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Tissue-Specific Lability and Expression of Avian Leukosis Virus Long Terminal Repeat Enhancer-Binding Proteins

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Avian leukosis virus (ALV) induces bursal lymphomas in chickens, after proviral integration next to the cellular *myc* proto-oncogene, and subsequent c-*myc* hyperexpression. Our previous work suggested that labile or short-lived cellular proteins interact with the viral long terminal repeat (LTR) enhancer, and binding of these proteins appeared to be essential for high rates of LTR-enhanced transcription (A. Ruddell, M. Linial, W. Schubach, and M. Groudine, J. Virol. 62:2728–2735, 1988). This lability is specific for B-lymphoid cell types, since T cells and fibroblasts show stable high rates of LTR-enhanced transcription and stable LTR-binding activity. Moreover, the lability of these proteins may be important in determining susceptibility to bursal lymphoma. In this study, we separated and characterized the labile and stable LTR-binding proteins and examined their lability and expression in different cell types. Gel shift and DNase I footprinting analyses indicated that at least five proteins interact with the 140-base-pair LTR enhancer region. These proteins were distinct by several criteria, including lability or stability after inhibition of protein synthesis, resistance to heat denaturation, chromatographic behavior, and expression in different cell types. Two binding proteins were present in many cell types and were specifically labile in B cells. A third binding protein showed hematopoietic-cell-type-specific expression and was also labile in B cells. These findings indicate that there is tissue-specific modulation of the lability and expression of ALV LTR-binding proteins, which may be important for regulation of LTR transcription enhancement and ALV bursal lymphomagenesis.

Avian leukosis virus (ALV) provides a model system for analysis of the role of viral and cellular factors in retroviral oncogenesis. ALV specifically induces bursal lymphomas in chickens, after proviral integration next to the *c-myc* proto-oncogene, resulting in c-*myc* hyperexpression (20, 33). Other ALV strains can induce erythroblastosis after integration next to c-*erb* (14) or rapid-onset B-cell lymphoma after integration next to c-*myb* (24, 35). Both the viral long terminal repeat (LTR) and viral structural genes (*env*, *pol*, and *gag*) can modulate viral infection and expression in different tissues (6, 7, 37). A strong LTR enhancer is important for oncogenesis, since endogenous viruses lacking the enhancer region are inefficiently expressed and rarely induce tumors (6, 37, 41).

Host cellular factors are also important in ALV oncogenesis. ALV infects many tissues, but the majority of the tumors are bursal lymphomas, induced in bursal stem cells present before 2 weeks posthatching (31). Infection of older birds does not induce tumors (8, 27). Moreover, some strains of birds are resistant to ALV lymphomagenesis, although they show high levels of viral infection in the bursa and other tissues (2, 13). Transplantation studies indicate that this host resistance is encoded by the target bursal cells (36). The first sign of ALV tumorigenesis is the appearance of transformed bursal follicles in susceptible birds a few weeks after infection (31), whereas transformed follicles are rarely found in resistant birds (3). Viral integration next to c-*myc* is observed in almost all transformed follicles (11), indicating that viral regulation of c-*myc* expression is an important early step in transformation.

Our recent experiments suggest that the host factors act by differentially regulating c-*myc* expression in target bursal cells. LTR-enhanced c-*myc* and viral gene transcription is specifically decreased 10- to 15-fold after inhibition of protein synthesis in bursal lymphoma cells, whereas LTR-enhanced transcription is unaffected by inhibition of protein synthesis in T cells or fibroblasts (26). These findings suggest that a short-lived protein regulates LTR enhancer activity in bursal lymphoma cells. Labile LTR-enhanced transcription is also observed in ALV-infected bursal cells before 2 weeks after hatching, the same age when birds are susceptible to ALV lymphomagenesis (38). LTR-enhanced transcription is stable in mature bursal cells, when birds are no longer susceptible to tumor induction. Labile transcription is also observed in bone marrow and spleen from young or mature birds. In contrast, lymphoma-resistant strains of birds show stable LTR-enhanced transcription in all hematopoietic tissues, including the immature bursa. Taken together, these data suggest that the labile character of LTR-enhanced transcription regulates ALV tumor induction in bursal cells. The resulting effects on c-*myc* oncogene expression (c-*myc* RNA and protein are also short lived [10, 19]) could play a critical role in tumor susceptibility.

Cellular sequence-specific DNA-binding proteins appear to be the determinants of viral and cellular enhancer activity (reviewed in references 23 and 28). These proteins could mediate the labile and stable patterns of LTR transcription enhancement observed in different cell types and strains. We previously used the gel shift assay (12, 17) to show that several DNA-binding proteins interact with the ALV LTR enhancer (38). A subset of the proteins is specifically labile in B cells (decreased in binding activity after inhibition of protein synthesis), whereas the binding activity of these proteins is stable in T cells and in fibroblasts. This lability strictly correlates with the lability of LTR-enhanced transcription in B-cell types, suggesting that labile protein expression is required for high rates of LTR enhancement in

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B cells. The proteins are expressed regardless of whether cells are infected with virus, indicating that they are under cellular control (38).

In this study, we characterized the labile and stable proteins interacting with the ALV LTR. DNase I footprinting and oligonucleotide gel shift analyses were used to determine the sequences involved in protein binding. Three labile and two stable proteins have been identified that interact with the LTR enhancer in bursal lymphoma cells. In contrast, the binding proteins are stable in T cells and in fibroblasts. One binding protein is restricted to hematopoietic cell types, indicating additional potential for tissue-specific regulation of LTR enhancer activity.

MATERIALS AND METHODS

Nuclear extract protein purification. S13 bursal lymphoma cells, MSB thymocytes (Marek's disease virus-transformed T cells [1]), and primary chicken embryo fibroblasts (CEF) were cultured with or without 0.1 mM emetine dihydrochloride (Sigma Chemical Co.) for 3 h as previously described (38). Nuclei were purified from 2 liters of S13 or MSB cultures or from 40 15-cm-diameter CEF plates, and proteins were sequentially extracted with 0.1 and 0.5 M NaCl as previously described (38). Proteins were precipitated in 45% $(NH_4)_2SO_4$ and suspended in buffer A (20 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid [HEPES; pH 7.9], 15% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium metabisulfite, 0.2 ng each of leupeptin and pepstatin per ml, 1 U of aprotinin per ml, 0.2 mM phenylmethylsulfonyl fluoride) plus 0.1 M NaCl. Protein concentration was determined by the Bradford assay (5).

The 0.5 M NaCl-extracted S13 protein was applied to a phosphocellulose column (Whatman P11) and eluted with 0.1 to 0.7 M NaCl-buffer A. The 0.4 to 0.6 M NaCl-phosphocellulose fractions were pooled and dialyzed against 0.1 M NaCl-buffer A. This fraction was further purified by 10 min of heat treatment at 85°C, followed by a 15-min centrifugation at 10,000 × *g* at 4°C, to remove precipitated protein. The supernatant was designated the heat-purified protein.

The phosphocellulose flowthrough protein was further purified by heparin-agarose chromatography (Organon Teknika), with 0.1 to 0.5 M NaCl-buffer A step gradient elution. The 0.25 to 0.35 M NaCl fractions were dialyzed against 0.1 M NaCl-buffer A and were designated the heparin-agarose-purified protein.

Preparation of DNA. The 245-base-pair (bp) *Mst*II-*Eco*RI fragment of an ALV LTR from BK25 bursal lymphoma cells was ligated to *Bam*HI linkers and cloned into pUC9. The sequence of this clone (provided by B. Steiner and B. Schubach) is almost identical to that of other Rous-associated virus-2 LTRs (4). The 245-bp LTR fragment was isolated by *Bam*HI digestion and electrophoresis on 8% polyacrylamide gels, eluted, and purified (29). DNA was ^{32}P labeled by Klenow reaction or by T4 polynucleotide kinase addition, followed by *Sph*I digestion and polyacrylamide gel purification. For DNase I footprinting assays, the ALV LTR plasmid was digested with *Eco*RI or *Hin*dIII, ^{32}P labeled by kinase addition, and digested with *Hin*dIII or *Eco*RI to give fragments labeled on the coding or noncoding strand, respectively.

Complementary LTR oligonucleotide sequences were generated by using an Applied Biosystems synthesizer, purified on 8 M urea–10% polyacrylamide gels, hybridized, and ^{32}P labeled by kinase addition or Klenow reaction (29). The oligonucleotide probes used are as follows:

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a1 5'-GAATGTAGTCTTATGCAATACTCTAATGCAATACT-3'
      3'-CATCAGAATACGTTATGAGATTACGTTATGCTTTA-5'
a3 5'-CATGCTTATGTAACGATGAGCTTCA-3'
       3'-AATACATTGCTACTCGAAGTGTACG-5'
b  5'-AAGGAGAGAAAAAGTACCGTGCATG-3'
   3'-TTCCTCTCTTTTTCATGGCACGTAC-5'
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Gel shift assay. Protein (2 to 8 μg) was incubated in a 15-μl reaction mix (100 μg of poly(dI-dc) · poly(dI-dC) [Pharmacia, Inc.] per ml, 10 mM Tris hydrochloride [pH 8], 50 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol) for 10 min on ice before addition of 0.01 to 0.1 ng of ^{32}P-labeled probe DNA (34). Reaction mixes were incubated at room temperature for 30 min, and protein-DNA complexes were resolved on 4% polyacrylamide gels in Tris acetate buffer (6.7 mM Trizma, 3.3 mM sodium acetate, 1 mM EDTA [pH 7.5]) at 25 mA for 60 to 90 min. For competition experiments, unlabeled double-stranded oligonucleotides (30- to 90-fold molar excess) were incubated with nuclear proteins on ice for 5 min before addition of ^{32}P-labeled probe. In some experiments, poly(dI-dC) was omitted from the reaction mix, and protein-DNA complexes were resolved on 0.375 M Tris glycine–10% polyacrylamide gels in 0.04 M Tris glycine buffer (pH 8.5) at 30 mA for 2 h (42).

DNase I footprinting. Gel shift reactions were scaled up to 60 μl, using ALV LTR DNA ^{32}P labeled on the coding or noncoding strand, and were treated with 40 to 100 ng of DNase I (Sigma) per ml in 5 mM $MgCl_2$–5 mM $CaCl_2$ for 1 min (15). Free DNA and DNA-protein complexes were separated on Tris acetate-polyacrylamide gels as described above. After autoradiography to locate bands, the free DNA and DNA-protein complexes were eluted from the gel, proteinase K digested, phenol extracted, and ethanol precipitated. DNA was resolved on 8 M urea–10% polyacrylamide gels along with purine cleavage products (29).

RESULTS

Separation and purification of ALV LTR-binding proteins. Our previous gel shift analyses (38) indicated that several proteins interact with the ALV LTR U3 enhancer (*Mst*II-*Eco*RI; Fig. 1A). The proteins interacting with the 150-bp AB restriction fragment (Fig. 1A) were labile in B-lymphoid cell types. In contrast, a single stable binding activity interacted with the 95-bp C restriction fragment (*Sph*I-*Eco*RI; Fig. 1A). These LTR-binding activities from S13 bursal lymphoma cells were separated by phosphocellulose chromatography. Several AB LTR-binding activities were differentially eluted from the column by a 0.1 to 0.7 M NaCl gradient, as detected by gel shift assay (Fig. 1B). The slowly migrating AB-binding proteins were enriched in the flowthrough (0.1 M NaCl), whereas the rapidly migrating AB-binding proteins eluted with 0.4 to 0.6 M NaCl, suggesting that at least two proteins interact with this region. The C-binding protein was enriched in the flowthrough, and a small amount eluted with 0.4 to 0.5 M NaCl, suggesting that the stable C-binding protein is also heterogeneous.

The slowly migrating AB-binding proteins (0.1 M phosphocellulose flowthrough) were purified about fivefold by 0.25 to 0.35 M NaCl elution from a heparin-agarose column (data not shown). The rapidly migrating AB-binding proteins (0.4 to 0.6 M NaCl-phosphocellulose fractions) were purified about fivefold by heat treatment. These proteins appear to be the only LTR-binding proteins that are resistant to heat denaturation (38). The AB LTR-binding activities of each preparation are shown in Fig. 1C. The differential purification and gel shift mobilities of the heparin-agarose- and

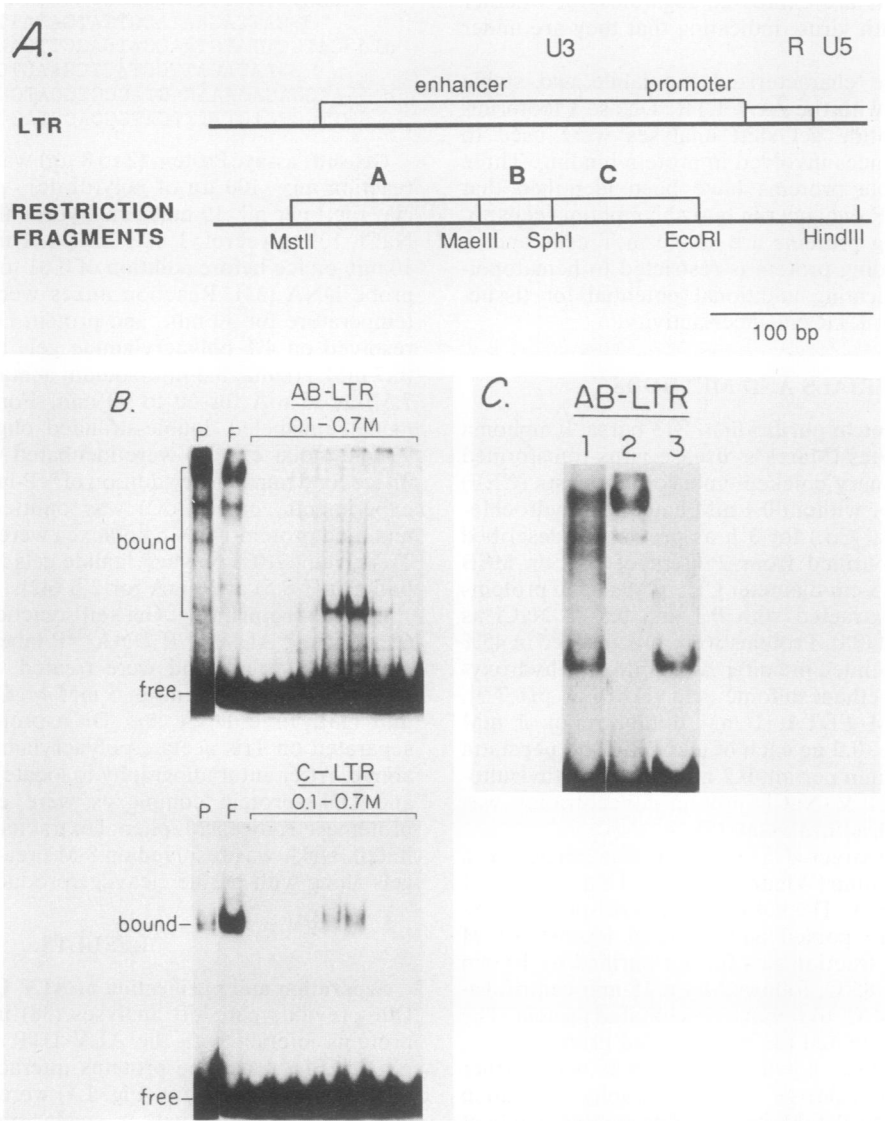

FIG. 1. Gel shift analysis of ALV LTR-binding protein purification. (A) Map of restriction fragment probes A, B, and C from the ALV LTR enhancer. (B) Gel shift analysis of phosphocellulose column fractionation of AB and C LTR-binding proteins from S13 cells. Protein (0.5 M NaCl extracted) was applied to the column in 0.1 M NaCl (lane P, precolumn protein), washed with 0.1 M NaCl (lane F, flowthrough), and eluted with 0.1 to 0.7 M NaCl. Samples (3 μg) of alternate fractions were incubated with ^{32}P-labeled AB or C LTR restriction fragments and resolved on Tris acetate-gels (see Materials and Methods). DNA-protein complexes (bound) and DNA (free) are indicated. (C) Analysis of 0.5 M NaCl-extracted crude nuclear protein (5 μg; lane 1), heparin-agarose-purified protein (2 μg; lane 2), and heat-purified protein (2 μg; lane 3) from S13 cells in Tris acetate-gel shift assays with the ^{32}P-labeled AB LTR restriction fragment.

heat-purified fractions indicated that at least two different proteins interacted with the AB LTR enhancer region.

Identification of LTR DNA-binding sequences. The LTR-binding sites of crude and purified nuclear proteins were identified by using the DNase I footprinting technique (15). Protein was incubated with ^{32}P-end-labeled LTR DNA (245-bp *Mst*III-*Eco*RI fragment; Fig. 1A), digested with DNase I, and separated on Tris acetate-polyacrylamide gels. DNA-protein complexes and free DNA were eluted from the gel, purified, and resolved on 8 M urea–10% polyacrylamide gels. The DNase I protection patterns of protein from S13 bursal lymphoma cells and CEF were compared, since these cell types express similar binding proteins except that the proteins are labile in S13 cells and stable in CEF (38).

The proteins interacting with the BC region of the LTR were preferentially enriched in 0.1 M NaCl nuclear extracts, as judged by gel shift assays (data not shown). Footprinting analysis using these extracts identified a large DNase I-protected region in the BC LTR region (Fig. 2). The protected sequences are illustrated in Fig. 3. Protein binding was much stronger on the coding strand than on the noncoding strand and was observed in S13 and in CEF extracts.

The proteins interacting with the AB LTR region were preferentially eluted from nuclei by 0.5 M NaCl (data not shown). The major DNase I-protected region obtained by using 0.5 M NaCl S13 and CEF extracts was observed at the 5′ border of the U3 region and designated a1 (Fig. 4). An adjacent, very weak binding site was designated a2. The protected sequences are illustrated in Fig. 3. CEF proteins consistently showed a stronger footprint than did proteins

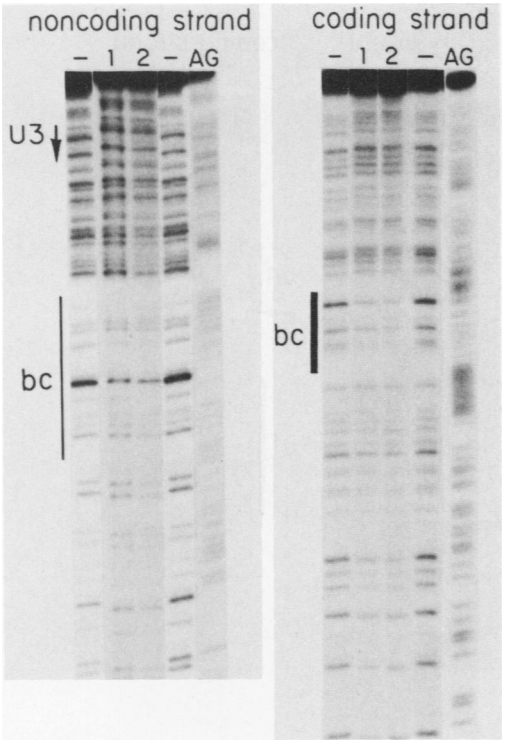

FIG. 2. DNase I footprinting of the AVL LTR, using 0.1 M NaCl nuclear extracts. The LTR probe was ^{32}P labeled on the noncoding or coding strand, bound to 0.1 M NaCl-extracted nuclear proteins, and digested with DNase I. DNA-protein complexes (S13, lane 1; CEF, lane 2) and free DNA (−) were separated by Tris acetate-gel electrophoresis, purified, and resolved on 8 M urea–10% polyacrylamide gels along with AG cleavage products. The bar indicates strongly DNase I-protected sequences, and the line indicates weakly protected sequences. The 5′ border of the U3 LTR is indicated.

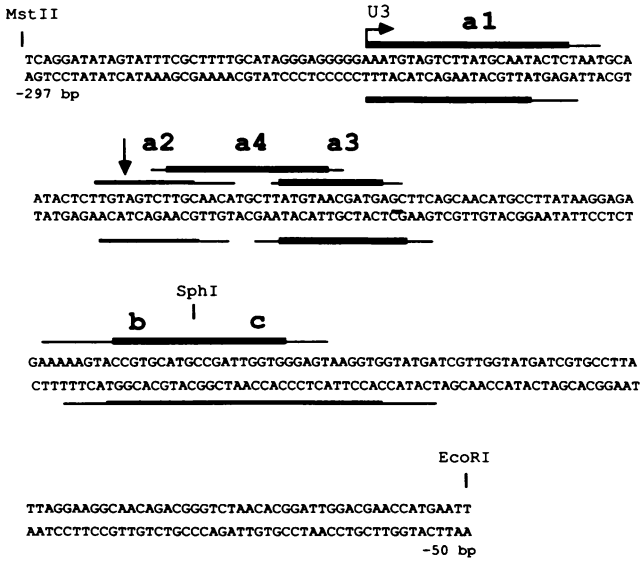

FIG. 3. Map of protein-binding sequences in the ALV LTR U3 enhancer. The DNA sequence of the BK25 ALV LTR between *Mst*II and *Eco*RI sites is shown. The 5′ border of the U3 region is indicated. Protein-binding sites are outlined with a bar (strongly protected regions) or line (weakly protected or uncertain regions). The distance from the transcription start site is shown in base pairs.

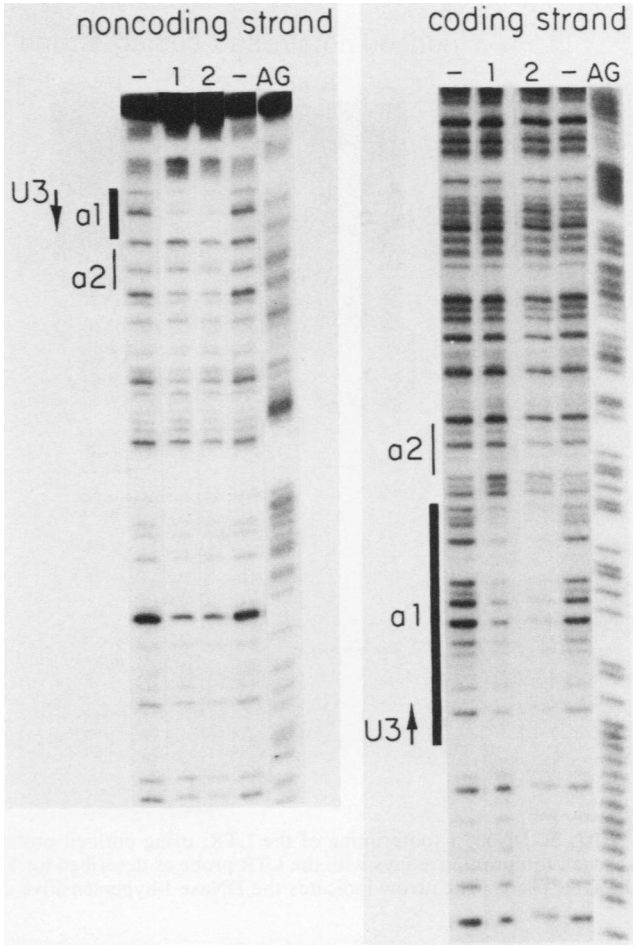

FIG. 4. DNase I footprinting of the LTR, using 0.5 M NaCl nuclear extracts. ^{32}P-labeled ALV LTR probe was bound to 0.5 M NaCl extracts from S13 cells (lane 1) or CEF (lane 2) and digested with DNase, and DNA-protein complexes and free DNA (−) were purified and analyzed on 8 M urea–10% polyacrylamide gels as described for Fig. 2.

from S13 cells, even though these cell types show similar gel shift LTR-binding activities (38).

The ALV LTR-binding sites of purified S13 proteins were also examined. The slowly migrating AB-binding protein in the heparin-agarose fraction footprinted a novel LTR region designated a3 (Fig. 5A). This footprint was not easily detected by using crude extracts, perhaps because binding at the a1 and a2 sites obscured footprinting of this adjacent site (Fig. 3).

The heat-purified AB-binding proteins footprinted the a1 site and a novel site, designated a4, observed primarily on the coding strand (Fig. 5B). A DNase I-hypersensitive site was observed between these protected regions. The a4 site footprinted by heat-purified protein overlapped the a3 site footprinted by heparin-agarose-purified protein (Fig. 3), suggesting that two distinct proteins can interact with these sequences.

Analysis of individual LTR protein-binding sites. We synthesized double-stranded oligonucleotides complementary to the a1-, a3-, and b-region binding sequences to further characterize the individual LTR-binding proteins by gel shift analysis (see Materials and Methods). The a1 probe interacted with several diffuse binding activities in S13 extracts

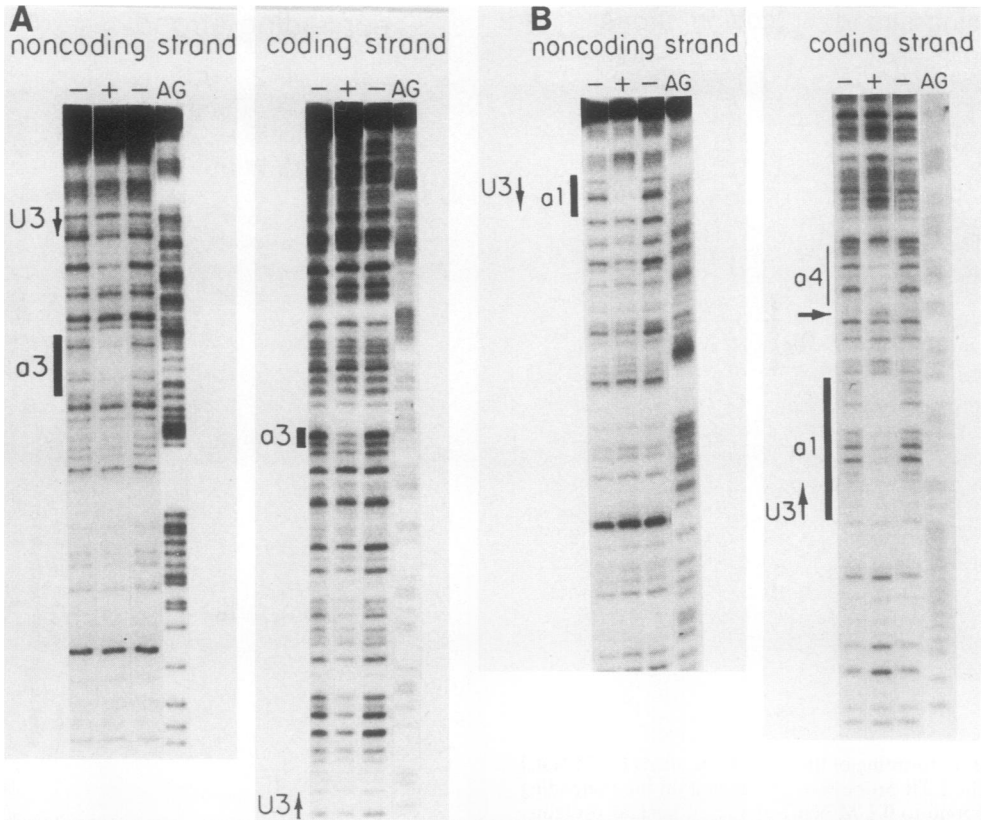

FIG. 5. DNase I footprinting of the LTR, using purified protein. (A) Heparin-agarose-purified protein from S13 cells (+) was used in DNase I footprinting assays with the LTR probe as described for Fig. 2. (B) Heat-purified protein from S13 cells (+) was used in assays with the LTR probe. The arrow indicates the DNase I-hypersensitive site.

(Fig. 6). This result suggested that the a1 probe contained more than one binding site, that a single binding protein is able to multimerize, or both. These binding activities were enriched after heat treatment but not after heparin-agarose purification (Fig. 6), in agreement with our finding that the heat-resistant protein primarily footprinted the a1 site (Fig. 5B).

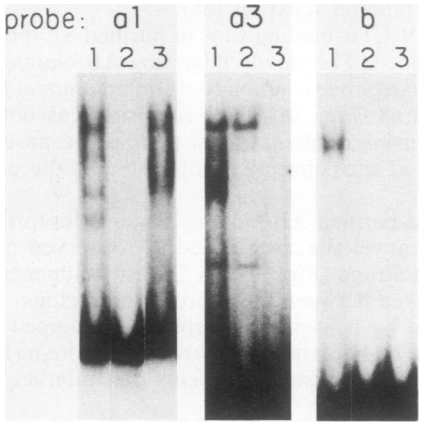

FIG. 6. Gel shift analysis of crude and purified B-cell proteins, using oligonucleotide probes. The a1, a3, and b ^{32}P-labeled LTR oligonucleotide probes were incubated with 8 μg of 0.5 M NaCl extract from S13 cells (lane 1), 3 μg of heparin-agarose-purified protein (lane 2), or 3 μg of heat-purified protein (lane 3) and resolved on Tris acetate-gels.

The a3 oligonucleotide probe includes the a3 binding site and most of the overlapping a4 binding site. The slowly migrating a3-binding protein was enriched by heparin-agarose chromatography (Fig. 6), in agreement with the DNase I footprinting data (Fig. 5A). In contrast, the rapidly migrating proteins, presumably interacting with the a4 site, were not enriched by heparin-agarose chromatography (Fig. 6).

The BC region of the LTR contains a large DNase I-protected sequence spanning the *Sph*I site (Fig. 3), which could involve the binding of more than one protein. We previously used gel shift analysis to identify a stable C-binding protein that binds to sequences 3′ to the *Sph*I site (38). We synthesized an oligonucleotide complementary to sequences 5′ of the *Sph*I site (b probe) to determine whether an additional protein binds to this sequence. There was a single b-binding protein in crude extracts, which was not enriched by heparin-agarose chromatography or by heat treatment (Fig. 6).

Identification of labile protein-binding sites in the LTR. We were interested in determining which of the AB LTR-binding sites interacts with labile proteins, since the labile character of these proteins appeared to be important for regulation of LTR transcription enhancement and bursal lymphomagenesis. We previously analyzed the overall lability of the proteins in a number of cell types and found that most of the AB-binding activity is labile in bursal lymphoma cells and other B-lymphoid cell lines, whereas it is stable in MSB T cells or in CEF (38). Figure 7 shows representative results obtained by using the a1 oligonucleotide probe in gel shift assays with equal amounts of extract from control cells or

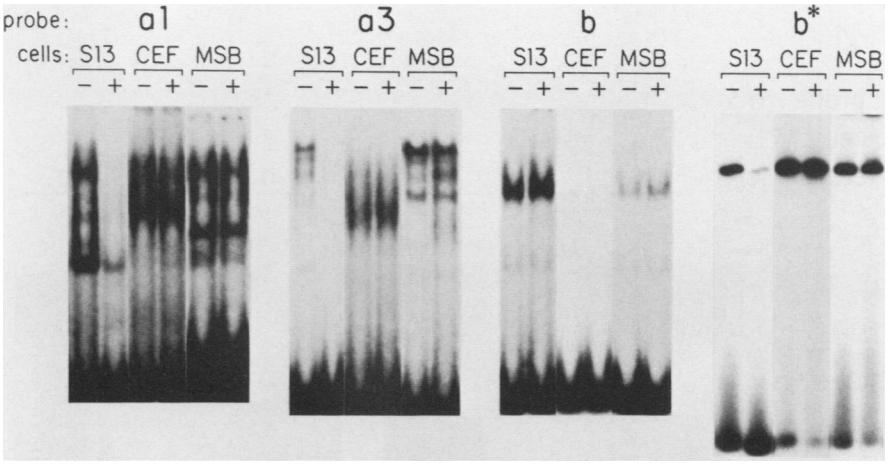

FIG. 7. Lability or stability of binding proteins in different cell types. Shown are Tris acetate gel shift analyses of 0.5 M NaCl-extracted proteins (8 μg) from control (−) or emetine-treated (+) S13, CEF, and MSB cells, using the ^{32}P-labeled a1, a3, or b oligonucleotide probe. Tris glycine gels were used to analyze b*-binding proteins (3 μg) interacting with the ^{32}P-labeled b oligonucleotide probe.

cells in which protein synthesis was inhibited by emetine treatment. The a1-binding proteins were labile in S13 cells; i.e., they showed decreased binding activity after inhibition of protein synthesis. The a1-binding proteins of CEF and MSB extracts were similar to those of S13 extracts. However, these binding proteins were stable after emetine treatment, and LTR-enhanced transcription is stable in these cells (38).

The a3-binding proteins were also labile in S13 B cells and stable in CEF or MSB cells (Fig. 7). The rapidly migrating proteins were expressed in all three cell types. However, the slowly migrating protein was not expressed in CEF.

The b-binding protein was stable after inhibition of protein synthesis in S13 cells, in contrast to the labile a1- and a3-binding proteins (Fig. 7). This b-binding protein was also stable in extracts from CEF and MSB cells (Fig. 7), although it appeared to be selectively degraded during preparation of extracts from these cells and was often barely detectable by gel shift assay.

We previously identified a b-binding protein that is labile after inhibition of protein synthesis in B cells (38). This activity was detected by using Tris glycine gels instead of the Tris acetate gels used for the experiments described above. Figure 7 shows representative data obtained by using the b oligonucleotide probe in Tris glycine gel shift assays (b*). The b*-binding activity was labile in S13 cells and was stable and strongly expressed in CEF and MSB cells. In contrast, the b-binding protein detected with Tris acetate gels was expressed at low levels in CEF and MSB cells (Fig. 7). These data suggest that two distinct b-binding proteins are detected in each gel system, one which is labile (b*) and one which is stable (b) in B cells.

Gel shift assays were used to assess the sequence specificity of the LTR-binding proteins by adding excess unlabeled homologous or heterologous oligonucleotide competitors to each assay. The a1-, a3-, b-, and b*-binding activities were all sequence specific, since binding was inhibited by the homologous oligonucleotide but not by heterologous oligonucleotides (data not shown). However, one exception was observed in competition assays using the a3 oligonucleotide probe, which supports the idea that two different proteins interact with this oligonucleotide sequence. Binding of the slowly migrating a3-binding protein of S13 cells (arrow in Fig. 8A) was specifically inhibited by the unlabeled a3 oligonucleotide but not by the a1 or b oligonucleotide. In contrast, binding of the rapidly migrating proteins (brackets in Fig. 8A) was inhibited by both the a1 and a3 oligonucleotides. These data support the observation that the heat-resistant a1-binding protein also interacts with the a4 site (Fig. 3) included within the a3 probe. Binding of the a1 protein of S13 and CEF was inhibited by the a1 oligonucleotide but was only partially inhibited by the a3 oligonucleotide (Fig. 8B), suggesting that these proteins interacted with the a1 probe more efficiently than with the a3 probe.

DISCUSSION

Several approaches have been used to separate and characterize five B-cell proteins interacting with a 140-bp region of the ALV LTR. Table 1 summarizes the data from a number of experiments. Each protein is distinct on the basis of sequence specificity, lability or stability after inhibition of protein synthesis, expression in different cell types, resistance to heat denaturation, and elution from phosphocellulose columns. This complex pattern of protein binding is common in viral enhancers. For example, five proteins interact with a 225-bp region of the human immunodeficiency virus LTR (16), and six proteins interact with the 75-bp repeat of the Moloney murine leukemia virus LTR (45). This density of protein-binding sites may ensure high rates of viral transcription and also could allow developmental modulation of enhancer function. The human immunodeficiency virus LTR-binding protein NF-κB is expressed at high levels in T cells only after activation (30). One Moloney murine leukemia virus LTR-binding protein is T cell specific (N. Manley and N. Hopkins, personal communication). In the case of ALV, the B-cell-specific lability of the a1-, a3-, and b*-binding proteins appears to be important for regulation of LTR transcription enhancement during lymphomagenesis. The a3-binding protein is restricted to hematopoietic cell types and could also modulate tissue-specific LTR enhancer function.

The a1 protein-binding site contains two sequence elements (TGTAGTCTT and TTATGCAAT; Fig. 3) that are related to the enhancer-CCAAT elements found in many viral enhancers and promoters (T[T/G]NNG[C/T]AA[T/G], where N is any nucleotide [18, 22, 40]). The a2 and a4 binding sites also include enhancer-CCAAT elements related

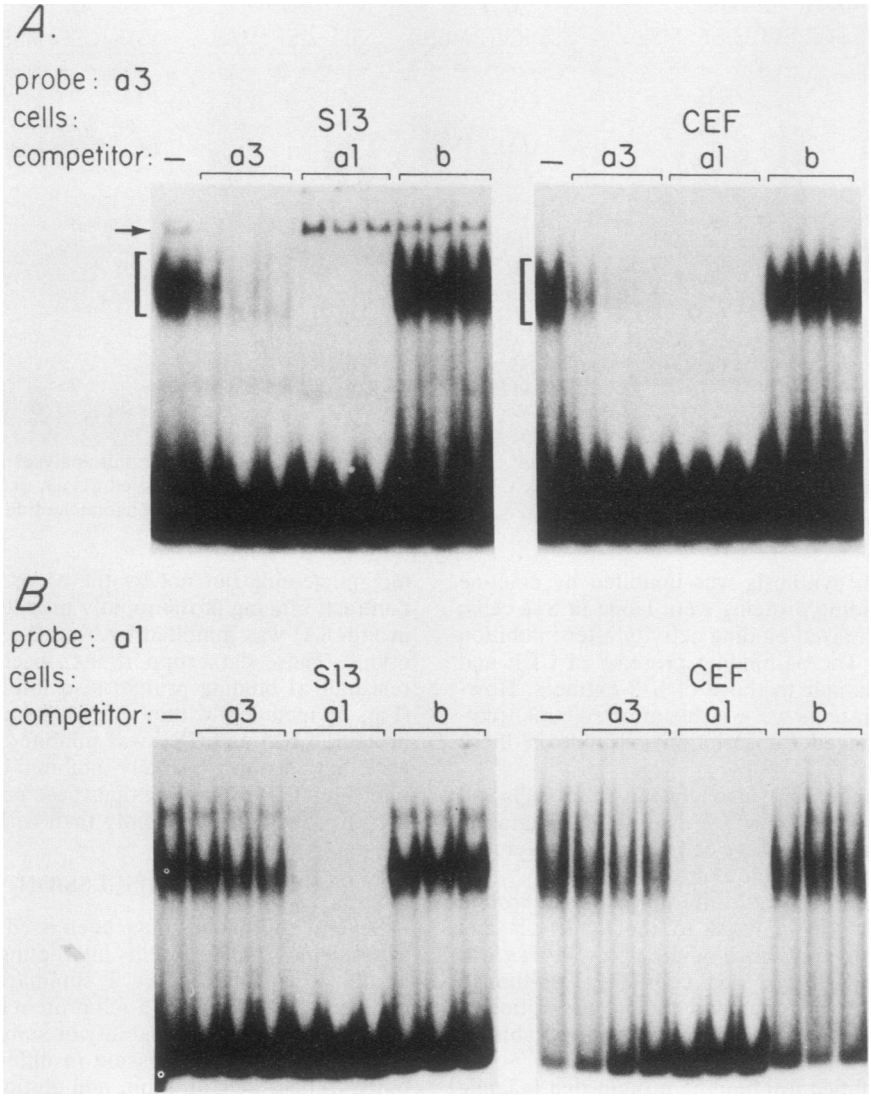

FIG. 8. Competition gel shift analysis of a1 and a3 oligonucleotide-binding proteins. (A) Tris acetate gel shift assays of a3 oligonucleotide-binding activity of 0.5 M NaCl-extracted S13 or CEF extracts (8 μg). No competitor (−) or 30-, 60-, and 90-fold molar excess of unlabeled a3, a1, or b oligonucleotide was added to each assay. The slowly migrating a3-binding protein is indicated by the arrow, and the rapidly migrating proteins are indicated by brackets. (B) Tris acetate gel shift assays of a1 oligonucleotide-binding activity of 0.5 M NaCl S13 or CEF extracts (8 μg).

to the a1 site sequences (TGTAGTCTT and TTATGTAAC, respectively; Fig. 3). This suggests that the same protein interacts with these four adjacent LTR sequence elements. These sequences are almost identical in the related Rous sarcoma virus Prague C (RSV) LTR (43, 46). A heat-resistant protein (C/EBP) that binds to the a1, a2, and a4 sites of the RSV LTR has been purified from rat liver (22). However, it is not yet known whether C/EBP is the a1-binding protein of B cells or whether there is a family of related CCAAT-enhancer-binding proteins (23).

Two distinct proteins interact with overlapping sequences in the a4 and a3 binding sites (Fig. 3). The RSV Schmidt-Ruppin A LTR contains a 5-bp deletion (ATGTA) in the a4 enhancer-CCAAT element relative to the ALV or RSV Prague C LTR (4, 46). This deletion abolishes heat-stable protein binding to the a4 site (40), whereas binding of the heparin-agarose-purified a3 protein is unaffected (data not shown). This suggests that the a4-binding protein interacts

TABLE 1. Characteristics of LTR-binding proteins

Protein	Lability in B cells[a]	Expression in[b]: S13 B cells	MSB T cells	CEF	Heat resistance[c]	NaCl concn (M) required for elution[d]
a1	+	+	+	+	+	0.4–0.6
a3	+	+	+	−	−	0.1
b	−	+	±	±	−	0.1
b*	+	+	+	+	−	0.1
c	−	+	+	+	−	0.1–0.5

[a] Effect of protein synthesis inhibition on LTR-binding activity in B cells. Lability (+) or stability (−) was measured by gel shift assays with oligonucleotide probes or LTR restriction fragments.

[b] Measured by gel shift assay.

[c] Measured by heat treatment of S13 nuclear extracts, followed by gel shift assay.

[d] 0.5 M NaCl-extracted S13 protein was bound to phosphocellulose and eluted with 0.1 to 0.7 M NaCl. Binding activity of column fractions was analyzed by gel shift assays.

with the enhancer-CCAAT element, whereas the a3-binding protein interacts with sequences 3′ of the element. These proteins could compete for binding to the overlapping a3 and a4 sites, to potentially modulate enhancer activity.

The DNase I-protected sequences surrounding the LTR *Sph*I site also interact with several proteins. Two distinct proteins that interact with sequences 5′ of the *Sph*I site were detected by using different gel shift conditions. The stable b-binding protein is strongly expressed only in B cells in Tris acetate gel shift assays, whereas the b*-binding protein is labile in B cells and is strongly expressed in all cell types in Tris glycine gel shift assays. These b-binding proteins may be uniquely sensitive to the different ionic conditions of Tris acetate and Tris glycine gels, since the a1- and a3-binding proteins show the same behavior in both gel systems (data not shown). Differences in binding-protein detection in different gel systems have also been observed for two of the Moloney murine leukemia virus LTR-binding proteins (Manley and Hopkins, personal communication) and for two c-*fos* serum response element-binding proteins (39). The stable C-binding protein binds to sequences 3′ of the *Sph*I site and appears to be distinct from the b- and b*-binding proteins, since it is stable and strongly expressed in all cell types (38). Moreover, the salt concentration required to elute the C-binding protein from phosphocellulose columns is different from that required to elute the b- and b*-binding proteins (Table 1). These data suggest that three proteins can interact with the BC LTR region.

The ALV and RSV LTR regions involved in enhancer function have been analyzed by DNA transfection assays, using normal and mutant LTR constructs in avian fibroblasts (9, 25, 32). These studies map the enhancer within the AB restriction fragment (Fig. 1A), between the 5′ U3 border and the *Sph*I site. This region includes the a1, a2, a3, a4, and b binding sites (Fig. 3). Although the deletions used in these studies were rather large, the data suggest that the entire protein-binding region is required for maximal enhancer activity.

The a1, a3, and b* LTR-binding proteins are specifically labile in B cells, in which inhibition of protein synthesis results in a large decrease in LTR-enhanced transcription (38). These binding proteins are stable or absent in T cells and fibroblasts, in which inhibition of protein synthesis does not affect LTR transcription enhancement. This strong correlation suggests that binding of some or all of the labile binding proteins is essential for LTR enhancer activity in B cells, whereas expression of the stable b- and c-binding proteins is not sufficient. Site-directed mutagenesis of each LTR-binding site and analysis of transcription in vivo will be required to determine the contribution of each protein to LTR enhancement.

Our previous experiments suggest that labile regulation of the LTR-binding proteins is important for ALV induction of bursal lymphoma (38). The cell-type-specific lability or stability of the a1-, a3-, and b*-binding proteins after inhibition of protein synthesis suggests that the half-lives of the proteins are differentially regulated or that the proteins are modified to an active form by a labile or stable enzyme, for example by phosphorylation or glycosylation (21, 44, 47). Purification of the labile binding proteins from B cells and comparison with the stable proteins of T cells will help define the factors regulating activity of the LTR-binding proteins and ALV induction of lymphoma.

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