

Identification of EFIV, a Stable Factor Present in Many Avian Cell Types That Transactivates Sequences in the 5' Portion of the Rous Sarcoma Virus Long Terminal Repeat Enhancer

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We define a protein complex present in avian nuclear extracts that interacts with the Schmidt-Ruppin strain of the Rous sarcoma virus (RSV) long terminal repeat (LTR) between positions –197 and –168 relative to the transcriptional start site. We call this complex EFIV and demonstrate that the EFIV protein(s) is present in several avian cell types examined, including B cells (S13 and DT40), T cells (MSB), and chicken embryo fibroblasts. We also report that the EFIV binding site activates transcription of reporter constructs after transfection into avian B cells and chicken embryo fibroblasts, demonstrating that the EFIV region constitutes a functional transactivator sequence. By chemical interference footprinting and mutational analyses we define the EFIV binding site as including the sequence GCAACATG, which is present in two copies between positions –197 and –168, as well as sequences that lie between the two repeats. Electrophoretic mobility shift competition experiments suggest that the EFIV protein(s) may be related to members of the CCAAT/enhancer-binding protein family of transcription factors that interact with different regions of the RSV and the avian leukosis virus (ALV) LTRs. However, as defined by differences in sensitivity to protein synthesis inhibitors and footprinting patterns, EFIV is clearly distinct from these previously defined LTR binding factors. In addition, the finding that EFIV binding activity is stable in B cells indicates either that the lability of all 5' LTR binding activities is not required for B-cell transformation by the ALV/RSV family of viruses or that nonacute transforming viruses that include an RSV LTR may use a mechanism to effect cellular transformation different from that proposed for ALV.

Animals infected with avian leukosis viruses (ALVs) develop primarily B-cell lymphomas after a 12- to 14-week latent period (8). An examination of bursal tumors from ALV-infected animals demonstrates that proviral DNA is integrated adjacent to the *c-myc* proto-oncogene and that *c-myc* is abnormally expressed under the control of sequences in the viral long terminal repeat (LTR)-associated enhancer (16). This enhanced expression of *c-myc* is a critical early event contributing to neoplastic transformation of avian B cells (8, 24).

Experiments with both avian and murine viruses have indicated that the absolute level of pathogenicity of a virus isolate as well as the spectrum of disease induced by different isolates is determined primarily, although not exclusively, by sequences within the viral LTR. Further investigation of murine and avian viral LTRs has indicated that the cell type specificity of tumor formation *in vivo* might be explained by relatively subtle cell-type-specific differences in LTR regulation dictated by cell-type-specific transcriptional transactivators. In the avian system, for example, Ruddell and colleagues have observed cell-type-specific differences in the binding of two ALV LTR enhancer binding activities called a1/EBP and a3/VBP (29, 30) (see Fig. 1). Each of these proteins is a member of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors (3, 37), and a3/VBP has been identified further as a previously identified protein called vitellogenin-binding protein involved in the transcriptional regulation of the

chicken vitellogenin gene (18). The sequence relationship between a3/VBP and a1/EBP with C/EBP family members is consistent with the fact that the a1/EBP and a3/VBP binding sites are coincident with three sites identified by Ryden and colleagues in the LTR of the Prague (Pr) strain of the Rous sarcoma virus (RSV) that specifically bind C/EBP α (31, 32).

Although a1/EBP is detected in all cell types examined, it is depleted specifically in extracts prepared from B cells treated with protein synthesis inhibitors such as emetine. Since a1/EBP is not depleted in extracts generated from emetine-treated chicken embryo fibroblasts (CEFs), a1/EBP is characterized as exhibiting tissue-specific binding lability. The a3/VBP also displays labile binding activity in emetine-treated B-cell extracts. These data have led to the proposal (21, 29, 30) that the labile binding activity of ALV a1/EBP and a3/VBP in B cells mediates a transient downregulation of the transcription of the abnormally expressed *c-myc* gene under the control of the ALV LTR. It was further proposed that this downregulation of *c-myc* is required for the transformation of B cells by ALV, perhaps by allowing further differentiation events which are required for transformation, or to allow escape from the apoptotic effects of *myc* overexpression (1, 7, 36).

We (40) and others (5, 6, 12, 13, 19, 20, 22, 25) have been interested in identifying enhancer sequences in the LTR of RSV. The RSV LTR is highly related by sequence to that of ALV, with the RSV LTR containing a few small deletions and several point mutations that differentiate it from the ALV LTR (28) (see Fig. 1). Current evidence indicates that the LTRs from these two virus types are also highly related biologically since a recombinant virus that contained the Schmidt-Ruppin (SR)-RSV LTR on an otherwise leukosis virus genome in-

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TABLE 1. Names, locations, and sequences of LTR-specific oligonucleotides

Oligonucleotide ^a	Location ^b	Sequence
EFIV oligonucleotides		
RSV EFIV short	-193 to -175	5'-CATGGTAACGATGAGTTAG-3'
RSV EFIV	-197 to -161	5'-GCAACATGGTAACGATGAGTTAGCAACATGCCTTACA-3'
RSV EFIV long	-206 to -158	5'-TGTAGTCTTGCAACATGGTAACGATGAGTTAGCAACATGCCTTACAAGG-3'
Other oligonucleotides		
RSV EFI	-155 to -121	5'-AGAAAAAGCACCGTGCATGCCGATTGGTGGGAAGTA-3'
RSV EFII	-229 to -192	5'-AATGTAGTCTTATGCAATACTCTTGTAGTCTTGCAACA-3'
RSV Sph	-175 to -147	5'-GCAACATGCCTTACAAGGAGAAAAAGC-3'
RSV PCR oligonucleotide	-209 to -196	5'-ttcgaaaagcttTCTTGTAGTCTTGC-3' ^c
ALV a3	-209 to -186	5'-CATGCTTATGTAACGATGAGCTTCAG-3'

^a Only the upper strand is shown for each oligonucleotide. The lower strands were the exact complement of strands shown here with no overhanging ends.

^b The nucleotide coordinates for the ALV LTR are different from those of the RSV LTR and were taken from reference 30.

^c Nucleotides shown in lowercase letters are not homologous to nucleotides of LTR sequences.

duced a pattern of B-cell disease analogous to that seen with ALV (17), demonstrating that the modest sequence variations between these two viruses do not affect their disease spectrum. Similar to results described above for ALV LTRs, several groups have demonstrated that the 5' portion of the SR- and Pr-RSV LTRs also binds members of the C/EBP family of transcription factors. Using avian nuclear extracts, Sears and Sealy (34, 35) have demonstrated the binding of the EFII factor to two sites at the extreme 5' end of the RSV LTR, with the 5' copy of the EFII repeat (positions -229 to -213) constituting the major binding site and with the 3' site exhibiting weaker binding. A recombinant form of the ALV LTR a1/EBP binding protein also interacts with sequences within this region of the SR-RSV LTR that are shared with ALV (37). Since the reported EFII and a1 binding sites overlap and since probes from these two LTRs show similar if not identical results by electrophoretic mobility shift assay (EMSA) (unpublished observations), it is likely that they represent the same cellular proteins. The EFII/a1 binding sites are also coincident with two binding sites (sites 1 and 2 for C/EBP α identified by Ryden and colleagues (31, 32) in the LTR from the Pr-C strain of RSV.

Smith and colleagues have also investigated the binding of the a3/VBP protein to the SR-RSV LTR and have found only one binding site for this factor (37). This a3/VBP binding site overlaps the 5'-most EFII/a1 binding site within sequences shared by ALV and RSV. The major a3/VBP site detected in the ALV LTR is not detected in the SR-RSV, a finding expected because of the absence of several nucleotides from SR-RSV that lie within the ALV a3/VBP binding site. Interestingly, the Pr-C strain of RSV does include the downstream a3/VBP binding site found in ALV; we currently do not know the significance of this difference between the Pr and SR strains of RSV in terms of LTR function or the potential differences in disease specificities of viruses that contain one or two copies of this binding site.

We have been investigating sequences and transactivators responsible for transcriptional regulation of the SR-RSV LTR and are particularly interested in determining the identities and natures of proteins required for pathogenesis. One important question we are investigating is whether the SR-RSV EBPs are regulated in a manner similar to that of the labile ALV binding activities that are of proposed importance for disease induction by this virus. Since ALV a3/VBP is one of the two LTR-binding proteins that exhibit labile binding activity proposed to be of importance for the specific transformation of avian B cells, we chose to examine the region of the SR-RSV LTR that lacks the 3'-most a3 binding site to determine

whether the SR-RSV LTR interacts with proteins that share a cell type distribution and lability similar to those of a3/VBP or whether these are characteristics unique to the ALV LTR-binding protein.

MATERIALS AND METHODS

Nuclear extract preparation. Extracts were prepared from S13 cells and from CEF cultures as previously described (15, 40) except that 0.6 M NaCl was used for nuclear extractions. Protein synthesis was inhibited in some cultures by treatment with 0.1 mM emetine for 3 h prior to harvesting. Cessation of protein synthesis was verified by quantitating the incorporation of [³⁵S]methionine into control and treated cultures. In most cases, counts incorporated into the emetine-treated cultures were inhibited approximately 95% relative to counts for the control, although no difference in EMSA results was obtained with one extract that showed only 80% inhibition.

Probe preparation, EMSA, and chemical footprinting. Oligonucleotides used for EMSAs and vector construction are shown in Table 1. In addition to oligonucleotides that include all or a portion of the EFIV binding site, other oligonucleotides used in these studies are the ALV a3 binding site (30); an RSV oligonucleotide that includes an inverted CCAAT box that binds EFI (9, 14), a YB-1 related factor (26); the RSV Sph probe, which includes a serum response factor (SRF)/CarG box binding site (40); and an oligonucleotide from the extreme 5' end of the RSV LTR that binds EFII (34). Table 1 also includes the sequence of the RSV PCR oligonucleotide that was used to generate the PCR long and short products (see Fig. 5B). The labeling and purification of oligonucleotide probes for EMSAs and for PCRs were conducted as previously described (40). The EFIV long and short PCR probes were generated by the amplification of portions of the RSV LTR defined by the RSV PCR primer on the 5' end and on the 3' end by either the EFIV or EFIV short oligonucleotides, respectively. Reaction conditions for PCRs, EMSAs, and methylation interference assays were as previously described (40). Probes were modified by diethyl pyrocarbonate (DEPC) essentially as described in reference 38.

To quantitate the apparent disassociation rate of EFIV from the wild-type and mutant binding sites, the indicated probes were labeled and incubated with nuclear extracts as described above for EMSAs. After the 20-min binding reaction, an aliquot of sample was loaded onto a running gel and a 500-fold molar excess of unlabeled, double-stranded wild-type EFIV oligonucleotide was added to each binding reaction mixture. Aliquots were removed and applied to the running gel at the indicated times. Bound probe was quantitated with a Molecular Dynamics PhosphorImager.

Plasmid construction. The parental LTR used for the construction of RSV-Luc was originally derived from the SR-A strain of RSV (11) and was excised from a previously described plasmid called pM-RSVNeo (15). The LTR fragment in pM-RSVNeo extended from an *Mst*II site (located 39 bp upstream of the LTR in the 3' untranslated region), which was converted to a *Bam*HI site through a *Bst*NI site in U5 (which was converted to a *Hind*III site). The LTR fragment was excised from pM-RSVNeo with *Bam*HI and *Hind*III and inserted into the *Bgl*II and *Hind*III sites within the polylinker of the luciferase expression vector pGL2 (Promega) to generate RSV-Luc.

Constructs that contained multiple copies of the EFIV binding site were generated by multimerizing the double-stranded oligonucleotides shown in Table 1 (see also Fig. 5B), which were then inserted into the polylinker upstream of the thymidine kinase (TK) promoter in pTK-Luc. This expression vector contained 166 nucleotides of the herpesvirus TK promoter on a *Bam*HI-*Hind*III fragment previously described (reference 23; the vector was kindly provided by H. Towle) that extends from positions -115 to +51, which was introduced into the *Bgl*II and *Hind*III sites of pGL2 (Promega). The contents and orientations of the

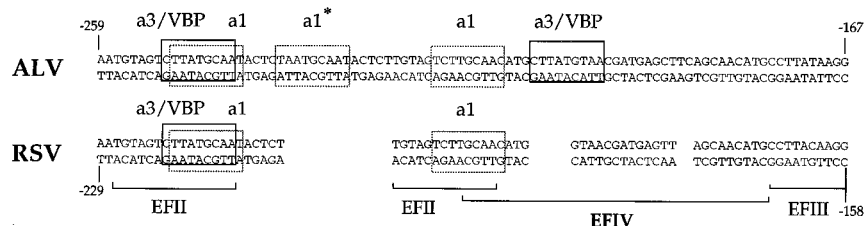


FIG. 1. Map of binding sites in the 5' ends of the ALV and RSV LTRs. The sequence of the RSV LTR was independently generated. The ALV sequence is from that of the BK25 isolate (30). Binding sites for a1/EBP are indicated by boxes drawn with dotted lines, while those for a3/VBP are depicted by boxes drawn with solid lines and their locations are positioned according to reference 37. The a1/EBP binding site indicated by the asterisk matches the a1/EBP consensus (3) and is included in the a1 DNase I footprinted region (3) but has not been characterized further with respect to a1 binding activity. The binding sites for EFII and EFIII are indicated by brackets, and their sequences were obtained from references 33 and 40, respectively. The relationship between EFII and a1/EBP has not been determined directly. The EFIV binding site is identified in this report.

wild-type and mutant EFIV binding sites in these constructs were verified by sequencing. At least two isolates of each construct were generated and tested in expression assays.

Transfection and luciferase reporter gene assay. The day before transfecting S13 cells, cultures were diluted to 2×10^5 cells per ml of growth medium, which consisted of Dulbecco modified Eagle medium supplemented with 10% tryptone phosphate broth (Difco), 5% fetal calf serum (Gibco), and 1% chicken serum (Gibco). On the day of transfection, 300 ng of the test construct was mixed gently with 224 μ l of TS (137 mM NaCl, 5 mM KCl, 0.3 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 25 mM Tris-HCl [pH 7.4], 1 mM MgCl_2 , 1 mM CaCl_2 (pH 7.4) which had been pre-warmed to 37°C, 12 μ l of 10 mg of DEAE-dextran (Pharmacia) per ml, and 25 ng of a plasmid that contained the cytomegalovirus (CMV) promoter driving the expression of the β -galactosidase gene (CMV- β GAL) (kindly provided by B. Van Ness) and then incubated for 5 to 10 min in a 37°C water bath. While the DNA solution was incubating, 8×10^5 cells per DNA sample were centrifuged for 5 min at 1,500 rpm in an Eppendorf Microfuge at room temperature. The cells were then washed once in approximately 0.5 ml of TS and centrifuged as described above. Following the TS wash, cells were resuspended in the warm DNA-DEAE-dextran solution, incubated in a 37°C water bath for 15 min, mixed gently, and then incubated for a further 15 min at 37°C. The samples were centrifuged, and the pellets were then resuspended in 0.5 ml of growth medium and transferred individually to 60-mm-diameter tissue culture plates containing 3.5 ml of growth medium, resulting in a final cell density of 2×10^5 cells per ml. Cultures were then incubated for 40 to 44 h before harvesting. CEFs were transfected as previously described (4) except that 5 μ g of the LTR constructs and 250 ng of CMV- β GAL were used per sample.

To quantitate the activity of different viral constructs, cells were harvested and luciferase activity was measured by the luciferase assay system (Promega) according to the manufacturer's instructions. To normalize for sample-to-sample variation that might occur in these experiments, all samples were cotransfected with the CMV- β GAL construct. β -Galactosidase activity was quantitated by the Tropix detection system according to the manufacturer's instructions. The relationships between constructs did not change as a result of this normalization.

RESULTS

Identification of the EFIV binding site. A sequence comparison of the SR-RSV (hereafter referred to as the RSV LTR unless otherwise noted) and ALV LTRs demonstrates that RSV lacks several nucleotides that lie within the major, 3' a3/VBP binding site of ALV (Fig. 1). In agreement with this finding, DNA probes that include this region from the RSV LTR do not bind a3/VBP (37), a result we have confirmed by EMSA using an oligonucleotide (the RSV EFIV short oligonucleotide listed in Table 1) that spans the region from -193 to -175 relative to the start site of transcription in the RSV LTR (data not shown). To determine if the region in RSV immediately downstream of the EFII binding sites might interact with different cellular proteins, a 37-bp oligonucleotide that spans the region from -197 to -161 (RSV EFIV; Table 1) was generated and used in an EMSA with extracts prepared from CEFs, from a B-lymphoma cell line (S13 cells), and from a chicken T cell line (MSB cells). Data obtained from all three cell types were indistinguishable; an example of those from CEFs is shown in Fig. 2. As shown in lane 1, the 37-bp oligonucleotide from RSV generated a shifted complex that ap-

peared as a doublet, indicating that this region from RSV interacts with cellular proteins. We have named this complex EFIV, consistent with the current nomenclature for other enhancer regions in the RSV LTR (2, 33). The EFIV complex migrated differently in EMSA gels relative to complexes generated with all other probes we have used from the RSV and ALV LTRs, including the RSV EFI, EFII, Sph, and the ALV a3 probes (Table 1) as well as the RSV EFIII (2) and the ALV a1 probes (data not shown and see below).

To determine if the EFIV interaction was specific, binding competition experiments were conducted with extracts prepared from S13 cells. As shown in Fig. 2, the addition of unlabeled competitor DNA identical to that of the input probe (lanes 2 and 3) led to the loss of complex formation when only a 10-fold molar excess of competitor DNA was added (compare lanes 1 and 2). In contrast, the addition of a 50- to 500-fold molar excess of an oligonucleotide from the EFI binding region of RSV had minimal effect on complex formation

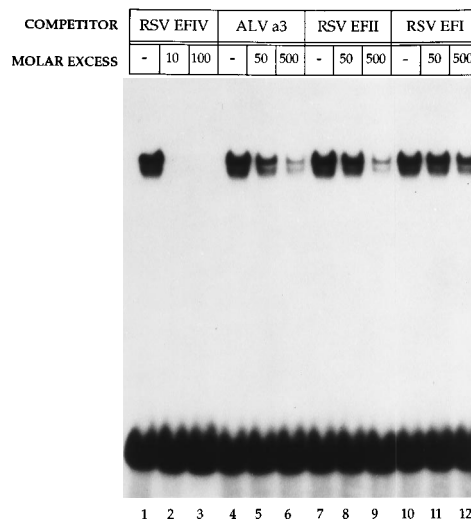


FIG. 2. The EFIV-protein complex represents a specific interaction. A constant amount (0.03 pm) of the end-labeled EFIV oligonucleotide was incubated with 7 μ g of protein extracted from S13 nuclei and 2 μ g of poly(dI) · poly(dC) in the absence (lanes 1, 4, 7, and 10) or presence of the following unlabeled competitor DNAs: lanes 2 and 3, a 10- and 100-fold molar excess of the EFIV oligonucleotide identical to the input probe; lanes 5 and 6, a 50- and 500-fold molar excess of the ALV a3 oligonucleotide; lanes 8 and 9, a 50- and 500-fold molar excess of the RSV EFII oligonucleotide; lanes 11 and 12, a 50- and 500-fold molar excess of the RSV EFI oligonucleotide. The locations and sequences of oligonucleotides used in this assay are listed in Table 1. The intensities of bands in control and experimental lanes were quantitated with a Molecular Dynamics densitometer after exposure of the dried gel to X-ray film.

(Fig. 2, compare lane 10 with lanes 11 and 12), indicating that the EFIV complex was specific.

To determine if the EFIV complex shared specificity with the ALV a3/VBP binding activity, we also used the ALV a3 probe shown in Table 1 as a competitor. The addition of a 50-fold molar excess of the ALV a3 oligonucleotide resulted in only an approximately 2-fold decrease in EFIV binding, while a 500-fold molar excess of this competitor resulted in an approximately 9-fold decrease in EFIV binding. These data indicate that the EFIV complex is distinct from that formed with the ALV a3 probe, in agreement with previous findings that indicated the RSV LTR lacked sequences in this region required for a3/VBP binding (37). The fact that the ALV a3 probe was able to partly compete for EFIV binding indicates that the binding protein(s) involved in a3 and EFIV complex formation may share a degree of similarity in binding specificities.

We also used as competitor DNA a 38-bp oligonucleotide (EFII; Table 1) from the 5' end of the RSV LTR that includes two sites that bind members of the C/EBP family, including EFII (34, 35), C/EBP α (31, 32), ALV a1/EBP, and a3/VBP (3, 37). When the RSV EFII oligonucleotide was used as the competitor DNA for EFIV binding, partial competition for EFIV formation was observed (Fig. 2, compare lane 7 with lanes 8 and 9), a result similar to that obtained with the ALV a3 probe. Again, the level of competition was significantly less than that seen with EFIV cold competitor. A similar result was obtained when the RSV EFII oligonucleotide was used as a probe in an EMSA and the EFIV oligonucleotide was used as the competitor (data not shown). Thus, as was seen with the ALV a3 oligonucleotide, sequences within the RSV EFII oligonucleotide share binding specificity with proteins that bind within the EFIV oligonucleotide.

Residues involved in EFIV binding mapped by chemical interference footprinting. To define guanine residues within the EFIV oligonucleotide involved in factor binding, we conducted methylation interference assays using extracts prepared from S13 cells and from CEFs as described in Materials and Methods. Identical results were obtained with either extract; those obtained with S13 cell extracts are shown in Fig. 3A. A comparison of the cleavage products generated from the free and bound fractions with the probe labeled on the upper strand revealed a depletion in the bound fraction of three guanine residues located at positions -190, -189, and -175, indicating that methylation of these residues interfered with protein binding. Results obtained with the lower strand revealed a depletion of two guanine residues at positions -185 and -174. An additional nucleotide, at position -171, was protected in some but not all experiments.

To more precisely define residues involved in EFIV binding, we also conducted footprinting experiments with DEPC, which preferentially modifies adenine residues relative to guanine residues. Results of these experiments obtained with extracts prepared from S13 cells are shown in Fig. 3B. As indicated, four bands specifically missing in the bound fraction indicated the lack of cleavage of adenine residues at positions -180, -176, -173, and -172 on the upper strand and of one adenine residue on the lower strand at position -188 (Fig. 3C).

Two binding sites for EFIV. An examination of the EFIV oligonucleotide revealed the presence of a sequence within the footprinted region (GCAACATG, positions -175 through -168) that was repeated at the 5' end of the EFIV oligonucleotide. The 3' copy of the motif, which begins at position -175, included three of the seven contacts on the upper strand and two of the three to four contacts on the lower strand of the EFIV oligonucleotide. The 5' copy, beginning at position

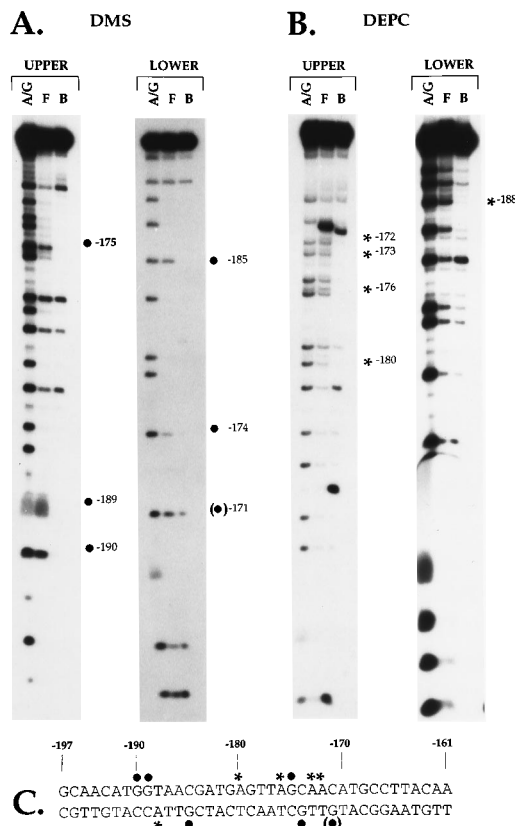


FIG. 3. Chemical interference footprints of the EFIV binding site. The EFIV oligonucleotide (Table 1) was labeled on either the upper or lower strand, treated individually with either dimethyl sulfate (DMS) (A) or DEPC (B) and incubated with proteins extracted from S13 nuclei. After electrophoresis, the free (F) and bound (B) fractions were excised from the gel, purified, cleaved with piperidine, and subjected to electrophoresis in denaturing acrylamide gels as described previously (40). The A/G ladders (A/G) were prepared in parallel also as described previously (40). The footprinted nucleotides are denoted by a dot (for guanine residues identified by DMS) or an asterisk (for adenine residues identified with DEPC), and their locations in the LTR are noted. (C) Sequence of the RSV LTR from positions -197 to -161 relative to the transcription start site. Footprinted nucleotides are depicted with the appropriate symbols, as noted above. The guanine residue in parentheses was identified in some experiments but not in others.

-197, included only one contact on the upper strand (Fig. 3C). This finding, together with the fact that the one residue identified in the 5' repeat did not correspond to a contact in the 3' repeat, suggested that the 5' copy did not interact with cellular proteins. However, the upstream copy of the motif was located at the extreme 5' end of the EFIV oligonucleotide, a position that might not allow efficient binding of EFIV. To test whether the 5' copy did include a functional EFIV binding site and to determine if both sites might bind EFIV simultaneously, two PCR products were generated from this region as described in Materials and Methods. One probe (PCR long) extended 12 bp further upstream than the original EFIV oligonucleotide and therefore included two copies of the GCAACATG repeat with flanking sequences. The second PCR product (PCR short) was 16 bp shorter than the PCR long product and was missing the 3' copy of the GCAACATG repeat. Each of these products was used in an EMSA to determine its ability to bind EFIV. As shown in Fig. 4A, the migration of the EFIV complex was indistinguishable among the EFIV oligonucleotide (lanes 1 and 2) and the PCR long (lanes 3 and 4) and short (lanes 5 and 6) products, although the amount of the complex

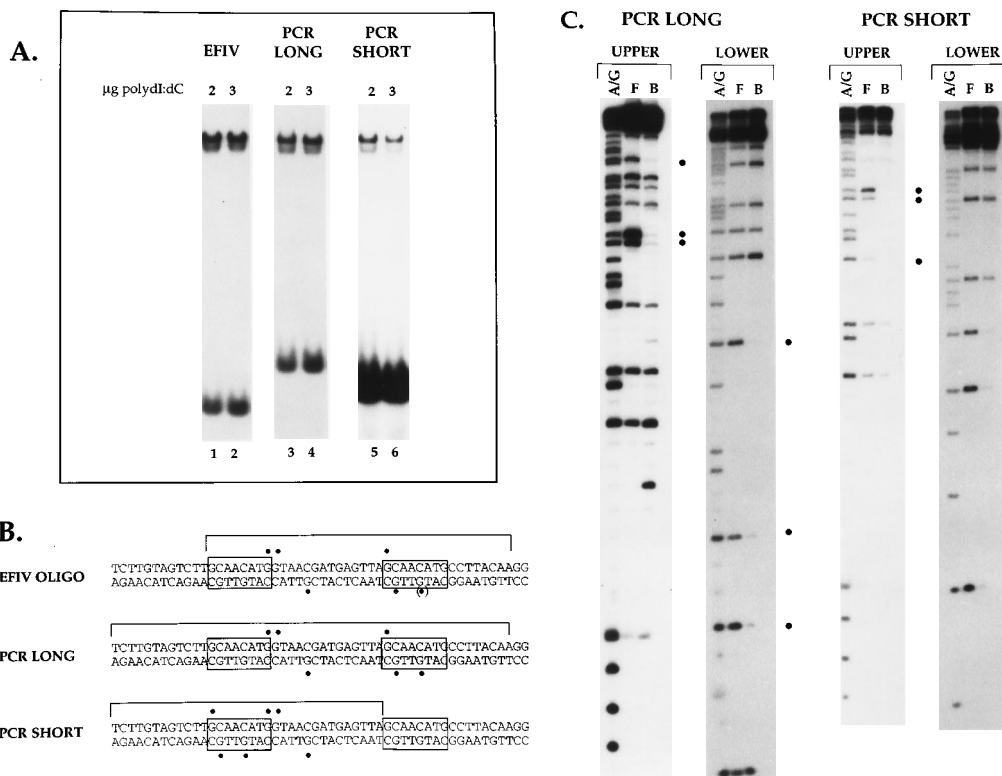


FIG. 4. Identification of a second EFIV binding site in the RSV LTR. The PCR long and PCR short probes were generated and labeled as described in Materials and Methods and subjected to EMSA (A) and methylation interference footprinting (C) as previously described (40). Protected residues within the bracketed regions in panels B and C are marked with dots. Lanes 5 and 6, which show binding to the PCR short probe, were obtained from a film that was exposed approximately four times longer than the film used for lanes 1 to 4, demonstrating the significantly weaker binding of EFIV to the 5' copy of the repeat present in the PCR short probe. The guanine residue in parentheses was identified in some but not all experiments. F, free fraction; B, bound fraction; A/G, A/G ladder.

seen with the PCR short probe was significantly less than that seen with the other two probes. The finding that the PCR short probe generated a complex that comigrated with EFIV suggested that the 5' copy of the repeat was able to bind EFIV. Interestingly, there was no evidence of a complex that migrated more slowly than EFIV with the PCR long probe, a result that would be predicted if both the 5' and 3' copies of the repeat were bound on one molecule.

To verify that the complex formed with the PCR short probe was due to EFIV binding and to determine if only one or both sites in the PCR long probe were able to bind EFIV when present on one molecule, methylation interference assays were conducted with both PCR products with extracts from S13 cells. As shown in Fig. 4C and depicted on the map in Fig. 4B, the methylation interference footprint obtained with the PCR long probe was the same as that seen with the EFIV oligonucleotide, indicating that when both copies of the repeat are present, binding to the 3' copy is preferred. When the PCR short probe was analyzed, EFIV binding over the 5' copy of the repeat was demonstrated by the fact that the three guanine residues mapped within the 3' copy of the repeat were also footprinted over the 5' copy of the repeat present in this probe. We interpret these data to indicate that the 5' copy of the repeat is able to bind EFIV but only when the 3' copy of the repeat is absent. Additional residues that lie between the 5' and 3' copies of the EFIV repeats were also footprinted when either copy of the repeat was bound. However, using the RSV EFIV short oligonucleotide (Table 1) that spans this internal region, we (data not shown) and others (37) have been unsuccessful in detecting direct binding to this site.

The EFIV binding site defines a transcriptional activator sequence. To determine if the EFIV binding site described above constitutes a transcriptional activating sequence, the EFIV oligonucleotide was multimerized and introduced into the pTK.Luc vector, which contains the TK promoter driving expression of the luciferase reporter gene as described in Materials and Methods. As shown in Fig. 5A, four copies of the wild-type EFIV oligonucleotide activated expression from the TK promoter four- to fivefold in CEFs and in the bursal lymphoma cell line S13. These data demonstrate that the EFIV binding site contains a functional transactivator sequence that is active in both avian B cells and fibroblasts. Analogous results were obtained with CEFs and S13 cells with constructs that contained two copies of the EFIV region (Fig. 5A and data not shown).

To investigate the role of the repeats and internal residues in the binding of EFIV and in transcriptional activation, the four mutant oligonucleotides shown in Fig. 5B were tested for binding activity and for the ability to activate transcription from the TK promoter. The mutant oligonucleotides contained mutations in either the 5' (m5') or 3' (m3') EFIV repeat, in the internal region (mI), or in both the internal region and the 3' repeat (mI3'). To define which portions of the EFIV binding site were critical for transcriptional activation, the four mutant oligonucleotides were introduced into the pTK.Luc vector as described in Materials and Methods. As shown in Fig. 5A, constructs that contained the m3' and m5' oligonucleotides, each of which contain one mutated EFIV repeat, showed an approximately twofold decrease in the level of expression relative to that of constructs that contained the wild-type EFIV

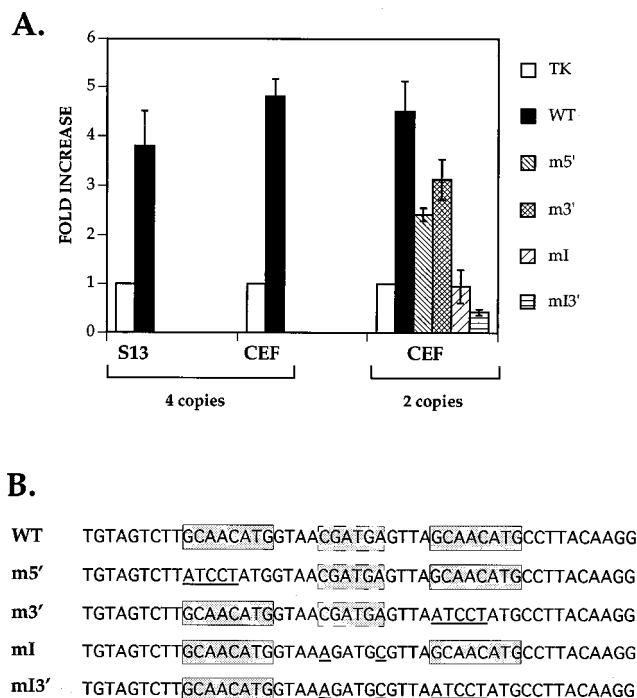


FIG. 5. The EFIV binding site includes transcriptional regulatory sequences active in CEF and S13 cells. The wild-type (WT) and mutant EFIV oligonucleotides shown in panel B were multimerized and introduced upstream of the TK promoter in TK.Luc as described in Materials and Methods. Constructs containing either two or four copies of the binding site were transfected into CEFs and S13 cells, and extracts were assayed for luciferase activity as described in Materials and Methods. Cultures were cotransfected with a CMV β -galactosidase plasmid as an internal control, and luciferase values were normalized to β -galactosidase activity. The relationship between constructs did not change as a result of this normalization. (B) The wild-type copies of the two EFIV repeats and the internal region are enclosed in stippled boxes, while mutated copies of these sequences are unboxed and the mutated residues are underlined.

region. These data demonstrate that each motif can function as an activator sequence and that maximal activity is obtained when both copies are present in the wild-type form. This result is not due to a simple copy number effect, since either two or four copies of the wild-type oligonucleotide gave the same level of activation in these assays (compare results obtained with two and four copies of the wild-type EFIV oligonucleotide in Fig. 5). Figure 5 also shows results obtained with constructs that contained mutations in two of the internal residues either alone (mI) or in combination with mutations in the downstream EFIV repeat (mI3'). As shown, the mI constructs showed no activation over that seen with the TK promoter alone, demonstrating that residues that lie between the two EFIV repeats identified as involved in EFIV binding by chemical interference assays are clearly involved in EFIV function. An additional mutation of the 3' copy of the EFIV repeat in construct mI3' reproducibly decreased levels of expression even further to levels below those seen with the TK promoter alone. Results analogous to those shown for CEFs were also obtained when these constructs were transfected into S13 cells (data not shown). These data demonstrate that both the EFIV repeats and nucleotides that lie between these two repeats are critical for the functional activity of this region.

Each of the mutant oligonucleotides was also tested for EFIV binding by both a direct binding assay and a binding disassociation assay. As shown in Fig. 6A, the m5' (lane 2), m3' (lane 3), and mI (lane 4) oligonucleotides each generated the

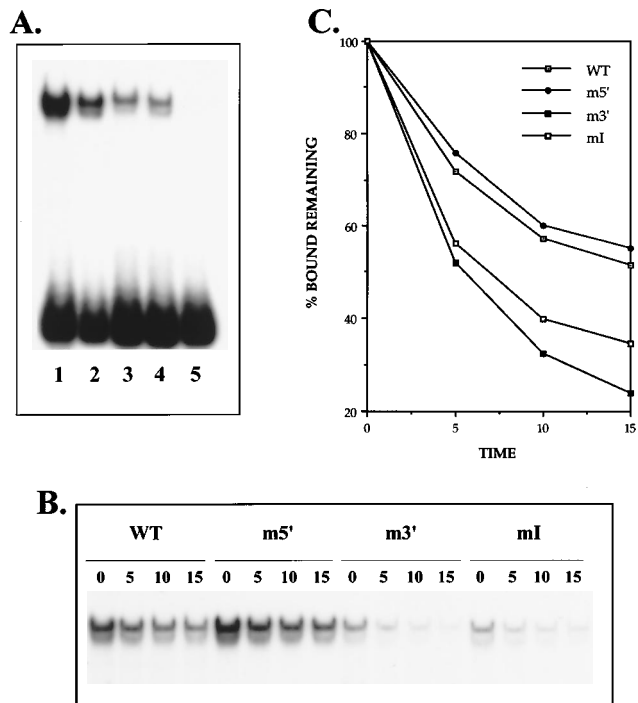


FIG. 6. Mutations within the EFIV binding site that affect function also affect protein binding activity. The wild type (WT) and the indicated mutant oligonucleotides shown in Fig. 5C were used in direct binding (A) and binding disassociation (B) experiments as described in Materials and Methods. (C) Results obtained in the binding disassociation experiment expressed as percentages of the bound fraction remaining after the indicated time (in minutes) had elapsed.

characteristic EFIV doublet seen with the wild-type probe (lane 1), although the amount of complex formed with the m3' and mI probes appeared significantly decreased. This decreased level of binding activity was a consistent finding, even in experiments such as that shown in Fig. 6 in which the probes were labeled by the same specific activity. No binding was detectable with the double mutant mI3' probe (Fig. 6, lane 5). To determine if the apparent differential binding activities of the four mutant oligonucleotides described above reflected a true difference in binding affinity, binding disassociation experiments were performed as described in Materials and Methods. As shown in Fig. 6B and graphed in Fig. 6C, these experiments gave results consistent with those of the direct binding assay. In particular, it was found that the m5' oligonucleotide, which contains an intact copy of the preferred, 3' EFIV repeat, bound EFIV in a manner indistinguishable from that of the wild-type oligonucleotide. The m3' oligonucleotide, which contains mutations in the preferred binding site, showed an apparent weaker binding affinity for EFIV, consistent with results obtained with the PCR short probe in Fig. 4A. Interestingly, the mI probe, which contains two mutations within the internal region, also showed weak binding activity for the EFIV probe, again supporting the functional data shown above that demonstrate the necessity of this internal region for EFIV binding and function.

The EFIV complex exhibits stable binding activity in CEFs and in avian B cells. Previous studies have indicated that the ALV a1/EBP and a3/VBP EBPs exhibit labile binding activity in avian B cell extracts but not in CEF extracts (29, 30). To determine if a similar lability would be observed with the RSV EFIV complex, extracts were prepared from CEFs and from S13 cells that had been treated with the protein synthesis

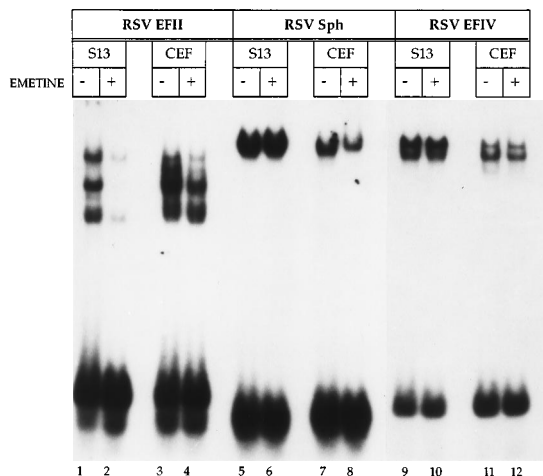


FIG. 7. The EFIV binding activity is stable in CEFs and S13 cells. Extracts were prepared from control cells (-) and from cells that had been treated with the protein synthesis inhibitor emetine (+) as described in Materials and Methods. These extracts were then used in EMSAs with the following probes: the RSV EFII probe, a 38-bp oligonucleotide from the extreme 5' end of the RSV LTR; the RSV Sph probe, a 29-bp oligonucleotide that contains a CArG box/EFIII binding site; and the EFIV oligonucleotide.

inhibitor emetine as described in Materials and Methods, and the presence or absence of different complexes was tested by EMSA as shown in Fig. 7. In agreement with results obtained by others (33–35), the RSV EFII probe generated a series of three bands when assayed with nuclear extracts prepared from either untreated avian CEFs or B cells (Fig. 7, lanes 1 and 3). We have observed the same pattern of shifted complexes with the ALV α 1 probe (data not shown). While at least two of the RSV EFII complexes were largely unaffected by emetine treatment of CEF extracts (Fig. 7, compare lanes 3 and 4), amounts of all three complexes were significantly reduced in extracts prepared from emetine-treated S13 cells (compare lanes 1 and 2). These data indicate that the RSV EFII binding activities show a pattern of emetine sensitivity in avian B cells similar to that reported for the α 1/EBP binding activity of ALV (29, 30). To demonstrate that this was a specific effect, the S13 and CEF control and the emetine-treated extracts were also tested with the RSV Sph probe, which binds EFIII, the avian CArG box-binding homolog of SRF (40). In agreement with reports that cycloheximide does not decrease and in fact increases the level of activation of genes regulated by SRF (39), we found that the EFIII complex was unaltered after emetine treatment of S13 cells (Fig. 7, lanes 5 and 6) and showed only a slight decrease after emetine treatment in the amount of extracts prepared from CEFs (lanes 7 and 8). Lanes 9 through 12 of Fig. 7 show results obtained with the RSV EFIV probe. As shown, approximately equal amounts of the EFIV complex were formed with control and emetine-treated extracts from S13 cells and CEFs, demonstrating that the formation of this complex is resistant to emetine treatment. Thus, EFIV is distinct from other 5' LTR-binding proteins of RSV and ALV in both cell type distribution and binding ability.

DISCUSSION

In this report we describe EFIV, an avian factor present in B cells, T cells, and CEFs that specifically interacts with an enhancer motif in the RSV LTR. The presence of a transcriptional activation sequence within the EFIV binding site was demonstrated directly with expression vectors introduced into

both avian B cells and CEFs. By chemical interference footprinting and mutagenesis we have identified residues important for factor binding and function. These residues include a sequence (GCAACATG) located from positions -175 through -168 that is repeated further upstream in the LTR at positions -197 through -190 as well as nucleotides between these two repeats. Interestingly, the repeats are separated by 14 nucleotides, so that if EFIV does bind at both repeats *in vivo*, the protein(s) would lie on opposite sides of a B form DNA helix. However, when both copies of the EFIV repeat were present on one DNA molecule, binding was observed only over the 3' copy of the element in *in vitro* binding assays, while the deletion or mutation of this 3' copy revealed binding over the 5' copy. These data indicate that the two sites do not represent equivalent binding targets for EFIV and that the 3' copy is the preferred site in these *in vitro* binding assays. The finding that binding over each site resulted in the same pattern of complexes in EMSAs and generated the same methylation interference footprint indicates that the same factor(s) binds at each of the EFIV repeats. It was further demonstrated that these repeats are important for transcriptional activation by the EFIV binding site, since the mutation of either element alone significantly decreased levels of activity.

Additional residues between the two EFIV repeats were also identified in chemical interference footprinting assays as being involved in EFIV binding, and the mutation of these residues abolished transcriptional activation by the EFIV region. These data suggest either that this internal region directly binds protein(s) required for EFIV function or that this region acts more indirectly by affecting either the stability of EFIV binding to the adjacent repeats or the structure and/or the conformation of the bound EFIV factors. We currently favor a more indirect role for this internal region on the basis of several findings. First, an oligonucleotide that includes only this internal region (the EFIV short oligonucleotide in Table 1) does not detectably bind protein under conditions that allow easy detection of EFIV binding to adjacent sequences. Second, the two nucleotides altered in the mI mutant were identified in chemical interference assays of the EFIV doublet detected with the wild-type probe. If these nucleotides directly bind a factor that is not bound by the repeat sequences, then the mutation of these nucleotides should disrupt the binding of this factor and thereby lead to the detection of a faster-migrating complex, a result not seen with EMSAs conducted with the mI oligonucleotide. This internal region is clearly important for EFIV function, however, since the mutation of only two nucleotides in this region abolished the transcriptional activation mediated by the EFIV region. It is possible that this region might, for example, serve to correctly position EFIV bound over the two repeats in relation to each other or, in the context of the intact LTR, relative to other factors bound in adjacent regions.

Portions of the EFIV site overlap sequences that bind the RSV EFII protein(s) (34, 35) and the ALV α 1/EBP and α 3/VBP proteins (3, 37), although the consensus binding motifs for each of these factors are different. Results obtained with EFIV demonstrate that it is distinct from RSV EFII and the ALV α 1/EBP and α 3/VBP proteins. This is indicated by the weak competition of these probes in electrophoretic mobility shift binding competition experiments and by the finding that EFIV generates a methylation interference footprint distinct from that described for the ALV binding factors (37). One point to note is that we have been unable to detect binding of EFII to the 3' EFII element that overlaps the upstream or 5' EFIV element, although we do see efficient binding of EFII to the 5' copy of its binding site (between the 5' end of the LTR

and position -213). Thus, sequences between positions -206 and -190 include the weaker, less preferred binding sites for both EFII and EFIV. If binding to the 3' copy of the EFII element was a high-affinity interaction, we would have expected to see complex formation with at least the EFIV mutant oligonucleotide which contains a reportedly functional binding site for EFII. These data support the findings of Sears and Sealy, who noted binding over the 3' copy of the EFII repeat in only one cell line tested, the avian bursal lymphoma cell line BK25, which contains relatively high levels of EFII (34).

Ryden and colleagues (31, 32) have identified four binding sites for C/EBP α in the 5' portion of the LTR from the Pr-C strain of RSV that match the C/EBP α consensus binding site (A/C/G)T(T/G)NNG(C/A/T)AA(T/G). Two of these sites (sites 1 and 2) also interact with three other C/EBP family members, a1/EBP (3), C/EBP β (35), and Ig-EBP (27). Site 3 overlaps the downstream ALV a3 binding site in the ALV LTR, which is deleted from the SR-RSV LTR. Thus, while the SR-RSV LTR is unable to bind a3 in this region, it is able to bind the EFIV proteins. The 3' EFIV binding site partially overlaps C/EBP α site 4, which was defined by Ryden and colleagues (32). However, the 3' EFIV binding site is a high-affinity binding site, which readily bound with crude nuclear extract, while it is a site having low-affinity for C/EBP α , which bound in this region only when high concentrations of purified protein were used (32). Thus we believe that EFIV is distinct from, but possibly related to, C/EBP α .

Two other reports have described factors that interact with sequences that overlap the EFIV binding site described here. Using oligonucleotides generated from the Pr-C strain of RSV, Kenny and Guntaka (19) described a factor, E2BP, that binds sequences that overlap the weak 5' copy of the EFIV repeat. No binding was detected over the strong 3' copy of the EFIV repeat. However, the Pr-C LTR contains a 1-nucleotide mismatch in the 3' EFIV repeat, so the significance of this finding is unclear. Two lines of evidence indicate that E2BP and EFIV are distinct. First, EFIV binding requires either a second copy of the EFIV repeat or defined sequences that lie between the EFIV repeats; we have been unable to detect binding to one EFIV repeat alone or to the 5' copy of the EFIV repeat when the 3' repeat and internal nucleotides are mutated. However, E2BP showed no such specificity and interacted equally well with oligonucleotides that contained either one or two copies of its binding site. Second, E2BP bound a sequence between positions -222 and -215 of the Pr-C LTR with a level of affinity equal to that seen with the site that overlaps the EFIV motif. However, direct binding and binding competition experiments have failed to reveal similar binding of EFIV to this E2BP motif, which is also present in the SR-RSV LTR used in these studies. It is possible that E2BP may represent a factor present in the quail, mouse, and rat cells lines tested by Kelly and Guntaka that is related to the EFIV factor we have detected in chicken cells; the answer to this question awaits cloning of the respective factors.

Goodwin (10) reported the binding of a factor (FI) from avian erythrocyte-derived extracts to sequences that also overlap the EFIV site described here. Figure 4C shows a sequence of the RSV LTR from positions -209 to -158 with the guanines and adenines involved in EFIV binding. The methylation interference footprint of the EFIV complex shown in Fig. 4C is similar to the FI footprint reported by Goodwin; DEPC footprinting experiments were not performed on the FI complex. In spite of the similarities of the methylation interference footprints, the FI complex appeared as a single complex in EMSA gels (10). It is therefore unclear if the erythrocyte FI complex is the same as one component of the EFIV doublet seen here

with CEF and S13 extracts. In these previous studies, it was noted that the FI binding site included an inverted repeat sequence located between positions -191 and -173 composed of the sequence



If this is the correct motif for FI binding, then this would indicate that FI is clearly distinct from EFIV, since this motif is not repeated again within sequences that comprise the upstream copy of the EFIV binding repeat. The determination of the relationship between these two factors and of the sequence requirements for binding will require more direct comparisons of factors in avian B cells and cells of the erythroid lineage.

Several of the factors that bind the ALV and RSV LTRs, including a1/EBP, a3/VBP, EFI, EFIII, and EFIV, have two or more binding sites in the LTR. Similar to results obtained by others who have introduced mutations into regions of the RSV LTR that include the a1/EBP and a3/VBP binding sites (5, 32), we found that specific mutations in the EFIV binding site resulted in only a modest decrease in levels of transcriptional activity within the context of an intact LTR (data not shown). Together, these mutation studies suggest that several of the enhancer motifs in the 5' portion of the LTR are functionally redundant, and mutations that affect more than one site within 5' enhancer sequences are required to see a significant effect on transcriptional activity. Interestingly, the ALV and RSV LTRs also contain two copies of the EFI and the EFIII/CArG box binding sites in the central portion of the LTR. However, these sites do not exhibit functional redundancy, since mutations in any one of these motifs abolish the activity of the RSV LTR (14, 15).

Previous studies by Smith and colleagues demonstrated that the binding of the 5' ALV LTR proteins a1/EBP and a3/VBP was labile specifically in B cells but not in other avian cell types. It was also reported that the expression of a3/VBP was limited to B cells, although more recent evidence indicates that the ALV a3-binding protein is identical to vitellogenin-binding protein (37), which is expressed in several avian tissues, including spleen, oviduct, testis, and liver tissues, and in CEFs (18). In addition to the lability of a1/EBP and a3/VBP binding observed in B cells, it was also demonstrated that LTR-driven expression was specifically downregulated in nuclei isolated from early bursal cells but not in nuclei from other cell types (21, 29). On the basis of these findings it was proposed that the cell-type-specific lability of 5' LTR binding activities is an important determinant for ALV-induced leukemogenesis in that it mediates a transient downregulation of LTR-driven *myc* expression in B cells of infected animals. Since deregulated *myc* expression has been linked to the induction of apoptotic cell death (1, 7, 36), this transient downregulation of *myc* expression might allow early B cells to escape apoptosis and undergo events required for neoplastic transformation.

In data presented here, we demonstrate that the RSV EFIV factor is present in many avian cell types and that it is not labile in B cells. This is in marked contrast to the reported B-cell-specific lability of the ALV a3/VBP. Despite these differences in the regulation of 5' LTR binding factors, it has been demonstrated that nonacute leukosis viruses that contain either the RSV or the ALV LTR induce the same spectrum of disease, primarily B-cell lymphoma. One interpretation of these data is that the B-cell-specific binding lability of the RSV EFII or the ALV a1/EBP factors might be either sufficient or of prime importance for cell-type-specific regulation of the LTR and for disease specificity and that lability and/or cell-type-specific ex-

pression of proteins that bind to the RSV EFIV or ALV a3 regions might be of minor importance in leukemogenicity. Alternatively, it is possible that while labile binding activity of both a1/EBP and a3/VBP might be important for ALV-induced disease, viruses that include the RSV LTR might utilize a distinct mechanism for disease induction. We are currently investigating these possibilities.

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