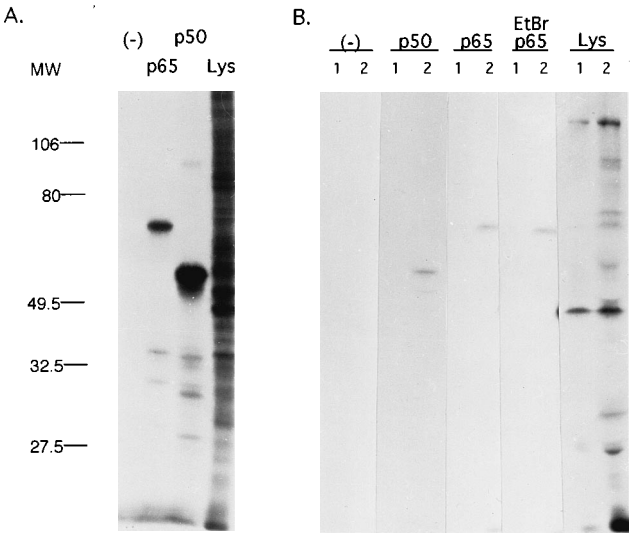

FIG. 8. A1/EBP interacts with p50 and p65 Rel family members in vitro. (A) ^{35}S-labeled in vitro transcription-translation reactions used a no-template DNA control (−), p65, or p50 expression plasmids. Samples were analyzed along with ^{35}S-labeled S13 bursal lymphoma cell lysate (Lys) by SDS-PAGE and fluorography. (B) ^{35}S-labeled in vitro transcription-translation reaction samples or labeled S13 cell lysate (as for panel A) was incubated with either bacterially expressed A1/EBP-TA- (lanes 1) or A1/EBP-conjugated (lanes 2) glutathione agarose beads. One p65 sample set was additionally incubated with ethidium bromide (EtBr p65). Binding proteins were eluted and analyzed by SDS-PAGE and fluorography. Molecular weight (MW) is expressed in thousands.

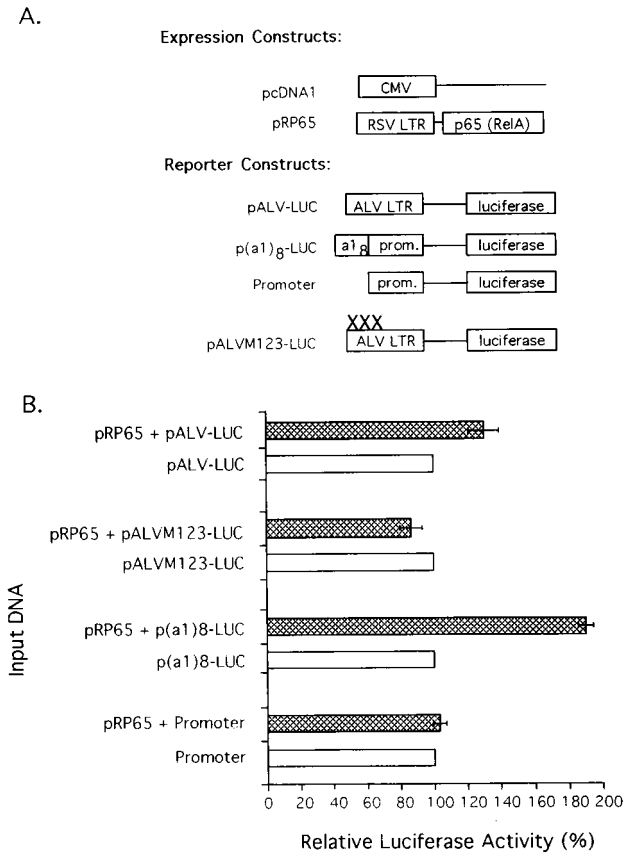

FIG. 9. p65 (RelA) enhances ALV LTR- and CCAAT/enhancer element-driven transcription. (A) Schematic of the pcDNA1 and pRP65 expression plasmids and the luciferase reporter plasmids used for transient cotransfection of DT40 B cells. (B) Results of transfection experiments with expression construct pcDNA1 or pRP65 and the pALV-LUC, pALVM123-LUC, $p(a1)_8$-LUC, or promoter-LUC reporter plasmids. Relative luciferase activities for transfection samples with pcDNA1 were arbitrarily set at 100%. Bars indicate standard errors ($n = 3$).

ments in p65-mediated transcriptional activation was more directly tested with reporter construct $p(a1)_8$-LUC, made by inserting eight copies of the a1 LTR binding site upstream of the ALV LTR promoter (bases −140 to +99) in the luciferase reporter plasmid (Fig. 9A). Cotransfection of pRP65 with this reporter construct shows a twofold activation of luciferase activity (Fig. 9B), indicating that p65 can act via the a1 LTR binding site in vivo to activate transcription. As a control, pRP65 was cotransfected with the ALV LTR promoter-driven reporter plasmid to determine if the effect of p65 is specific for enhancer-driven transcription. p65 expression had no significant effect on ALV LTR promoter-driven luciferase expression (Fig. 9B), further indicating that the activation effect observed for p65 is specific for the LTR CCAAT/enhancer elements. The effects of p65 expression on CCAAT/enhancer element-driven transcription appear to occur indirectly via a p65-C/EBP interaction, as p65 was unable to directly bind these CCAAT/enhancer elements in gel shift assays (data not shown).

DISCUSSION

The ALV and RSV LTRs are strong transcriptional enhancer and promoter units that may have evolved to utilize some of the more general cellular transcription factors to achieve high-level transcription in many cell types. The LTR enhancers contain multiple overlapping CCAAT/enhancer elements that are recognized by C/EBP-related transcription factors. These proteins may act in concert to influence LTR transcriptional enhancement by forming heterodimers, or they may bind independently. A1/EBP and VBP (27, 54) are good candidates to mediate the widespread high levels of LTR-enhanced transcription, as they are ubiquitously expressed. Other C/EBP family members that can also bind these motifs show tissue-restricted expression and are apparently not expressed in B cells (1, 6, 29). Moreover, both the long and short forms of C/EBPβ repress transcription mediated by LTR CCAAT/enhancer elements (52). The Ig/EBP gene is thought to encode a truncated basic region-leucine zipper protein that also represses LTR CCAAT/enhancer element-driven transcription (3, 38), suggesting that other transcriptional activator proteins must mediate the high rates of LTR-enhanced retroviral transcription observed in vivo. A1/EBP is nearly identical to Ig/EBP in the basic region and leucine zipper domains, while the amino-terminal region diverges from that of Ig/EBP (8). This longer cDNA could encode an amino-terminal transactivating region in addition to the basic region and leucine zipper domains found in Ig/EBP, and this region could activate transcription from the ALV and RSV LTR CCAAT/enhancer elements. The observed 40-kDa size of the A1/EBP protein supports the hypothesis that this C/EBP-related factor could encode a larger transactivating protein. Until the full-length coding sequence of A1/EBP is obtained, however, the transactivating potential of this protein cannot be fully assessed.

NF-κB/Rel–C/EBP interactions which may have important effects on ALV and RSV LTR-enhanced transcription have recently been described. The effects of these interactions on transcriptional activity were found to be dependent upon binding site context, and this effect was not dependent on DNA binding by both factors (56). If CCAAT/enhancer sites are present, as would be the case for the LTR, a C/EBP–NF-κB interaction can lead to transcriptional activation; however, if κB-binding sites are present, an NF-κB–C/EBP interaction usually results in decreased transcriptional activity. This result suggests that the transcriptional effect of this interaction depends upon the nature of the interacting proteins and the context of the binding site. We found that A1/EBP interacts with a 70-kDa Rel-related family member in avian cells and also with murine p65 (RelA) and p50 in vitro. This Rel-related protein is a component of the a1 LTR binding complex as detected by gel shift assay, and the presence of this Rel factor in the complex appears to be due to protein-protein interactions, as its presence does not appear to be mediated by direct binding of the Rel protein to CCAAT/enhancer elements. Cotransfection of p65 (RelA) with the LTR CCAAT/enhancer element-driven reporter constructs leads to the activation of transcription, further suggesting that a Rel-A1/EBP interaction could have important effects on ALV and RSV LTR-enhanced expression. We do not yet know which of the Rel family members participates in this interaction, as all of these factors share the Rel homology domain region. The avian p65 and c-Rel proteins are both approximately 70 kDa in size (26), so either or both could be the 70-kDa Rel species. The finding that A1/EBP can interact with murine p50 in vitro suggests that this factor could also be involved in LTR binding.

NF-κB was first believed to be a B-cell-specific, DNA-binding protein that binds a *cis* regulatory element within the enhancer of the Ig(κ) light-chain gene (53). Although its natural occurrence appears to be restricted to mature B lymphocytes, NF-κB activity can be induced in pre-B cells and other cells by treatment with bacterial LPS, phorbol esters, interleukin-1, tumor necrosis factor alpha, calcium ionophores, lectin, anti-

gen, and DNA-damaging agents such as UV light (5). NF-κB and C/EBP factors are involved in the regulation of many genes, including those encoding Igs, cytokines, and acute phase response proteins (1, 19). Interactions of A1/EBP with Rel family members could influence the transcriptional activity of CCAAT/enhancer elements in the viral LTR or in cellular genes and could also potentially influence the activity of genes containing NF-κB binding sites.

A1/EBP and the Rel-related factor both contribute to the labile LTR CCAAT/enhancer element binding activity in B cells and the stable binding activity in T cells, suggesting that these interactions may be differentially regulated in each cell type. These factors could potentially regulate the lability of LTR-enhanced transcription and the ALV lymphoma susceptibility of immature B cells. We previously proposed that the labile LTR-enhancing factors may be down-regulated early in B cell development so that lower levels of LTR-driven c-*myc* expression permit target cell survival and further steps in oncogenesis (7). In lymphoma-resistant chicken strains, the observed stable high levels of LTR-driven transcription could eliminate these tumor target cells through the cytotoxic effects of c-*myc* hyperexpression. Examination of the developmental expression and activity of C/EBP and NF-κB family members should give insight into the genetic basis for the labile regulation of LTR-enhanced c-*myc* transcription in immature B cells and for the susceptibility of these cells to ALV tumor induction.

ACKNOWLEDGMENTS

We thank Dennis McCance, Stephen Dewhurst, Sally Kent, and Sheila Curristin for advice and Kelly Bird, Joseph Petrosino, and Thanh Vu for excellent technical assistance. We also thank Mark Hannink for providing the R2 antiserum.

This work was supported by a Leukemia Society of America Special Fellowship and NIH grant CA54768 to A. Ruddell. W. J. Bowers was supported by NIH grant AI07362, and L. A. Baglia was supported by NCI grant CA09363.

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