Functional and Defective Components of Avian Endogenous Virus Long Terminal Repeat Enhancer Sequences

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Oncogenic avian retroviruses, such as Rous sarcoma virus (RSV) and the avian leukosis viruses, contain a strong enhancer in the U3 portion of the proviral long terminal repeat (LTR). The LTRs of a second class of avian retroviruses, the endogenous viruses (ev) lack detectable enhancer activity. By creating ev-RSV hybrid LTRs, we previously demonstrated that, despite the lack of independent enhancer activity in the ev U3 region, ev LTRs contain sequences that are able to functionally replace essential enhancer domains from the RSV enhancer. A hypothesis proposed to explain these data was that ev LTRs contain a partial enhancer that includes sequences necessary but not sufficient for enhancer activity and that these sequences were complemented by RSV enhancer domains present in the original hybrid constructs to generate a functional enhancer. Studies described in this report were designed to define sequences from both the ev and RSV LTRs required to generate this composite enhancer. This was approached by generating additional ev-RSV hybrid LTRs that exchanged defined regions between ev and RSV and by directly testing the requirement for specific motifs by site-directed mutagenesis. Results obtained demonstrate that ev enhancer sequences are present in the same relative location as upstream enhancer sequences from RSV, with which they share limited sequence similarity. In addition, a 67-bp region from the internal portion of the RSV LTR that is required to complement ev enhancer sequences was identified. Finally, data showing that CArG motifs are essential for high-level activity, a finding that has not been previously demonstrated for retroviral LTRs, are presented.

The long terminal repeats (LTRs) of avian exogenous retroviruses, such as Rous sarcoma virus (RSV) and the avian leukosis viruses (ALVs), contain a strong enhancer that is required for high-level expression from the viral promoter and that can augment transcription from a number of heterologous promoters in different cell types (8, 9, 13, 15, 22, 24, 26, 29, 44, 45). In ALVs, the LTR-associated enhancer is also an important contributor to cellular transformation, since it is responsible for the increased transcription of cellular oncogenes adjacent to sites of provirus integration (18, 23, 31). The LTRs of a second class of avian retroviruses, the avian endogenous viruses (ev) (1, 21, 32), are distinct from those of the exogenous viruses in that they lack a detectable LTR-associated enhancer (5, 8–10, 29, 44). The absence of a strong enhancer in ev LTRs has been correlated with the low oncogenic potential of ev relative to exogenous viruses (6). Sequence comparisons have demonstrated major differences between ev and exogenous virus LTRs (19, 20, 37, 38), although it is not known which of these differences is responsible for their distinct enhancer activities.

We have previously demonstrated that ev LTRs, although lacking detectable enhancer activity, contain sequences that are able to functionally replace, in an orientation-independent manner, essential enhancer sequences in the RSV LTR, with which they share limited sequence similarity (5). This was demonstrated by constructing ev-RSV hybrid LTRs that replaced sequences from the RSV LTR required for enhancer function with sequences from the ev U3 region. The ability of the ev U3 region to restore high-level transcriptional activity to the enhancer-deleted RSV was not explained by differences in the ability of ev and RSV promoters

to respond to enhancers placed in *cis*. In agreement with previous data (8–10, 29, 44), it was also found that the same ev U3 fragment that was able to restore transcription to the enhancer-deficient RSV LTR was unable to enhance transcription from an unrelated promoter, that of the herpesvirus thymidine kinase gene. A hypothesis proposed to explain these data was that ev LTRs contain motifs necessary but not sufficient for enhancer function and that these motifs can be complemented by sequences from the RSV LTR present in the hybrid constructs to generate a functional enhancer element. Experiments presented in this report were designed to define sequences from both the ev and RSV LTRs required to generate this composite enhancer.

Sequences from RSV that were included in the hybrid LTR constructs mentioned above and that were therefore candidate sequences required to complement ev enhancer sequences extended downstream of an *Sph*I site at −141 from the start site of transcription. At least three proposed components of the RSV enhancer have been identified within this region. These include two inverted CCAAT boxes centered at positions −67 and −131 (−67 and −131 CCAAT boxes) that bind a nuclear factor(s) that will be referred to as EFI, with the −131 site showing an apparent higher affinity for this protein(s) (12, 14, 16, 30, 33, 34, 39). Mutational analyses have demonstrated that each of these CCAAT motifs is required for maximal transcription from the RSV LTR (16). Also included in this region is a CArG box, defined by the sequence $CC(A-T rich)_6GG$, centered at position −100. This motif binds a factor called EFIII (2), which is the proposed avian homolog of the serum response factor, a positive regulatory protein that mediates transcriptional upregulation of the c-*fos* serum response element and other CArG-containing elements in response to a variety of growth factors and mitogens, including serum (42). CArG boxes are also requisite transcriptional regulatory sequences

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found upstream of other cellular genes, including the vertebrate actin genes and the murine immediate-early gene *zif268* (42). Sequence comparisons demonstrate that ev LTRs lack motifs analogous to the −67 inverted CCAAT box and the −100 CArG box. In addition, although ev LTRs do contain an inverted CCAAT box at the same relative location as the −131 CCAAT box in RSV, previous studies indicated that the ev CCAAT box does not detectably bind RSV CCAAT box binding factor EFI (46). Thus, the two inverted CCAAT boxes and the −100 CArG box motifs in RSV are all candidate sequences required to complement ev enhancer sequences in hybrid LTRs.

The portion of the RSV enhancer that was deleted during construction of hybrid LTRs, and which therefore was functionally replaced by ev U3 regions, extended from the 5′ end of the LTR at position −229 through the *Sph*I site at −141. This 88-bp fragment can enhance transcription from at least some heterologous promoters (8) and the deletion of sequences within this region can abrogate transcriptional activation (8, 22, 24, 29), demonstrating the necessity of 5′ proximal LTR sequences for enhancer function. Protein binding studies with the RSV LTR and the highly related LTR of ALV have identified at least three sites within this region that bind avian nuclear proteins. These include a second CArG box-EFIII binding site centered at position −162 (46) and two poorly characterized regions that bind avian nuclear proteins that have been named EFII (39), FIII (14) or a1 (33, 34), and a3 (33, 34). Proteins that interact with the a1 and a3 binding regions show B-cell-specific lability in cells from chickens susceptible to virus-induced lymphomagenesis and have been implicated in the regulation of tumorigenesis by ALVs (33, 34).

The ev U3 fragment inserted in place of the upstream RSV enhancer sequences extended from the 5′ end of the ev LTR through an *Acc*I site at −50. This region of the ev LTR contains a binding site for a CCAAT/enhancer-binding-protein (c/EBP)-like heat-stable protein whose role in transcriptional regulation has not been determined (36). Sequence comparisons and protein binding studies have also demonstrated the presence of a CArG box-EFIII binding site at the same relative position as the RSV −162 CArG box (46), suggesting that this motif is one component of ev enhancer sequences. Finally, ev LTRs also contain an inverted CCAAT box at the same relative position as the RSV −131 CCAAT box-EFI binding site although, as mentioned above, the ev CCAAT box does not detectably bind the EFI factor (46). Aside from these CArG and CCAAT motifs, however, ev LTRs show extensive divergence from RSV within 5′ LTR sequences, and the identity and location of ev sequences that are functionally equivalent to the RSV 5′ enhancer sequences have yet to be determined.

To define sequences from the ev LTR that can functionally substitute for the upstream RSV enhancer domains and to define sequences from RSV required to complement ev enhancer sequences, additional hybrid LTRs that exchanged defined sequences between ev and RSV were constructed. In addition, the requirement for specific motifs for high-level transcription from hybrid LTRs was tested directly by site-directed mutagenesis. Results obtained indicate that enhancer sequences from the ev LTR are located in the same relative position as the upstream enhancer domains from RSV. This finding was unusual because of the limited degree of sequence similarity between ev and RSV LTRs in this region. A 67-bp region from the RSV LTR that was required to complement ev enhancer sequences was also identified. Additional data showing that CArG motifs are essential for

high-level activity of these retroviral LTRs, a finding that has not been previously demonstrated, are presented.

MATERIALS AND METHODS

Generation of ev-RSV hybrid LTRs. The parental plasmids used for construction of ev-RSV hybrid LTRs have been previously described (5) and are called pM-RSVNeo and pRAV-0Neo. pM-RSVNeo contains the 3′ LTR from the Schmidt-Ruppin A strain of RSV and was derived from pRSVNeo (15). The LTR in pRAV-0Neo was generated from the 3′ LTR of ev-2 (kindly provided by P. Tsichlis and J. Coffin). In each case, the LTR fragment extended from an *Mst*II site (which was converted to a *Bam*HI site) located 39 bp upstream of the LTR in the 3′ untranslated region through a *Bst*NI site in U5 (which was converted to a *Hind*III site). These LTRs were linked to the neomycin resistance (Neo^r) gene encoding sequences as previously described (5).

The basic strategy used to generate hybrid LTRs is shown in Fig. 1. First, overlapping regions from pRAV-0Neo and from either pM-RSVNeo (for hybrids I, II, and III) or hybrid II (for hybrids II-A and II-B) were amplified separately by polymerase chain reaction (PCR). The overlap between ev and RSV sequences either was due to naturally occurring regions of similarity between these two LTRs (for hybrid II) or was generated by using hybrid oligonucleotides as internal primers (for all other hybrid constructs). The products of these reactions were then mixed and subjected to a second round of amplification with only the external primers (labeled A and D in Fig. 1) to generate hybrid LTRs whose junction was defined by the identity of the internal primers used in initial reactions. The PCR-generated LTRs were then digested with *Bam*HI (which cleaves upstream of the LTR at the converted *Mst*II site in the 3′ untranslated region) and *Hind*III (which cleaves within U5 at the converted *Bst*NI site) and inserted in place of the RSV LTR in pMRSVNeo. Parental, mutant, and chimeric LTRs were sequenced by dideoxy sequencing (Sequenase), and at least two isolates of each plasmid were tested in primer extension assays. Some isolates of hybrid II contained a T to C mutation at position −51 that did not affect the transcriptional activity of individual isolates (see Fig. 6B). This alteration was also present in hybrid II LTRs that contained mutations in the ev CCAAT box and ev CArG box.

The external 5′ primer (primer A) used in the first and second amplification reactions was a universal sequencing primer (5′-GTAAAACGACGGCCACT-3′), while the external 3′ primer (primer D) was a neomycin specific primer (5′-CGTACTGCCTAACTTGTACC-3′). The sequences of internal primers (primers B and C) used to generate each of the hybrid LTRs are shown in the lower portion of Fig. 1. Hybrids I, II, and III contained one ev-RSV crossover point that is shown in Fig. 1 and 2. Hybrid II was used to generate additional hybrids (II-A and II-B) with a second crossover point also shown in Fig. 1 and 2.

Site-directed mutagenesis. Hybrid II was used to introduce site-specific mutations into the ev CArG box, the RSV −100 CArG box, and the ev CCAAT box. This was accomplished by using mutant oligonucleotides that contained specific alterations described below as internal primers in PCR amplifications.

Transfection, RNA isolation, and primer extension. Hybrid, mutant, and wild-type LTR constructs were transfected into QT35 cells, a chemically transformed quail embryo fibroblast cell line (28), and RNA was isolated after 48 h as previously described (5). In some cases, a plasmid that
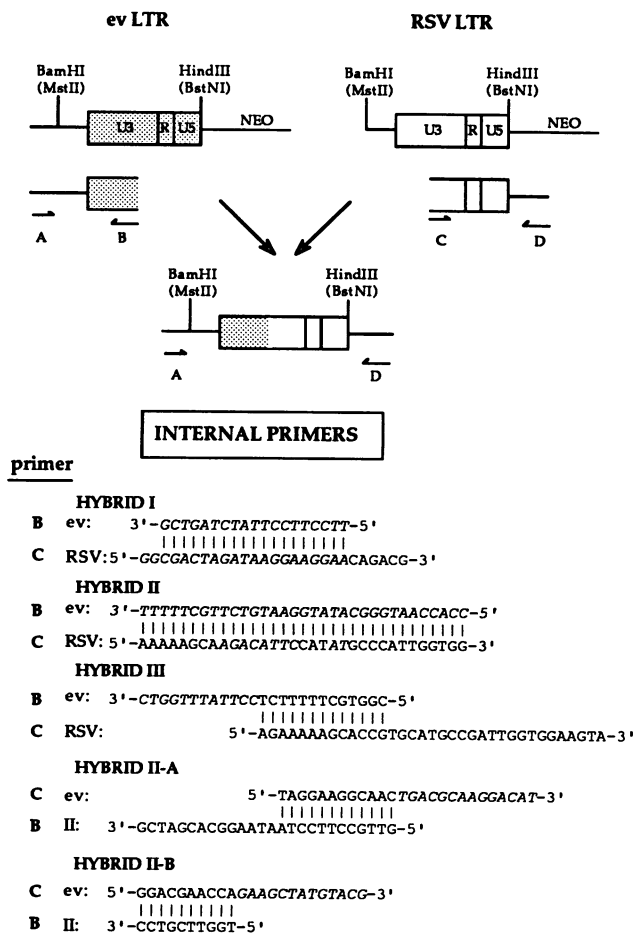
ev LTR   RSV LTR

primer

INTERNAL PRIMERS

HYBRID I

B ev: 3'-*GCTGATCTATTCCTTCCTT*-5'
          |||||||||||||||||||
C RSV: 5'-*GGCGACTAGATAAGGAAGGAACAGACG*-3'

HYBRID II

B ev: 3'-*TTTTTCGTTCTGTAAGGTATACGGGTAACCACC*-5'
          ||||||||||||||||||||||||||||||||
C RSV: 5'-*AAAAAGCAAGACATTCCATATGCCCATTGGTGG*-3'

HYBRID III

B ev: 3'-*CTGGTTTATTCCTCTTTTTCGTGGC*-5'
          ||||||||||||
C RSV: 5'-AGAAAAAGCACCGTGCATGCCGATTGGTGGAAGTA-3'

HYBRID II-A

C ev: 5'-TAGGAAGGCAAC*TGACGCAAGGACAT*-3'
                ||||||||||||
B II: 3'-GCTAGCACGGAATAATCCTTCCGTTG-5'

HYBRID II-B

C ev: 5'-*GGACGAACCAGAAGCTATGTACG*-3'
         ||||||||||
B II: 3'-CCTGCTTGGT-5'

FIG. 1. ev-RSV hybrid LTRs: construction and sequence of primers. The top portion shows the strategy for constructing ev-RSV hybrid LTRs. To generate ev-RSV hybrid LTRs that contained one crossover point (hybrids I, II, and III), internal oligonucleotide primers (B and C) listed in the lower portion of the figure and external primers (A and D) described in Materials and Methods were used to amplify discrete regions of each LTR in separate reactions. The sequences of internal primers used in these reactions were such that they resulted in a sequence overlap between ev and RSV LTRs. This overlap either was due to naturally occurring regions of similarity between these two LTRs (hybrid II) or was generated by using primers that contained an artificial junction of ev and RSV sequence information (all other hybrids). The products of these initial amplifications were then mixed, subjected to a second round of amplification with only the external primers, and cleaved with *Bam*HI and *Hin*dIII. The resultant hybrid LTR fragment was then inserted into a neomycin expression construct as described in Materials and Methods. Hybrids II-A and II-B were constructed by the same strategy, except that the hybrid II template was used in initial amplification reactions in place of the RSV LTR. The sequences of internal primers used in initial amplification reactions are shown in the lower portion of the figure. ev sequences are shown in italics, and RSV sequences are in roman type; vertical lines between the sequences indicate identity. Hybrids I, II, and III contained one crossover point between ev and RSV sequences shown in the figure, while hybrids II-A and II-B contained a second crossover; only the second crossover points in these hybrids are shown. The locations of crossover points within the context of intact ev and RSV LTRs are also shown in Fig. 2.

contained the cytomegalovirus (CMV) promoter (7) driving expression of the Neo[r] gene (kindly provided by M. Linial) was cotransfected with experimental constructs as an internal control. Transcripts generated from both the CMV- and LTR-containing vectors were detected by primer extension as previously described (5) by using the 22-nucleotide neomycin-specific primer described above. Correctly initiated transcripts from the LTR promoters are 77 nucleotides in length, while those from the CMV promoter are 90 nucleotides in length. Transcript levels were quantitated from autoradiograms by using computer-assisted video densitometry (25) as previously described (5).

EMSA. Nuclear extracts were prepared from QT35 cells as previously described (46), except that 0.5 M NaCl was used for nuclear extractions. Electrophoretic mobility shift assays (EMSA) and methylation interference assays were conducted as previously described (46), with approximately 0.002 pmol (10,000 cpm) per reaction. The sequences of the 5' (upper) strands of oligonucleotides used in Fig. 6A that contained the wild-type CArG motifs were as follows: for the RSV −100 CArG box, 5'-CGATCGTGCCTTATTAGG AAGGCAAC-3'; for the ev CArG box, 5'-TCTAAAGACCA AATAAGGAAAAAGC-3'. Oligonucleotides that contained the mutated CArG motifs (named GArC in Fig. 6) were identical to those listed above except for the CArG box mutations described below. For use in EMSA, oligonucleotides were labeled on one strand with T4 polynucleotide kinase (Bethesda Research Laboratories) and [γ-^{32}P]ATP (New England Nuclear), made double stranded by incubating with the complementary strand, and purified as previously described (46).

DNA fragments that were generated for EMSA shown in Fig. 5B extended from what in RSV is defined as the −131 CCAAT box-EFI binding site and the −100 CArG box-EFIII binding site. For generation of end-labeled DNA fragments, 20 pmol of one of the oligonucleotide primers listed below was labeled on its 5' end with T4 polynucleotide kinase (Bethesda Research Laboratories) and [γ-^{32}P]ATP (New England Nuclear). Conditions for PCR amplification and purification of DNA fragments were as previously described (46). The templates and primer pairs used to generate these DNA fragments were as follows: for the RSV LTR, 5' primer 5'-AGAAAAAGCACCGTGCATGCCGATTGGTGGAA GTA-3' and 3' primer 5'-CGATCGTGCCTTATTAGGAAG GCAAC-3'; for the ev LTR, 5' primer 5'-AAAAAGCAAG ACATTCCATATGCCCATTGGTGG-3' and 3' primer 5'-CGACTAGATAAGGAAGGAA-3'). For the hybrid II LTR, the 5' primer was the same as that used for the ev LTR while the 3' primer was the same as that used with the RSV LTR. For the hybrid II template that contained a mutant CCAAT box, the 5' primer was the same as that used for the ev template except that it contained the CCAAT box mutations described below, while the 3' primer was the same as that used with the RSV LTR. Amplification reactions were conducted as previously described (46), and products were purified by acrylamide gel electrophoresis.

RESULTS

ev enhancer sequences are located in the same relative position as upstream RSV enhancer domains. Figure 2 shows a sequence comparison of the RSV and ev U3 regions aligned to show maximum homologies. Also shown in the figure are previously described factor binding motifs within each U3 region. Figure 3 shows a diagrammatic representation of the RSV (pM-RSVNeo) and ev (pRAV-0Neo) LTRs
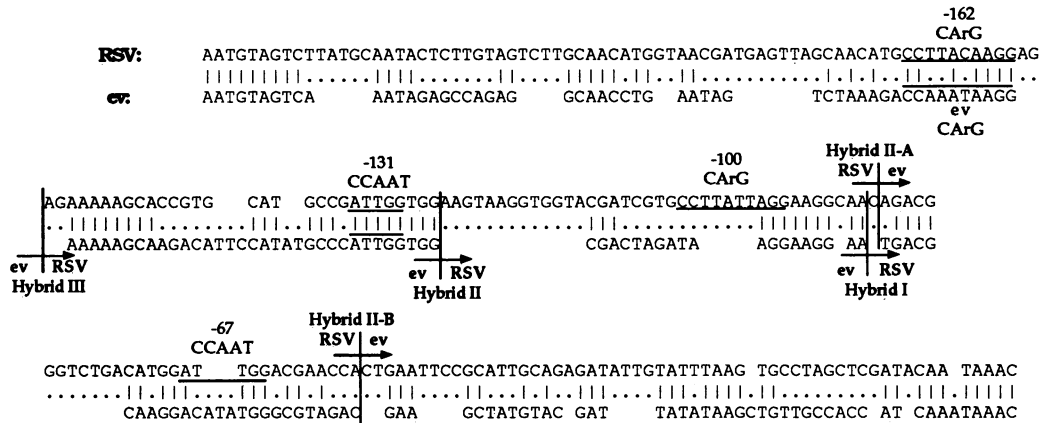
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                                                                                    -162
                                                                                    CArG
RSV:    AATGTAGTCTTATGCAATACTCTTGTAGTCTTGCAACATGGTAACGATGAGTTAGCAACATGCCTTACAAGGAG
        IIIIIIIII......IIII........II....IIIII.II..II...........I.I.I..II..I.IIII..
ev:     AATGTAGTCA    AATAGAGCCAGAG   GCAACCTG  AATAG       TCTAAAGACCAAATAAGG
                                                                              ev
                                                                              CArG

                              -131                          -100        Hybrid II-A
                              CCAAT                         CArG          RSV| ev
     AGAAAAAGCACCGTG  CAT  GCCGATTGGTGGAAGTAAGGTGGTACGATCGTGCCTTATTAGGAAGGCAACAGACG
     ..IIIIIIII........III..III.IIIIIIII.............III........IIIIIII.II..IIII
        AAAAAGCAAGACATTCCATATGCCCATTGGTGG            CGACTAGATA    AGGAAGG AA TGACG
     ev I RSV                          ev I RSV                         ev I RSV
     Hybrid III                        Hybrid II                        Hybrid I

              -67       Hybrid II-B
              CCAAT      RSV| ev
     GGTCTGACATGGAT    TGGACGAACCACTGAATTCCGCATTGCAGAGATATTGTATTTAAG TGCCTAGCTCGATACAA TAAAC
     .......II.III....III.II.I....II.III....II...I.I..III....III.IIII.II..........II.III.IIIII
     CAAGGACATATGGGCGTAGAC GAA   GCTATGTAC GAT    TATATAAGCTGTTGCCACC AT CAAATAAAC
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FIG. 2. Sequence comparison of ev and RSV U3 regions. Sequences of the RSV and ev-2/RAV-0 U3 regions are from previously published sequences (20, 37, 38) and were independently confirmed. Vertical lines between the sequences represent identity, while dots represent mismatches. Gaps were introduced to allow maximum alignment between the two sequences. Previously described protein binding motifs within each LTR are underlined (RSV) or overlined (ev). Designated lines identify crossover points of hybrid LTRs. Note that for the hybrid II-A and II-B LTRs, which were generated from hybrid II, only the second crossover point is labeled.

and the previously described ev-RSV hybrid LTR (ori hybrid) that demonstrated the presence of enhancer sequences in ev U3 regions (5). As shown, this hybrid LTR contained RSV sequences downstream of an SphI site at −141 and therefore included the −131 CCAAT motif and sufficient flanking DNA to retain EFI binding activity (39), the −100 CArG box-EFIII binding site, the −67 CCAAT box-EFI binding site, and downstream promoter sequences. The ev fragment in these original hybrids extended from the 5' end of the LTR through an AccI site at −50. This region contains the ev CArG box-EFIII binding site (46), a c/EBP-like binding site (36), and the ev CCAAT box, which does not detectably bind EFI (46). Other ev sequences included in this hybrid have not been characterized with respect to protein binding and/or transcriptional regulatory activity. To determine whether ev enhancer sequences were present at the same relative location as the RSV upstream enhancer domains or whether additional 3' sequences such as the ev c/EBP-like binding site might also be required for maximal transcription of hybrid LTRs, additional LTRs that simply replaced increasing amounts of information from the 3' portion of the ev LTR with the similarly positioned sequences from RSV were constructed. The sequence of crossover points for each hybrid is shown in Fig. 1 and 2 and depicted diagrammatically in Fig. 3.

As shown, hybrid I contained the ev CArG box-EFIII binding site, the ev CCAAT box, and additional upstream ev sequences of unknown function linked to RSV sequences that included the −67 CCAAT box-EFI binding site and the RSV promoter. The RSV-derived sequences in hybrid II extended further upstream than those in hybrid I to include the −100 RSV CArG box-EFIII binding site and an additional 13 nucleotides upstream of this site in place of the corresponding sequences from the ev LTR, while hybrid III contained an additional replacement of the ev CCAAT box region with sequences from RSV. At least two isolates of each LTR were inserted in place of the RSV LTR in an RSV-neomycin vector and transfected into avian fibroblasts, and transcription from the control and experimental plasmids was monitored by primer extension with a neomycin-specific primer as described in Materials and Methods.

Results obtained with these hybrids and control constructs

are shown in Fig. 4. In agreement with previous results, the intact RSV LTR (pM-RSVNeo) was transcribed efficiently in this system, while the deletion of essential enhancer sequences upstream of the SphI site resulted in virtual elimination of transcription from the pS-RSVNeo construct. This low level of transcriptional activity is approximately equal to that seen with an intact ev LTR (pRAV-0Neo). In contrast, the original ev-RSV hybrid LTR gave rise to easily detectable levels of transcripts, demonstrating the ability of ev sequences to restore transcriptional activity to the enhancer-deleted RSV LTR. Quantitation of autoradiograms demonstrated that this hybrid was 20- to 25-fold higher in transcriptional activity than the intact ev LTR or the deleted RSV LTR and was only 2-fold lower in activity than an intact RSV LTR.

Figure 4 also shows results obtained with hybrids I, II, and III. Hybrid I, which contained the RSV promoter and −67 CCAAT box linked to upstream sequences from the ev LTR, gave rise to barely detectable levels of extension product, which is analogous to results obtained with an intact ev LTR. The inactivity of hybrid I indicates that substitution of the ev promoter with that from RSV is not sufficient to complement ev enhancer sequences, a result consistent with previous data that indicated that the promoter is not the defective component within the ev LTR (5). These data also indicate that the presence of a promoter-proximal CCAAT box, which is missing in intact ev LTRs, does not result in high-level transcription in conjunction with upstream ev sequences.

Hybrid II, which included RSV sequences that extend in the 3' direction from a site 13 nucleotides upstream of the RSV −100 CArG box-EFIII binding site, was transcribed efficiently in this system, giving rise to a level of transcripts that was either equal to or within twofold of that seen with the original ev-RSV hybrid LTR. The finding that in some experiments, such as that shown in Fig. 4, hybrid II gave rise to a slightly lower level of transcripts than the original ev-RSV hybrid LTR suggests that the additional ev or RSV sequences included in the original hybrid might be required for maximal activity. However, the high-level transcriptional activity of hybrid II demonstrates that the primary ev enhancer sequences are present at the same relative location
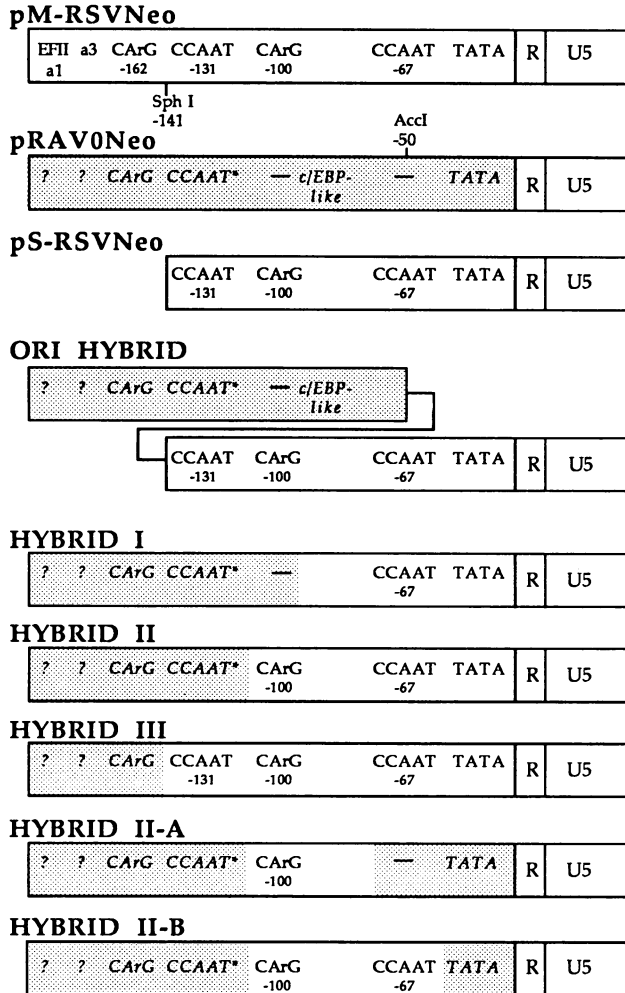
FIG. 3. Content of ev-RSV hybrid LTRs. pM-RSVNeo, pS-RSVNeo, and pRAV-0Neo have been previously described (5) and represent an intact RSV LTR, an RSV LTR deleted for sequences upstream of the *Sph*I site at −141, and an intact ev LTR inserted into a neomycin-based expression vector, respectively. The construct labeled ori hybrid has also been previously described and includes ev U3 sequences that extend from the 5' end of the LTR through an *Acc*I site at −50 inserted into the pS-RSVNeo vector. The construction of hybrids I, II, III, II-A, and II-B shown in the figure is described in the legend to Fig. 1 and in Materials and Methods. These LTRs represent PCR-generated ev-RSV hybrid LTRs that contain either a single crossover point (hybrids I, II, and III) or two crossover points (hybrid II-A, and II-B) that were defined by the identity of internal primers used in amplification reactions. The location and identity of previously described factor binding sites in ev and RSV LTRs are shown. RSV motifs are depicted in roman type, while those from the ev LTR and shown in italics and are shaded.

as the upstream enhancer sequences from RSV and also demonstrate that the promoter-proximal c/EBP-like binding site in the ev LTR is not required for high-level transcriptional activity in the context of these hybrid LTRs. With the exception of a CArG box-EFIII binding site and an inverted CCAAT box, which are present in both the ev and RSV LTRs, other sequences within this functionally exchangeable 5' portion of ev and RSV LTRs share only limited sequence similarities (Fig. 2).
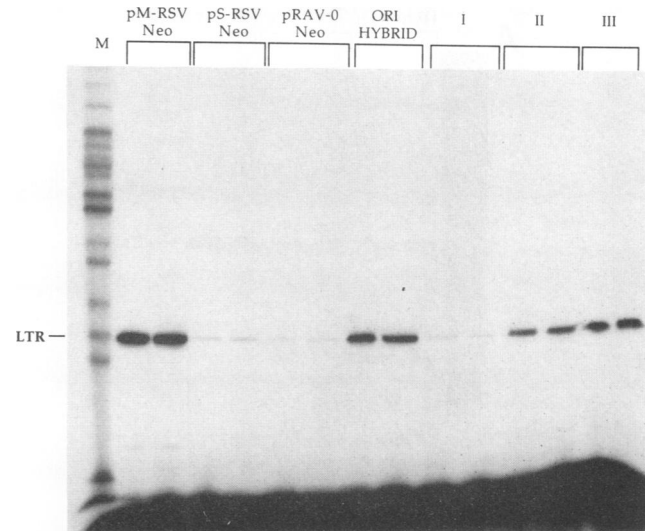
FIG. 4. Transcriptional activity of hybrids I, II, and III. Indicated plasmids (10 μg each) were transfected into avian fibroblasts, and total RNA was harvested after 48 h. Twenty micrograms of each RNA sample was then used in a primer extension assay with a Neo[r] gene-specific primer as previously described (5). For hybrids I, II, and III, each lane represents results obtained with different isolates of each construct. The 77-nucleotide LTR extension product is indicated. The lane marked M contains pBR322 *Msp*I-digested DNA as a marker. The relative levels of extension product generated from pRAV-0Neo and hybrids I, II, and III were analogous when a CMV-neomycin vector was cotransfected as an internal control (data not shown).

Hybrid III, which exchanged ev sequences with RSV sequences that include and extend downstream of the RSV −131 CCAAT box-EFI binding site, was approximately equal in transcriptional activity to hybrid II, which contained the ev CCAAT box. Previous studies indicated that the ev CCAAT box does not detectably bind the RSV CCAAT box binding factor EFI or any other nuclear protein (46). Given the fact that mutations in the RSV −131 CCAAT box that abrogate EFI binding can result in a significant decrease in transcription from the RSV LTR (16), it was surprising that hybrids II and III exhibited equivalent transcriptional activities. The basis for this apparent discrepancy was investigated further, as described below.

Sequences within the ev CCAAT box are required for high-level transcriptional activity of the hybrid II LTR. One possible explanation for the similar transcriptional activities of hybrid III, which contained the RSV −131 inverted CCAAT box, and hybrid II, which contained the ev inverted CCAAT box, was that in contrast to results obtained with an intact RSV LTR (16), a functional upstream CCAAT box may not be required for high-level transcription in the context of these hybrid LTRs. To investigate this possibility, the ev inverted CCAAT box in hybrid II (5'-ATTGG-3') was mutated to 5'-AATCG-3' as described in Materials and Methods and the transcriptional activity of this LTR was monitored by primer extension as described above. In these and the following experiments, a CMV-neomycin vector was included in transfections as an internal control; the presence of this vector did not alter the relative activities of the pRAV-0Neo and hybrid I, II, and III templates (data not shown).

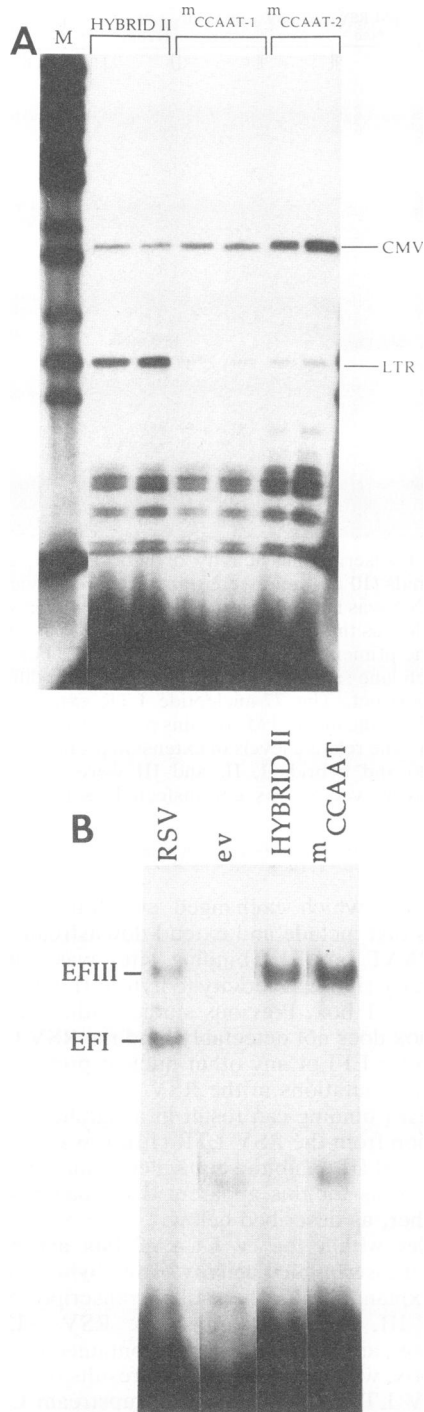As shown in Fig. 5A, transcription from hybrid II LTRs

FIG. 5. ev CCAAT box: protein binding activity and role in transcription. (A) Transcriptional activity of hybrid II LTRs with a mutated ev CCAAT box. Mutations described in the text were introduced into the ev CCAAT box in the hybrid II LTR. Two different isolates of this construct (^{m}CCAAT-1 and ^{m}CCAAT-2), in parallel with the parental hybrid II plasmid, were transfected into avian fibroblasts, and transcription was monitored by primer extension. Ten micrograms of a CMV-neomycin vector was cotransfected with each sample as an internal control. The LTR (77-nucleotide) and CMV (90-nucleotide) extension products are marked. (B) DNA fragments that extend from what in RSV is defined by the −131 CCAAT box-EFI binding site and the −100 CArG box-EFIII binding site were generated from the RSV, ev, hybrid II, and

that contained the mutant ev CCAAT box (^{m}CCAAT) was decreased approximately 20-fold relative to that seen with the unmutated hybrid II plasmid. These results are consistent with the requirement for an upstream CCAAT box for high-level transcription in the context of hybrid LTRs. They are not, however, consistent with our previous results showing that the ev CCAAT box does not detectably bind EFI. One possible explanation for this apparent discrepancy was that the ev CCAAT box may be able to bind EFI in the context of hybrid II because of the location of the crossover point in this hybrid, which placed a 13-bp RSV sequence that is missing in ev LTRs downstream of the ev CCAAT box (Fig. 2). To investigate this possibility, DNA probes that extended from what in RSV is defined by the −131 CCAAT box-EFI binding site and the −100 CArG box-EFIII binding site (Fig. 2) were generated from RSV, ev, hybrid II, and hybrid II ^{m}CCAAT LTR templates and used in EMSA as

As shown in Fig. 5B, the RSV-derived probe generated two DNA-protein complexes designated EFI and EFIII. The identity of these complexes was verified by methylation interference assays (46 and data not shown). The ev-specific probe failed to generate a complex corresponding to EFIII binding, a finding consistent with the absence of a CArG motif in ev LTRs at a position corresponding to the RSV −100 CArG box-EFIII binding site. The ev-specific probe also failed to generate an EFI complex, which is in agreement with previous results that demonstrated the lack of detectable EFI binding to the ev CCAAT box (46). The fragment generated from hybrid II formed an EFIII-DNA protein complex, a result that was predicted because of the presence of the RSV −100 CArG box-EFIII binding site in this construct. However, we failed to detect an EFI-DNA complex with this probe, indicating that the presence of RSV sequences downstream of the ev CCAAT box in hybrid II does not detectably increase the affinity of this motif for EFI. As predicted, the hybrid II ^{m}CCAAT-specific fragment also failed to form an EFI complex. Thus, although sequences within the ev CCAAT motif are required for maximal transcriptional activity of these hybrid LTRs, an upstream, high-affinity EFI binding site is not required.

CArG box-EFIII binding sites are required for transcriptional activity of hybrid LTRs. All LTRs tested that showed high transcriptional activity, including the intact RSV LTR and hybrids II and III, contained two CArG box-EFIII binding sites. In RSV, these motifs are centered at positions −100 and −162. In hybrids II and III, the RSV −162 motif was replaced by the conserved CArG box found in ev LTRs. Previous studies have demonstrated that the −162 RSV CArG box is located within sequences required for full enhancer function (8, 22, 24, 29). Similarly, Cullen and coworkers (8) have demonstrated that deletions that remove the RSV −100 CArG box result in an approximately 80% decrease in transcription from the RSV LTR. In each of these cases, however, deletions were not confined to the CArG boxes but included additional sequences flanking these motifs. It was therefore unclear whether each of these

CArG boxes is required for full activity of the RSV LTR or whether the ev CArG box is required for high-level transcription in the context of the hybrid LTRs. To investigate these questions, mutations were introduced individually into the ev CArG and the −100 RSV CArG motif in the context of hybrid II by using oligonucleotide-directed mutagenesis as described in Materials and Methods, and the effects of these mutations on transcription were analyzed after transfection into avian fibroblasts. In each case, mutations that changed the terminal two cytidine residues on each end of the motif to guanine residues were introduced, thus altering the consensus 5′-CC(A/T-rich)$_6$GG-3′ sequence to 5′-GG(A/T-rich)$_6$CC-3′. Studies with other CArG motifs have demonstrated that these mutations abrogate serum response factor binding and significantly decrease CArG-mediated transcriptional activation (42).

To verify the loss of EFIII binding activity of these mutant CArG motifs, mutant (GArC) and wild-type (CArG) oligonucleotides that define the ev CArG and RSV −100 CArG-EFIII binding site regions described in Materials and Methods were tested by EMSA. As shown in Fig. 6A, both of the oligonucleotides that contained a wild-type CArG motif generated a single DNA-protein complex in EMSA that represents EFIII binding as defined by methylation interference assays (46 and data not shown). As expected, however, oligonucleotides that contained the ev GArC or the RSV GArC sequence failed to generate this distinct complex, verifying the importance of the 5′ cytidine dinucleotide within the CArG motif for EFIII binding.

Figure 6B shows the results of a primer extension assay conducted with RNA isolated from avian fibroblasts transfected with the wild-type hybrid II construct and with hybrid II constructs that contained either the ev or the RSV −100 GArC mutation. As shown, each of the mutant hybrids showed an at least 20-fold decrease in transcriptional activity compared with the parental hybrid II construct. These data support the hypothesis that two CArG motifs are required for high-level LTR-directed transcription, a result not previously demonstrated for retroviral LTRs, and identify the ev CArG motif as one component of the ev enhancer.

Identification of sequences from RSV required to complement ev enhancer sequences. Data presented above indicate that the RSV −100 CArG box-EFIII binding site is required for high-level transcription in the context of hybrid LTRs. To determine whether this portion of the RSV LTR was sufficient to complement upstream ev enhancer sequences or whether a promoter-proximal CCAAT box-EFI binding site, which is also missing in ev LTRs, might be additionally required for complementation, hybrids II-A and II-B shown in Fig. 3 were constructed. These hybrids contained a second crossover point to replace downstream RSV sequences in hybrid II with those from the ev LTR. The crossover point in hybrid II-A was downstream of the RSV −100 CArG box-EFIII binding site and therefore resulted in the replacement of the RSV −67 CCAAT box-EFI binding site and RSV promoter with the corresponding 71 bp from the ev LTR. The RSV contribution to this hybrid therefore included the −100 CArG box-EFIII binding site and an additional 13 bp upstream of this site, sequences that are not represented in ev LTRs. The second crossover point in hybrid II-B was downstream of the RSV −67 CCAAT box and therefore resulted in the generation of an LTR that contained the RSV −100 CArG box and −67 CCAAT box linked to the ev promoter and upstream enhancer sequences.

Transcription from each of these constructs was monitored by primer extension as described above. As shown in
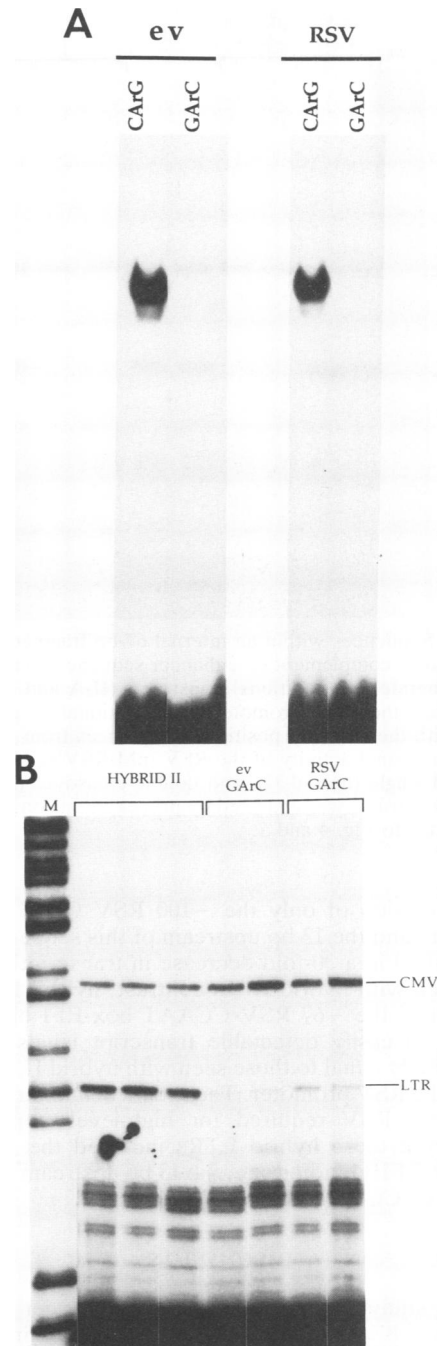

FIG. 6. LTR CArG motifs: protein binding and role in transcription. (A) Wild-type (CArG) and mutant (GArC) oligonucleotides from ev and RSV LTRs described in Materials and Methods were incubated with nuclear extracts prepared from QT35 cells in the presence of 5 μg of salmon sperm DNA as a nonspecific competitor, and the resultant DNA-protein complexes were resolved after electrophoresis in polyacrylamide gels (5). The prominent complex seen with the CArG-containing oligonucleotides represents EFIII binding as determined by methylation interference assays (46 and data not shown). (B) The transcriptional activity of hybrid II constructs and hybrid II constructs that contained the ev GArC or RSV −100 GArC mutation were tested by primer extension analysis after transfection into avian fibroblasts as described in the legends to Fig. 4 and 5. The three lanes labeled hybrid II show results obtained with three isolates of this construct, two of which are wild type in sequence and one of which contains a T to C mutation at position −51.
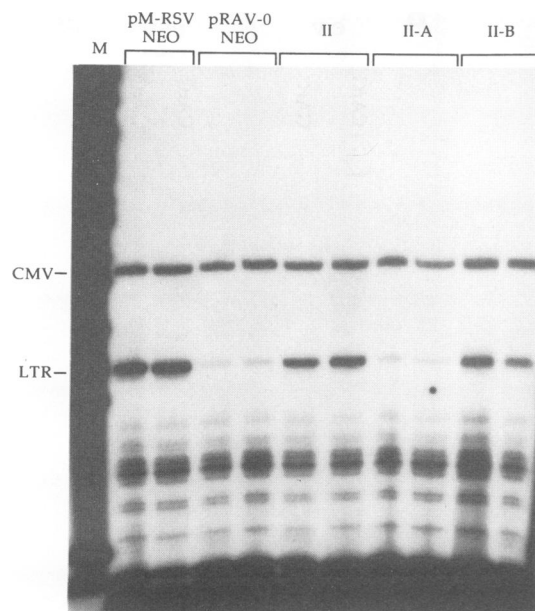
FIG. 7. Sequences within an internal 67-bp fragment from RSV are required to complement ev enhancer sequences. Hybrid II was used to generate two additional constructs (II-A and II-B; Fig. 3) that replaced the RSV promoter and additional 3′ proximal sequences with the similarly positioned sequences from the ev LTR. The transcriptional activity of the RSV (pM-RSVNeo), ev (pRAV-0Neo), and single (hybrid II)- and double-crossover (hybrids II-A and II-B) constructs was analyzed by primer extension as described in the legends to Fig. 4 and 5.

Fig. 7, inclusion of only the −100 RSV CArG box-EFIII binding site and the 13 bp upstream of this sequence (hybrid II-A) resulted in a 30-fold decrease in transcription relative to that seen with hybrid II. In contrast, hybrid II-B, which also included the −67 RSV CCAAT box-EFI binding site, gave rise to easily detectable transcript levels that were approximately equal to those seen with hybrid II, which also included the RSV promoter. These data demonstrate that the region from RSV required for high-level transcriptional activity from these hybrid LTRs included the −100 RSV CArG box-EFIII binding site, the 13 bp upstream of this site, and the −67 CCAAT box-EFI binding site.

DISCUSSION

In this study, we have localized enhancer sequences within the 5′ 87 bp of the ev LTR and have defined at least two transcriptional control sequences from the RSV LTR that are required to complement ev enhancer sequences. Data obtained with hybrid II-B demonstrated that the replacement of an internal portion of the ev LTR with sequences from RSV resulted in an approximately 15-fold increase in transcription relative to that seen with an intact ev LTR. The 67-bp sequence from the RSV LTR that was present in hybrid II-B contained two known transcriptional regulatory motifs, a CCAAT box and a CArG box. Mutation of the −100 RSV CArG box in hybrid II demonstrated the necessity of this motif for transcriptional activity of the hybrid LTR, and the requirement for a promoter-proximal CCAAT box for maximal transcription from the RSV LTR has been shown previously (16). Each of these motifs is missing in ev LTRs, indicating that the primary defect in ev

LTRs lies within this internal region. It is currently not known whether additional sequences within this 67-bp fragment from RSV, such as the 13 bp upstream of the −100 CArG box, might also contribute to activity, a possibility currently under study. It should be noted that the level of transcripts encoded by the hybrid II-B construct was approximately twofold lower than that seen with an intact RSV LTR. It is not clear whether this reflects the fact that the upstream ev enhancer sequences are less active than those from RSV or whether the location of crossover points selected to make the hybrid LTRs might have disrupted uncharacterized motifs required for full ev enhancer function.

A CArG box and a CCAAT box were also present in the portion of the ev U3 region that could functionally replace upstream RSV enhancer sequences, and mutational analyses demonstrated the importance of these motifs for high-level transcription from the hybrid LTRs. Since upstream RSV enhancer sequences that were replaced by ev sequences in the transcriptionally active hybrid LTRs also included a CArG and a CCAAT box, these data indicate, first, that two copies of each of these motifs are required for maximal transcription from both the RSV and hybrid LTRs and, second, that there may have been a simple exchange of these upstream RSV motifs with the corresponding ev sequences in the hybrid LTRs. However, differences in the relationship between protein binding activity and function of the ev CCAAT box relative to that reported for the RSV −131 CCAAT box were seen. In particular, we have been unable to detect binding of the EFI factor to the ev CCAAT box by EMSA with DNA fragments in which the conserved pentanucleotide was flanked either by ev sequences or by downstream sequences from RSV as it is positioned in the transcriptionally active hybrid II construct. These findings may reflect the fact that the ev CCAAT box can bind EFI but at a level below that required for detection in EMSA and that this level of binding may be sufficient for activity. This possibility is at least partially supported by studies with the RSV CCAAT box, in which it was shown that two different point mutations that eliminated EFI binding to the −131 CCAAT box as determined by EMSA led to only a 2- to 3-fold decrease in transcription from the RSV LTR, while a deletion of the pentanucleotide resulted in an approximately 20-fold decrease in transcription (16). In these studies, it was also demonstrated that although the mutant RSV CCAAT motifs tested were unable to detectably bind EFI by EMSA, they could compete for EFI binding to the RSV −131 CCAAT box when added to binding reaction mixtures at a concentration that was fivefold higher than that required for competition by the wild-type sequence. These findings indicate that the mutant CCAAT motifs could bind EFI with a relatively low affinity and that this reduced level of binding activity resulted in only a modest reduction in transcription. Similar binding competition experiments that we have conducted with an oligonucleotide that includes the ev CCAAT box have demonstrated that it is unable to compete for EFI binding to the RSV −131 CCAAT box, even when added at concentrations 20-fold higher than that required for competition by the RSV CCAAT box oligonucleotide (4). However, we have recently found that a DNA fragment that includes the ev CCAAT box flanked by downstream RSV sequences is able to detectably compete for binding of the EFI factor to the RSV −131 CCAAT box when a large (500 to 2,000) molar excess of the fragment is used in binding competition experiments (4). We are currently investigating the relative affinity of EFI for the ev and RSV CCAAT

motifs by more rigorous means to determine whether this apparent low level of binding might reasonably be expected to occur in vivo. An alternative explanation for our findings is that the CCAAT box region of the ev LTR may not bind EFI but may interact with another protein(s) required for high-level transcription from hybrid LTRs. In this case, mutations that were introduced into the ev CCAAT box might have inadvertently altered residues required for binding of this proposed factor. Attempts to identify such a factor(s) with the ev, hybrid II, and ^mCCAAT hybrid II templates have been unsuccessful to date. Additional experiments will therefore be required to resolve this question.

As noted above, the second ev motif identified that was required for high-level transcription from hybrid LTRs was a CArG box located in a position analogous to the −162 RSV CArG motif. Thus, all LTRs tested that exhibited high-level transcriptional activity, including the RSV LTR and hybrids II, III, and II-B, contained two CArG motifs. Multiple CArG box motifs are also present in the regulatory regions of several cellular genes (42). Work from several laboratories has demonstrated that many CArG motifs interact with a protein indistinguishable from serum response factor (3, 27, 43). However, recent studies indicate that at least some CArG boxes interact with distinct or additional proteins, some of which exhibit different affinities for individual CArG motifs and show differences in cell-type-specific or developmentally regulated binding activity (11, 17, 35, 41; for a review, see reference 42), suggesting that they are involved in different types of CArG box-mediated regulation. Previous studies have demonstrated that the ev CArG box and the −100 and −162 CArG boxes from RSV all bind EFIII (2, 46), the proposed avian homolog of serum response factor (2). We have recently identified an additional cellular protein of approximately 50 kDa that also binds an oligonucleotide containing the RSV −162 CArG box but not the ev CArG or the RSV −100 CArG (40), raising the possibility that the RSV −162 CArG box, like other CArG motifs associated with cellular genes mentioned above, may bind additional proteins that contribute to its regulation. In this case, the regulatory contributions of the RSV −162 CArG and the ev CArG may not be equivalent under all growth conditions, a possibility currently under study.

Deletion studies with RSV have demonstrated that the 5′ proximal LTR sequences upstream of the −162 CArG box that were replaced with ev sequences in hybrid constructs are essential for enhancer function (8, 22, 24, 29). Although the precise motifs included in these enhancer sequences have not been identified by site-directed mutagenesis, EMSA, DNase I footprinting, and methylation interference assays define at least two regions within 5′ proximal enhancer sequences that interact with avian nuclear proteins. Interestingly, the patterns of DNA-protein complexes detected by EMSA with probes from this region show cell-type-specific variation (14, 33, 34, 39), suggesting that the exogenous virus enhancer may be subject to differential regulation in specific cell types. In support of this hypothesis, ALV enhancer binding proteins designated a1 and a3 by Ruddell and colleagues show cell-type-specific differences in binding activity that have been correlated with the oncogenic spectrum of ALV in vivo (33, 34). A sequence comparison of the ev and RSV and ALV 5′ enhancer sequences shows that ev LTRs lack sequences included in the a1 and a3 binding sites. We are currently defining the 5′ proximal enhancer motifs within the ev LTR upstream enhancer sequences and the relationship between ev and RSV enhancer binding proteins. The availability of hybrid LTRs that are tran-

scribed efficiently yet which contain enhancer motifs from the weakly oncogenic ev LTR will also provide a system to directly investigate the relationship among enhancer activity, the identity and function of distinct enhancer motifs, and, by inserting hybrid LTRs into replication-competent virus, the role of specific enhancer motifs in oncogenesis.

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