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Lability of Leukosis Virus Enhancer-Binding Proteins in Avian Hematopoeitic Cells

A. RUDDELL,[1] M. LINIAL,[1] W. SCHUBACH,[2] AND M. GROUDINE[1,3]*

Division of Basic Sciences, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, Washington 98104[1]; Department of Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794[2]; and Department of Radiation Oncology, University of Washington School of Medicine, Seattle, Washington 98195[3]

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Bursal lymphomas induced by avian leukosis virus (ALV) are characterized by integration of long terminal repeat (LTR) enhancer sequences next to the *myc* proto-oncogene and by subsequent *myc* hyperexpression. Nuclear runoff transcription analyses have shown that protein synthesis inhibition specifically decreases transcription of LTR-enhanced genes in bursal lymphoma cell lines (M. Linial, N. Gunderson, and M. Groudine, Science 230:1126–1132, 1985). Here, we show that LTR-enhanced transcription is also labile in nontransformed bursa, bone marrow, and spleen but not in other ALV-infected tissues from lymphoma-susceptible chickens. The bursal cells demonstrated this lability of LTR-enhanced transcription only at an early stage of development, when chickens are susceptible to ALV-induced lymphomagenesis. Mature bursal cells show stable LTR transcription enhancement (unaffected by inhibition of protein synthesis) and are not susceptible to lymphomagenesis. In lymphoma-resistant chicken strains, LTR-enhanced transcription was stable in all tissues during development. These data suggest that lability of LTR transcription enhancement in hematopoeitic cells is involved in susceptibility to lymphomagenesis, and we propose a model for the action of these labile enhancing factors. Gel shift analysis of nuclear proteins from lymphoma cells indicated that four or more binding proteins specifically interact with the three LTR enhancer regions. These proteins can be separated by their differential sensitivity to heat treatment or protein synthesis inhibition. The lability of a subset of these binding proteins correlates with lability of LTR-enhanced transcription in certain lymphoid cell types, suggesting that these proteins are essential for LTR transcription enhancement.

The avian retroviruses provide a well-characterized system for studying the role of viral enhancer sequences in oncogenesis. Acute leukemia viruses that contain a viral *myc* oncogene rapidly induce leukemia and other tumors, whereas avian leukosis viruses (ALV) which do not contain an oncogene induce B-cell lymphomas more slowly, after a rare integration of the proviral LTR next to the cellular *myc* gene (20). In both cases, the resulting *myc* hyperexpression is regulated by enhancer domains in the U3 region of the long terminal repeat (LTR), which are usually integrated 5′, but can also be 3′, to c-*myc* in either orientation (12, 29, 36). Elevated *myc* levels are also observed in plasmacytomas in mice and Burkitt's lymphomas in humans (43, 45).

Analyses of ALV-induced bursal lymphoma cell lines homozygous for the *myc* allele containing proviral sequences have shown that the normal c-*myc* 5′ DNase I-hypersensitive sites disappear after ALV integration and new hypersensitive sites appear within the adjacent U3 LTR (41). Since hypersensitive sites are often indicative of protein-binding sites (e.g., see reference 14), these results suggest that the interaction of chromosomal proteins with the LTR enhancers is important in the hyperexpression of *myc* and viral genes (*gag*, *env*, and *pol*). Inhibition of protein synthesis in ALV bursal lymphoma cells causes a specific 5- to 10-fold reduction in *myc* and viral gene transcription (29). Under these conditions, initiation of c-*myc* transcription shifts from the LTR promoter back to the normal c-*myc* promoter, and the LTR hypersensitive site diminishes. These data suggest that a short-lived regulatory protein interacts with the LTR to enhance the transcription of linked genes. ALV-infected fibroblasts also show high rates of viral transcription, but in these cells LTR-enhanced transcription is not affected by protein synthesis inhibition. This suggests that the labile protein is limited to specific cell types or that its stability varies in different tissues.

ALV infects many cell types in bone marrow, blood, and bursa but induces tumors primarily in bursal cells (3). These clonal tumor cells produce immunoglobulin M (8, 11, 33). Tumor induction is possible only when birds are infected with ALV before hatching or within 2 weeks after hatching, while stem cells are homing to the bursa to differentiate and before the immune system is able to eliminate ALV-infected cells (3, 10, 39). This implies that only a specific subset of developing B cells supports tumor formation within the bursal environment.

Different strains of chickens show varying susceptibility to lymphomagenesis. SC and line $15I_5 \times 7$ birds show 50 to 100% tumor incidence after ALV infection, while the FP and line 6_3 strains develop tumors very rarely (0 to 3%). The time course and levels of viral infection are similar in all of these strains, although the FP strain shows heterogeneity in the extent of viremia (2, 16). These data suggest that cellular factors, different in each strain, play an important role in the development of lymphoma. These factors are required in target bursal cells, since bursal transplantation experiments show B-cell autonomy of susceptibility and resistance traits (38).

In this study, we used in vitro nuclear runoff transcription assays to determine the effect of inhibition of protein synthesis on LTR transcription enhancement in vivo. We found that in lymphoma-susceptible birds, LTR-enhanced transcription was labile in developing bursa, spleen, and bone marrow during the first 2 weeks after hatching, when birds are sensitive to ALV induction of lymphoma. In contrast,

* Corresponding author.

the factors enhancing transcription in lymphoma-resistant chicken strains were stable in hematopoeitic tissues throughout development. These data led us to propose a model for the role of labile LTR-enhancing factors in susceptibility to lymphomagenesis.

We used the gel shift electrophoresis assay (15, 17) to identify labile and stable LTR-binding proteins in nuclear extracts from bursal lymphoma cell lines. We found that several LTR-binding proteins interacted with three regions in the ALV LTR. A subset of these gel shift binding activities was labile in bursal lymphoma cells (decreased after inhibition of protein synthesis). The tissue-specific lability or stability of these binding proteins correlated with the lability or stability of LTR-driven nuclear runoff transcription in the different cell lines, suggesting that these proteins are involved in LTR transcription enhancement.

MATERIALS AND METHODS

Cell culture. S13, 243L1, and BK25 cell lines were cultured from bursal lymphomas of RAV-1- or MCAV-A-infected chickens (22, 28, 35) and grown in RP9 medium (Dulbecco modified Eagle medium supplemented with 5% calf serum, 1% heat-inactivated chick serum, and tryptose phosphate broth [GIBCO Laboratories]). The KBMC cell line was derived from avian reticuloendotheliosis virus strain T-infected bone marrow of SC chickens (26) and grown in RPMI (with supplements as described above). The SC3 cell line was cloned from avian reticuloendotheliosis virus strain T-infected spleen cells of SC chickens (Linial and Ruddell, unpublished data) and grown in RP9 medium. Marek's disease thymocytes (1) were grown in RPMI with supplements. Chicken embryo fibroblasts were obtained from 10-day-old embryos, infected with ALV at the first passage, and grown in CM medium (F10 supplemented with tryptose phosphate broth, 10% calf serum, 2% chick serum, vitamins, and folic acid; 27). Cells were used in the log phase of growth (10^6 cells per ml), and in some cases they were incubated in 0.1 mM emetine dihydrochloride (Sigma Chemical Co.) for 3 h.

Tissue preparation. Ten-day-old SC Hyline or FP chicken embryos were injected with ALV (supernatant from S13 bursal lymphoma cells) and sacrificed at different times after hatching. Tissues were minced and gently homogenized, and lymphoid cells were purified by centrifugation through Ficoll-Paque (Pharmacia, Inc.), followed by culturing in CM medium with or without emetine at 5×10^6 cells per ml. Transformed peripheral macrophages were prepared from 5-day-old SC chickens as previously described (27), by infecting blood cells with MC29 leukemia virus (supernatant from MC29-infected quail fibroblasts) and passaging them several times (27).

Nuclear runoff transcription. Nuclei were prepared from control or emetine-treated cells, stored at −70°C (in 40% glycerol, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 50 mM Tris hydrochloride, pH 8.0), and tested in nuclear runoff transcription assays (29). ^{32}P-labeled transcription products were hybridized to slot blots containing pUC-, *myc*-, *gag*-, *env*-, *fos*-, and glyceraldehyde-3-phosphate dehydrogenase gene-containing plasmids (29). By Northern (RNA) blot analysis, the *fos* plasmid detects several RNA species, in addition to *fos* mRNA, under high-stringency hybridization conditions. These RNAs are transcribed by RNA polymerase II, as measured by α-amanitin sensitivity in runoff assays. Thus, although the identities of all transcripts that hybridize to the *fos* plasmid are not known, they serve as an additional control in runoff assays.

Nuclear extracts. Nuclear protein extracts were prepared from control or emetine-treated cell lines as previously described (44), with the following modifications. Cells were homogenized in buffer A (1 mM EDTA, 0.25 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 0.5 mM spermidine, 0.15 mM spermine, 0.2 ng of leupeptin per ml, 1 U of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride, 7 mM β-mercaptoethanol, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.9)–0.5 M sucrose–50 mM NaCl–0.25% Triton X-100 and centrifuged, and the nuclear pellet was rinsed twice. Nuclei were extracted with 5 pellet volumes of buffer A–25% glycerol–0.1 M NaCl for 20 min and centrifuged. The pellet was extracted with 5 pellet volumes of buffer A–25% glycerol–0.5 M NaCl for 25 min and spun, and the supernatant was precipitated by addition of $(NH_4)_2SO_4$ to 45% saturation. The precipitate was resuspended in buffer A–25% glycerol–0.1 M NaCl, insoluble material was removed by centrifugation at 17,000 × *g* for 10 min, and protein was stored at −70°C. Protein concentration was determined by the Bradford assay (6). For heat treatment experiments, S13 nuclear extract was heated at 85°C for 5 min and insolubilized protein was removed by centrifugation at 17,000 × *g* for 15 min.

Preparation of DNA. The ALV LTR adjacent to the c-*myc* gene was cloned from a genomic library made from bursal lymphoma line BK25 cells. The U3 region of the LTR (*Mst*II-*Eco*RI 245-base-pair [bp] fragment) was subcloned by addition of *Bam*HI linkers and ligation into the *Bam*HI site of pUC9. This LTR fragment was purified by restriction digestion and separation on polyacrylamide gels (30) and was used to generate *Sph*I or *Mae*III subfragments, separable by polyacrylamide gel electrophoresis. DNA was end labeled with ^{32}P-labeled nucleotides by kinase addition or Klenow reaction and purified by polyacrylamide gel electrophoresis. The 35-bp complementary b oligonucleotides (upper-strand sequence, GTTATAAGGAGAGAAAAAGTACCGTGCAT GCCGAT) were synthesized with an Applied Biosystems synthesizer, and full-length oligonucleotides were purified on a sequencing gel (30). The annealed oligonucleotides were end labeled with [γ-^{32}P]-ATP by using polynucleotide kinase. The adenovirus major late promotor (MLP)-containing plasmid (from A. Sennear) was digested with *Hin*dIII and *Xho*I for purification of the MLP enhancer fragment (7). pSV2CAT was used to prepare a 270-bp partial digest fragment including the simian virus 40 enhancers (*Hin*dIII-*Sph*I fragment; 18). pAdBalI (from A. Sennear) contains the adenovirus E1A enhancer (270-bp *Eco*RI-*Bal*I fragment; 21).

Gel shift assay. Samples of nuclear extracts were mixed with 2 μg of poly(dI-dC)–poly(dI-dC) (Pharmacia) in buffer for 10 min on ice before addition of about 0.5 ng of ^{32}P-labeled DNA fragments and, in some cases, unlabeled competitor fragments (37). Twenty-microliter reactions were incubated at room temperature for 30 min and resolved on 4% polyacrylamide gels in TAE buffer (6.7 mM Tris acetate, 3.3 mM sodium acetate, 1 mM EDTA, pH 7.5) at 200 V for 90 min. ^{32}P-labeled oligonucleotide gel shift binding activity [minus poly(dI-dC)–poly(dI-dC)] was assayed with 10% polyacrylamide gels in 0.375 M Tris glycine buffer (pH 8.8) (40).

RESULTS

Developmentally regulated lability of LTR-driven transcription. Using nuclear runoff assays, we previously showed that inhibition of protein synthesis results in a rapid and specific

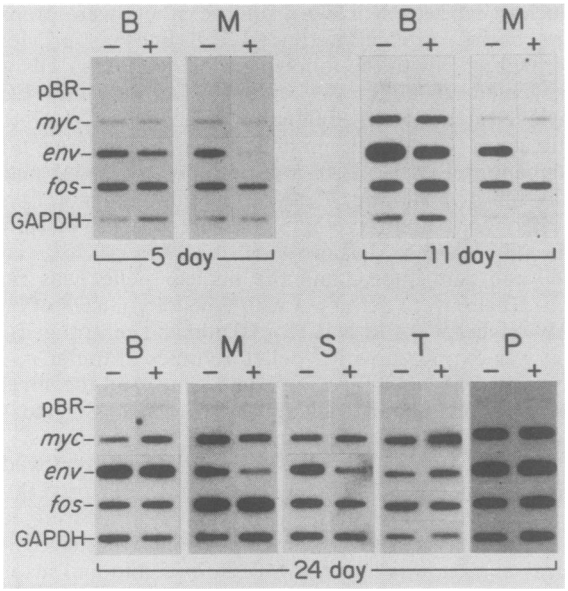

FIG. 1. Lability of LTR-enhanced transcription in lymphoma-susceptible SC chickens. Lymphocytes were isolated from ALV-infected tissues at different ages after hatching and cultured with (+) or without (−) emetine (see Materials and Methods). Nuclei were prepared and assayed for runoff transcription by hybridization of equal amounts (counts per minute) of labeled transcripts to plasmid slot blots, followed by autoradiography. B, Bursa; M, bone marrow; S, spleen; T, thymus; P, MC29-transformed macrophages in culture. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

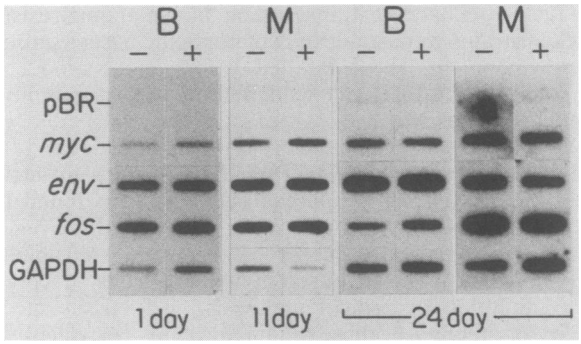

FIG. 2. Stability of LTR-enhanced transcription in lymphoma-resistant FP chickens. Lymphocytes from ALV-infected tissues were cultured with or without emetine. LTR-driven transcription was assayed by nuclear runoff transcription and slot blot hybridization as described in the legend to Fig. 1.

5- to 10-fold decrease in LTR-enhanced transcription in bursal lymphoma cell lines but not in ALV-infected T cells or fibroblasts (29). This observation led us to analyze LTR-driven viral transcription in different tissues from ALV-infected birds to determine whether LTR-enhanced transcription is also labile in vivo. We were particularly interested to determine whether this lability is specific to bursal cells or whether it is also found in other hematopoietic tissues. To assay the lability of LTR transcription enhancement, lymphoma-susceptible SC strain embryos were injected with ALV and tissues were dissected from the birds at different ages. Lymphocytes were prepared and incubated for 3 h with or without emetine (emetine treatment resulted in approximately 95% inhibition of protein synthesis [data not shown]). Rates of LTR-driven viral gene transcription were compared by runoff transcription of isolated nuclei and hybridization of ^{32}P-labeled transcripts to plasmid slot blots (29).

LTR-driven viral *env* transcription occurred at a relatively high rate in control cells from a number of tissues, relative to *myc* or glyceraldehyde-3-phosphate dehydrogenase sequences (Fig. 1). This suggests that ALV infection is widespread in chickens infected as 10-day-old embryos. Viral *env* transcription was specifically decreased two- to threefold by emetine treatment of bursal cells at 5 and 11 days after hatching, whereas emetine had no effect on *env* transcription in 24-day bursas. Transcription of the viral *gag* gene showed similar emetine effects (data not shown). In contrast, transcription of sequences hybridizing to the glyceraldehyde-3-phosphate dehydrogenase gene- and *fos*-containing plasmids was stable after emetine treatment (the latter probably representing hybridization of nonspecific α-amanitin-sensitive transcripts; see Materials and Methods). *myc* transcription was not affected by emetine treatment in these tissues, since few cells had LTR sequences integrated next to c-*myc*. Throughout development, bone marrow cells showed a larger (5- to 10-fold) decrease in *env* transcription after emetine treatment. Mature 24-day spleen cells also showed labile *env* transcription, whereas thymus and MC29-infected macrophages showed stable *env* transcription. These data indicate that the lability of LTR transcription enhancement is both tissue specific and developmentally regulated in the bursa. This labile effect was observed with several inhibitors of translation (emetine, cycloheximide, and anisomycin; data not shown), suggesting that it is caused by decreased protein synthesis rather than by side effects of drug treatment.

Lability of LTR-enhanced transcription and susceptibility to ALV-induced lymphoma. The lability of LTR-enhanced transcription in bursal cells during the first 2 weeks after hatching correlated with the time of susceptibility to ALV lymphomagenesis (10). In contrast, bursal cells from mature (24-day-old) birds demonstrated a stable transcription pattern, and at this age ALV infection rarely leads to lymphoma. Further evidence suggesting that stage-specific lability of LTR-driven transcription correlates with susceptibility to lymphoma comes from comparison of LTR-driven transcription in different chicken strains. The SC strain is susceptible to ALV-induced lymphomas (>50%), whereas the FP strain is resistant (0%) (2). Viral *env* nuclear runoff transcription was stable in bursa and bone marrow from FP birds throughout development (Fig. 2), in contrast to the regulated pattern of lability observed in SC chickens (Fig. 1). Analyses of nuclear runoff transcription in line $15I_5 \times 7$ lymphoma-susceptible and line 6_3 lymphoma-resistant chickens also showed labile and stable bursal LTR-driven transcription, respectively (data not shown). These findings add to the correlation of lability of LTR-driven transcription with susceptibility to lymphomagenesis.

Binding of several proteins to ALV LTR enhancers. The emetine sensitivity of LTR transcription enhancement in hematopoeitic cells may be due to the interaction of short-lived proteins with LTR enhancer sequences. We used the gel shift assay to determine whether there are sequence-specific binding proteins which retard the migration of ^{32}P-labeled LTR DNA sequences on polyacrylamide gels (15, 17). We tested the gel shift binding activity of different fragments of the U3 LTR region with 0.5 M salt-extracted nuclear proteins from ALV-infected S13 bursal lymphoma cell lines. Figure 3 shows the restriction map of the 245-bp

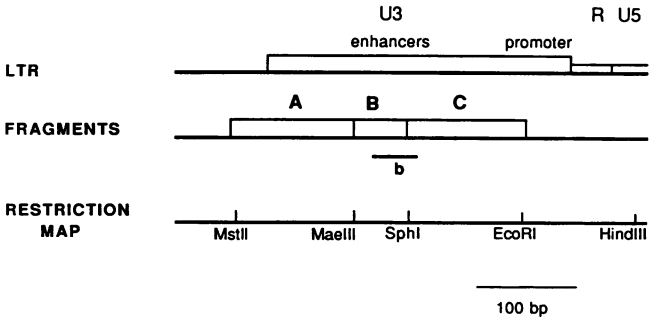

FIG. 3. Restriction map of ALV LTR enhancers. Enhancer and promotor regions of the U3 region of the BK25 LTR are shown as boxes. The A, B, and C restriction fragments and the enzymes used to generate them are shown beneath. The position of the b oligonucleotide probe is shown as a dark line.

LTR U3 region (ALV sequences cloned from bursal lymphoma line BK25; see Materials and Methods). The ALV enhancer and promotor regions were not precisely mapped, but homologous U3 sequences in the Rous sarcoma virus (RSV) LTR have been analyzed in DNA transfection studies (12, 24, 34). The RSV B region (24) contains the strongest enhancing activity and is nearly identical to the sequence of our A and B restriction fragments (5, 24). The C restriction fragment may also contribute to enhancer and promotor function.

Several binding complexes were detected by using the whole U3 enhancer (A, B, and C) in the gel shift assay (Fig. 4). Four major DNA-protein complexes were also found with the 150-bp AB subfragment, whereas the 95-bp C fragment showed a single major binding complex and the promoter region (*Eco*RI-*Hin*dIII; Fig. 3) showed negligible binding. Proteinase K treatment abolished gel shift complexes, while RNase A treatment had no effect (data not shown). These data suggest that several proteins interact with the LTR enhancer regions. The same gel shift pattern was observed when RSV LTR fragments, which are very similar in sequence to the ALV LTR, were used (data not shown). The A region showed a diffuse pattern of binding which includes three or more complexes. The B region also bound proteins, as deduced from comparison of BC and C gel shifts, because the BC region showed one or two binding complexes in addition to that of the single C-binding complex (Fig. 4). This B binding was further mapped by using a

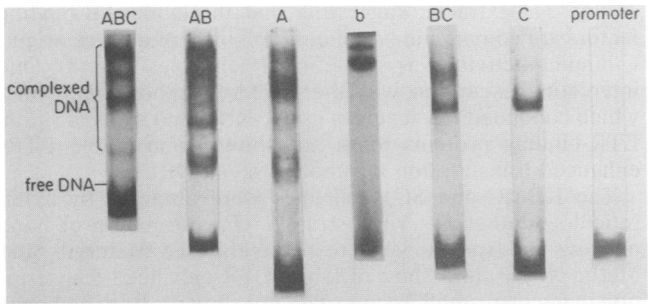

FIG. 4. Gel shift analysis of LTR-binding proteins. Nuclear extract protein from S13 bursal lymphoma cells (5 μg) was incubated with ^{32}P-labeled LTR restriction fragments and poly(dI-dC–poly(dI-dC), and DNA-protein complexes and free DNA were resolved on polyacrylamide gels. ^{32}P-labeled b oligonucleotide was tested in a modified gel shift assay (see Materials and Methods).

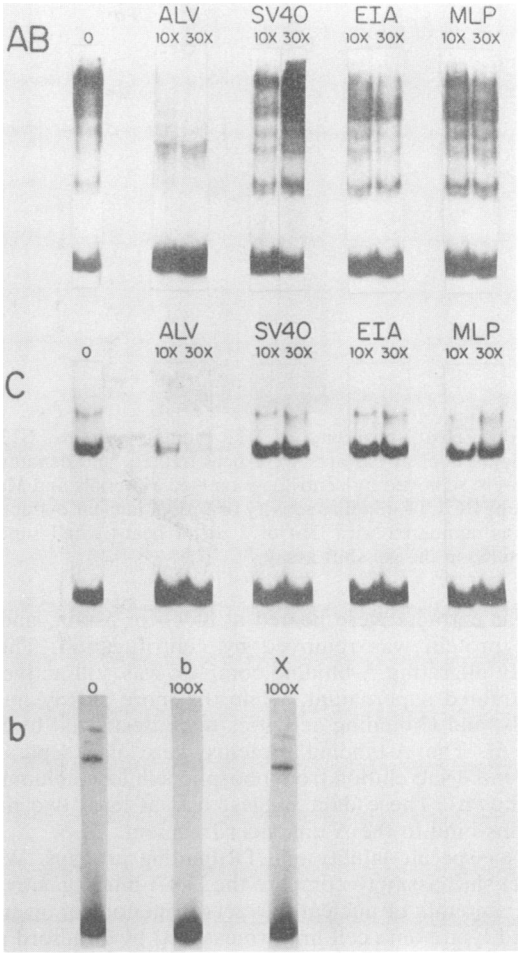

FIG. 5. Competition analysis of LTR-binding specificity. Unlabeled DNA fragments were added to gel shift assays with ^{32}P-labeled LTR AB and C fragments. Competitor fragments tested were the 245-bp BK25 ALV LTR, the 270-bp simian virus 40 (SV40) enhancer, the 270-bp adenovirus E1A enhancer (EIA), and the 450-bp adenovirus MLP sequences at 10- and 30-fold molar excess. The double-stranded oligonucleotides tested were the b LTR and X (mouse serum amyloid A gene) sequences at 100-fold molar excess.

35-bp double-stranded oligonucleotide of B sequences (b, Fig. 3) and testing for binding in a modified gel shift assay (40). Two binding complexes were observed when this b probe was used (Fig. 5).

The LTR enhancer-binding proteins appear to be sequence specific, because unlabeled ALV LTR DNA added to the gel shift assay readily competed for LTR binding, except for a small amount of diffuse A binding (Fig. 5). Simian virus 40 or adenovirus major late promoter (MLP) enhancer sequences did not compete with AB or C complex formation, whereas adenovirus E1A enhancer sequences competed slightly for AB binding. The b oligonucleotide-binding activities are also specific, since competition with unlabeled b oligonucleotide abolished binding, whereas an unrelated oligonucleotide (from mouse serum amyloid A gene) did not compete (Fig. 5).

The complex gel shift pattern observed with the A LTR fragment could represent binding of multimeric forms of a single binding protein, or several different proteins could be involved. These A-binding proteins can be distinguished by their stability after heat treatment. S13 bursal lymphoma

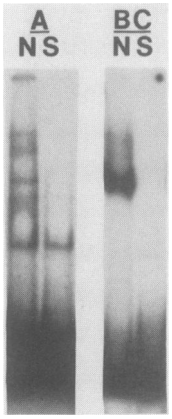

FIG. 6. Heat sensitivity of LTR-binding proteins. S13 bursal lymphoma nuclear extracts were heat treated, and denatured proteins were removed by centrifugation (see Materials and Methods). The A or BC LTR-binding activity of 4 μg of nuclear extract protein (N) was compared with that of 2 μg of heat-treated supernatant protein (S) in the gel shift assay.

nuclear extracts were heated at 85°C for 5 min, and denatured protein was removed by centrifugation. The most rapidly migrating A-binding complex was still active in the heat-treated supernatant, while the more slowly migrating A-, B-, and C-binding activities were destroyed by heating (Fig. 6). The A-binding proteins were also separated by differential salt elution from phosphocellulose columns (data not shown). These data suggest that at least two different proteins bind to the A enhancer fragment.

Tissue-specific lability of LTR-binding proteins. We used the gel shift assay to compare the DNA-binding activities of equal amounts of nuclear extracts from control or emetine-treated lymphoma cell lines (measured by Bradford protein assay) to determine whether any of the LTR-binding proteins were labile. Most of the A LTR binding activity was labile in bursal lymphoma line 243 cells (with the exception of the most rapidly migrating heat-stable A-binding protein), showing an approximate 5- to 10-fold decrease in LTR-binding activity in emetine-treated cells (Fig. 7A). The B LTR-binding activities also appeared to be labile, as shown by comparison of BC- and C-binding complexes or use of the b oligonucleotide probe. This AB-binding lability correlates with the 5- to 10-fold decrease in LTR-driven transcription seen after emetine treatment of these cells (measured by nuclear runoff transcription assays; 29). In contrast, C-binding activity was stable after emetine treatment (Fig. 7A).

Two controls demonstrated that the decrease in AB-binding activity after emetine treatment is specific. (i) The protein concentration of each extract (determined by Bradford assay) was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining (32). Equal amounts of silver-stained protein were observed in extracts from control and emetine-treated cells, confirming that the Bradford assay accurately measures the protein concentration of each extract (data not shown). (ii) The gel shift binding of the adenovirus MLP-binding protein with the 450-bp MLP sequence (7) was compared in control and emetine-treated extracts. This factor has a relatively long half-life, since 3 h of emetine treatment did not affect MLP-binding activity (Fig. 7A). These data indicate that factors binding to the AB LTR sequences show a specific lability relative to the C LTR- or MLP-binding factors. The labile AB-binding proteins could have a very short half-life

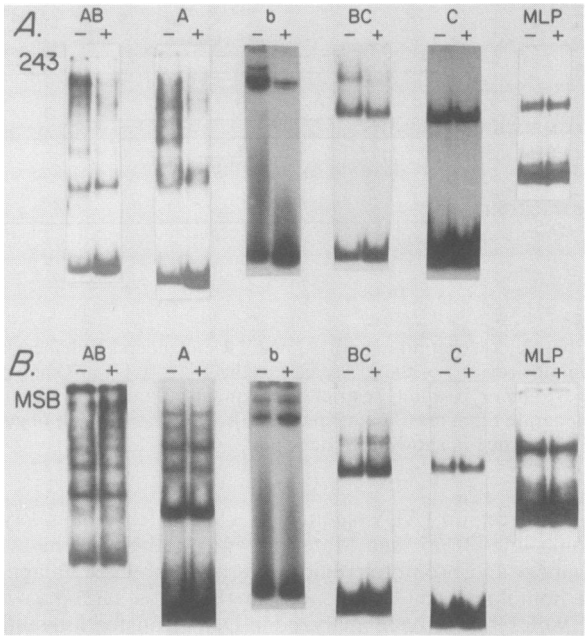

FIG. 7. Comparison of the labilities of LTR-binding proteins in B and T cells. The 243 bursal lymphoma (A) and Marek's disease thymocyte T-cell (B) lines were incubated with (+) or without (−) emetine for 3 h before extraction of nuclear proteins. The binding activity of 5 μg of protein was tested in gel shift assays with ^{32}P-labeled A, B, or C LTR fragments, b LTR oligonucleotide, or adenovirus MLP sequences.

or could require continuous modification to an active binding form by a labile enzyme.

The gel shift assay was used to compare the labile and stable binding activities in additional cell lines to determine whether the lability or stability of LTR-driven transcription measured by nuclear runoff assays (29) correlates with the lability or stability of LTR-binding proteins. We previously showed that the Marek's disease thymocyte T-cell line (1) exhibits stable LTR-driven transcription in nuclear runoff assays (29). The gel shift binding pattern of Marek's disease thymocyte T-cell nuclear extracts was very similar to that of bursal lymphoma cells (Fig. 7A), but the A-, B-, C-, and MLP-binding activities were all stable after emetine treatment (Fig. 7B). Figure 8 shows representative analyses of additional cell lines. The labile AB-binding pattern of S13 and BK25 bursal lymphoma cell extracts was similar to that of line 243 extracts, suggesting that the labile AB-binding factors are common in lymphoma cell lines of diverse origin. C-binding activity was stable in all cell types (Fig. 8). One interesting exception was the BK3A lymphoma cell line, which contained low levels of c-*myc* RNA and showed stable LTR-binding proteins (data not shown) and stable LTR-enhanced transcription in runoff assays (29).

The KBMC and SC3 cell lines were obtained by avian reticuloendotheliosis virus strain T transformation of bone marrow and spleen cells, respectively (see Materials and Methods; 26), and showed labile LTR-enhanced transcription in nuclear runoff assays (data not shown). Both cell lines showed a pattern of labile gel shift complexes similar to that of lymphoma cells (Fig. 8), suggesting that labile AB-binding proteins are common in hematopoietic cells, in agreement with the analysis of nuclear runoff transcription in spleen and bone marrow cells (Fig. 1). In contrast, chicken embryo

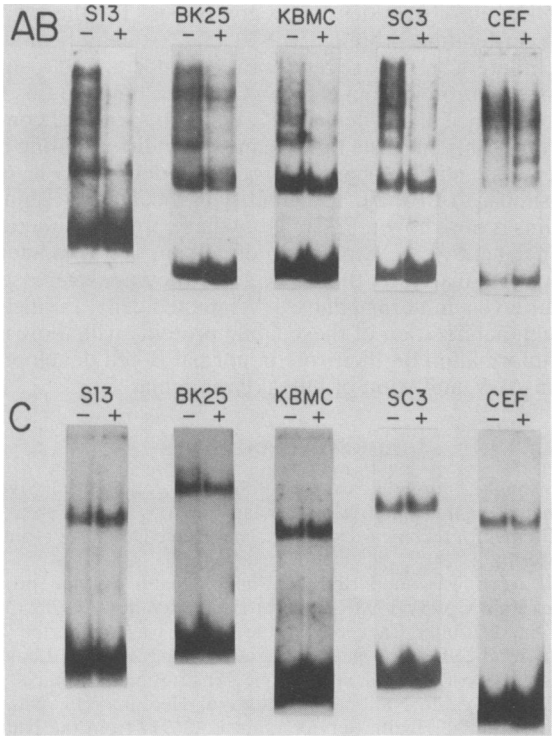

FIG. 8. Cell type specificity of labile LTR-binding proteins. Five micrograms of nuclear proteins from control and emetine-treated cells was analyzed for AB or C LTR-binding activity in the gel shift assay. The origin of each cell line is described in Materials and Methods. CEF, chicken embryo fibroblasts.

fibroblasts showed stable A, B, and C LTR-binding activity (Fig. 8) and nuclear runoff transcription (29).

Minor variations in AB-binding activity were observed in each cell line, which could represent small differences in the abundance or activity of proteins in different cell lines. The most striking difference observed was in the lability or stability of AB-binding proteins. Data from a number of independent experiments with all of the DNA test fragments are summarized in Table 1. A roughly 5- to 10-fold decrease in A- and B-binding activity (except for the fastest-migrating A-binding activity) was observed in the hematopoeitic cell lines but not in T cells or fibroblasts. The C- and MLP-binding factors were stable in all cell types. This lability of AB-binding proteins strictly correlates with the lability of LTR-driven transcription in hematopoeitic cells, suggesting that these proteins are essential for high rates of LTR-enhanced transcription.

TABLE 1. Lability of LTR-driven transcription and LTR-binding proteins in different cell types

Cell line (type)	Lability of[a]:				
	LTR-driven transcription[b]	LTR-binding proteins[c]			
		A	B	C	MLP
S13 (B lymphoma)	L	L	L	S	S
243 (B lymphoma)	L	L	L	S	S
BK25 (B lymphoma)	L	L	L	S	S
KBMC (bone marrow)	L	L	L	S	S
SC3 (spleen)	L	L	L	S	S
MSB (thymus)	S	S	S	S	S
CEF (fibroblast)	S	S	S	S	S

[a] L, Labile; S, stable; CEF, chicken embryo fibroblasts.
[b] Measured by nuclear runoff transcription.
[c] Measured in gel shift assay.

DISCUSSION

Labile LTR-binding proteins and susceptibility to lymphoma. We defined a labile characteristic of LTR transcription enhancement which correlates with the susceptibility of cells to ALV tumorigenesis. In lymphoma-sensitive chickens, developing bursal cells showed labile LTR-driven transcription before 2 weeks of age, during which time the birds are susceptible to ALV lymphomas (Fig. 1). In contrast, bursal cells from mature 24-day-old birds showed stable LTR transcription enhancement and were refractory to lymphomagenesis. Bone marrow- and spleen-derived lymphoid cells from mature birds showed labile transcription, whereas thymus cells and macrophages showed stable transcription, suggesting that this characteristic is differentially regulated in hematopoietic cells. Since bone marrow and spleen contain many different hematopoietic precursors (46), we do not know which cell types are the source of labile LTR-driven transcription. However, the strong lability of LTR-driven transcription in these tissues suggests that most infected cells express labile factors.

Lymphoma-resistant birds showed stable LTR-driven transcription in bursa and bone marrow (Fig. 2), in contrast to the labile transcription pattern observed in lymphoma-sensitive chickens (Fig. 1). This correlation of labile LTR-driven transcription and sensitivity to lymphomagenesis supports the hypothesis that labile enhancement of LTR-driven transcription is important in lymphoma induction (see below). It is not understood what additional effects this difference in stability has in cells of resistant chickens. Lymphoma-sensitive and -resistant strains were healthy and showed similar levels of ALV infection (3, 16; Fig. 1 and 2). Thus, the lability or stability of LTR-binding factors might not result in a phenotypic difference, except in the induction of bursal tumors. Alternatively, these strains could show differences in B-cell development that have not yet been detected.

Role of labile factors in ALV lymphomagenesis. These data have led us to propose a model for the role of labile LTR-enhancing proteins in ALV lymphomagenesis. After ALV infection of a B stem cell, in rare cases the proviral LTR integrates next to the c-*myc* gene. The resulting hyperexpression of the *myc* gene, driven by the interaction of regulatory proteins with LTR enhancers, may be important in proliferative expansion of these cells (4, 33). In chickens sensitive to lymphoma, the factors regulating LTR-driven transcription have a very short half-life (20 to 30 min by nuclear runoff analysis; data not shown), and their lability appears to be developmentally regulated in the bursa. Because *myc* RNA and protein also have a very short half-life (15 to 20 min; 13, 19), a transient decrease in the level of these labile enhancing factors would rapidly lead to a reduction of *myc* gene products to normal levels (29), which could allow the pre-B cells to leave the proliferative compartment, home to the bursa, and differentiate. A return to high levels of the labile enhancing factors and then of *myc* protein levels might again disrupt the developmental program and promote proliferation and further steps in oncogenesis.

Central to this model is the notion that there is a transient down regulation of the labile LTR-enhancing factors during B-cell development of lymphoma-sensitive birds. In con-

trast, in lymphoma-resistant strains, constitutive expression of stable LTR-enhancing factors would give continuously high *myc* levels in rare cells having the LTR integrated next to *myc*, and this would promote proliferation rather than differentiation (e.g., see references 4, 9, and 25). This might prevent the progenitor cells from proper development within the bursal environment, which is required for ALV tumorigenesis (38), or it could promote immune elimination of these abnormal cells (3). Chronic *myc* hyperexpression in lymphoma-resistant birds could also be cytotoxic, since infection of murine spleen cells with *myc*-containing virus causes considerable cell death (50 to 70%; 47) and high levels of *myc* expression are toxic in hamster ovary cells (48). All of these effects of *myc* in early B cells would be reduced in lymphoma-sensitive birds, as down regulation of *myc* expression by labile enhancing factors could allow some differentiation. Analysis of the expression of the labile LTR-enhancing factors and of the *myc* gene in ALV-infected cells during bursal development will be important in testing this model.

Labile and stable LTR-binding proteins. Our data suggest that labile or short-lived proteins interact with the LTR to enhance c-*myc* transcription in a way that promotes tumorigenesis. We used the gel shift assay to show that a number of factors specifically interact with LTR enhancer sequences (Fig. 7 and 8). The A region showed several binding activities, which appear to be due to at least two different proteins, resolved by phosphocellulose chromatography (data not shown), by their heat sensitivity (Fig. 6), and by their sensitivity to inhibition of protein synthesis (Fig. 7). The B region bound one or two labile proteins, whereas the C region showed a single stable binding activity (Fig. 7). The lability of the B-binding and more slowly migrating A-binding proteins correlated with the lability of LTR-driven transcription observed in nuclear runoff assays (Table 1). These data suggest that the labile AB-binding proteins are necessary for LTR transcription enhancement, whereas the stable, most rapidly migrating A- and C-binding proteins are not sufficient. DNA transfection experiments with different RSV constructs also indicate that the homologous A and B restriction fragments act as strong transcription enhancers in this related virus (24, 34). One caveat of this hypothesis is that we did not directly demonstrate a role of these binding proteins in ALV LTR-driven transcription. However, the strict correlation of the lability of AB-binding proteins in different cell types, measured by the gel shift assay, with the lability of LTR-driven transcription, measured by nuclear runoff assay, (Table 1) suggests that these proteins are important for enhancer function.

The LTR-binding proteins were expressed in all avian cell types examined, with minor variations (Fig. 7 and 8). The most striking difference observed was in the overall lability of the more slowly migrating A- and B-binding proteins. This lability of AB-binding proteins appeared to be restricted to bursal, bone marrow, and spleen cells, whereas these proteins were stable in T cells and fibroblasts (Table 1), suggesting that the lability of the proteins is differentially regulated in certain lymphoid cells. This lability could represent binding proteins with a short half-life, or perhaps the proteins are constitutively expressed and modified to an active binding form by a short-lived factor (e.g., by phosphorylation; 31).

Two RSV LTR-binding proteins have been identified in quail embryo fibroblasts (42), which are similar to the ALV LTR-binding proteins of chicken bursal lymphoma cells. These quail proteins interact with RSV sequences homologous to the ALV A, B, and C restriction fragments, as measured by DNase 1 footprinting (42). The A region of ALV and RSV includes a recognition site for the rat liver enhancer-binding protein EBP-20 (5, 23). This heat-stable protein interacts with several viral enhancers, and a similar heat-stable protein from chicken liver also binds to this RSV LTR sequence (T. Ryden and K. Beemon, personal communication). This suggests that the most rapidly migrating heat- and emetine-stable A-binding protein we detected (Fig. 6 and 7) is similar to EBP-20. The identity of the labile AB-binding proteins is not known. Presumably these proteins function in enhancement of the expression of cellular genes in addition to those regulated by the LTR, since they are expressed at similar levels in normal and ALV-infected cells. Purification and characterization of these labile proteins will allow us to gain information on their role in normal B-cell development and in ALV induction of bursal lymphoma.

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