Activation of an Endogenous Retrovirus Enhancer by Insertion into a Heterologous Context

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An enhancer element is located in the U3 portion of exogenous avian retrovirus long terminal repeats (LTRs). A similar element has not been detected in the LTRs of ev-1 and ev-2, two avian endogenous viruses (evs) that normally are not expressed in vivo. Experiments were initiated to determine whether minor nucleotide differences in the U3 region of a previously untested ev that is ubiquitously expressed in vivo (ev-3) might confer enhancer function on the LTR of this provirus. This question was addressed by inserting U3 regions from ev-3 and from ev-1 and/or ev-2 both upstream of the herpesvirus thymidine kinase gene promoter and in place of the major enhancer domains of the Rous sarcoma virus LTR and determining their relative effects on transcription. U3 regions from all evs tested were unable to enhance transcription from the thymidine kinase gene promoter, indicating that nucleotide differences in the ev U3 regions do not affect their relative enhancer function and therefore are unlikely to play a role in their differential expression in vivo. Unexpectedly, however, all ev U3 regions were able to augment transcription in an orientation-independent manner in the ev-Rous sarcoma virus hybrid LTRs. Further experiments conducted to determine why this enhancer activity is not detectable in intact ev LTRs demonstrated that it was not due to removal of repressor sequences in the ev fragments used that might normally be present in intact ev LTRs. The lack of detectable enhancer activity in intact ev LTRs also was not explained by a defect in ev promoters that makes them unresponsive to enhancers in *cis*. These experiments therefore identify sequences that, although unable to function detectably as enhancers in their natural context, can function efficiently in a heterologous context. Data are discussed in terms of the modularity of enhancer elements and possible interactions between enhancers and promoter-specific sequences.

The long terminal repeats (LTRs) of exogenous avian retroviruses, such as Rous sarcoma virus (RSV) and the avian leukosis viruses, contain a strong enhancer that is active in many different cell types (10, 15, 28, 29, 36, 46). The LTRs of these viruses are divided into three regions: U3, R, and U5 (reviewed in reference 5). Both the enhancer and the promoter for transcription of viral RNA are located in U3; the U3-R boundary defines the transcription start site (45). Regions within U3 that are required for enhancer function (from the 5' end of the LTR at position -229 from the start site of transcription to an *Sph*I site at -141 [10, 28, 29, 36, 46]) or promoter function (from an *Eco*RI site at -50 to +1 [10, 13, 33, 36, 47]) have been identified. In different assay systems, the approximately 90-bp region between the *Sph*I (-141) and *Eco*RI (-50) sites has been described as part of the enhancer (3, 28, 36, 46) or part of the promoter (10, 29), suggesting that this region may contain sequences that are required for both. This type of organization is similar to that of several other genes in which components of the enhancer and promoter are intermingled upstream of the transcription start site (1). Candidate *trans*-acting proteins involved in transcription regulation of exogenous retrovirus LTRs have been identified in various avian cell types (3, 12, 14, 18, 38, 39, 40, 43).

The LTRs of a second class of avian retroviruses, the avian endogenous viruses (evs) (6, 37), are organized similarly to those of exogenous viruses (22–24, 41, 44). However, sequence comparisons of exogenous virus and ev LTRs show that although the R and U5 regions are virtually identical, the U3 regions of these two virus groups are highly divergent (23, 42, 44). In particular, ev U3 regions are shorter and lack sequences identified in exogenous viruses that are essential for enhancer function. Previous studies using cloned LTRs from two endogenous proviruses (ev-1

and ev-2) demonstrate that these LTRs are from 10- to 100-fold less active transcriptionally than exogenous virus LTRs (9–11, 36). These LTRs also are unable to augment transcription from several heterologous promoters, including those from the β -globin gene (9) and from simian virus 40 (SV40) (46). Therefore, unlike the LTRs of exogenous viruses, the LTRs of ev-1 and ev-2 do not contain detectable enhancer activity either in their natural context or in conjunction with heterologous promoters. The precise nucleotide differences in ev LTRs relative to exogenous virus LTRs responsible for this difference in enhancer function have not been defined.

Avian evs are present in the germ line of most chickens and are inherited as normal cellular genes (21, 24, 37). Some of these elements are transcribed from their inherited chromosomal locations, while others are silent (2, 21). The two proviruses used to define the absence of detectable enhancer activity in ev LTRs, ev-1 and ev-2, are examples of proviruses that normally are not transcribed in vivo. The LTRs of ev-1 and ev-2 are transcriptionally competent, however, since cloned copies of these proviruses are expressed after transfection into cells in culture (9, 11). In addition, these proviruses can be activated in vivo by treating cells which contain them with nucleotide analogs such as bromodeoxyuridine (37) or 5-azacytidine (7, 20). Under these conditions, ev-2 gives rise to an infectious virus called Rous-associated virus-0 (RAV-0); the LTRs of ev-2 and RAV-0 are identical (23, 41). Previous studies have indicated that the expression of individual evs correlates with the site of provirus integration (7, 8, 21, 25, 26, 41) and with the pattern of proviral DNA methylation acquired during gametogenesis (19). However, sequence analysis of the LTR of an ev that normally is expressed in vivo (ev-3) showed minor nucleotide variations relative to the LTRs of ev-1 and ev-2. Since

even single point mutations can alter the activity of transcriptional control elements, it was possible that the sequence differences in the LTR of ev-3 compared with those of ev-1 and ev-2 might affect their relative enhancer function and thereby contribute to the differential expression of these elements *in vivo*. Experiments in this report were therefore initiated to investigate this question. When tested in conjunction with the herpesvirus thymidine kinase (TK) gene promoter, there was no evidence of enhancer activity in the ev-3 LTR, indicating that the expression of this element *in vivo* is not attributable to the presence of a U3-associated enhancer. In the course of these studies, however, an unexpected result was obtained: U3 regions from all evs functionally replaced essential enhancer sequences in the RSV LTR in an orientation-independent manner. Possible explanations for the appearance of such enhancer activity, which is not evident in intact ev LTRs, are discussed.

MATERIALS AND METHODS

Vector construction and generation of U3 fragments. (i) **RSV-Neo vectors.** The parental plasmid used for all vector constructions was pRSVNeo (16), kindly provided by C. Gorman. This plasmid contains the 3' LTR from the Schmidt-Ruppin A strain of RSV (extending from a *Pvu*II site in the viral *src* gene through a *Bst*NI site at position +32 in U5) that drives expression of the neomycin resistance gene. The Neo^r gene coding sequences are followed by a splice site and a poly(A) addition site derived from SV40. In the original construction of this vector, the *Bst*NI site in U5 was converted to a *Hind*III site and linked to the coding portion of the neomycin resistance gene. To facilitate insertion of enhancer fragments into RSV-Neo-based vectors, the entire insert from pRSVNeo was excised and inserted into the polylinker of Bluescript (Stratagene). This was accomplished by cleaving pRSVNeo with *Nde*I and *Bam*HI, which cut immediately upstream and downstream of the insert, respectively. After conversion of the *Nde*I site to a *Bam*HI site, the 2.9-kbp insert was introduced into the *Bam*HI site of the Bluescript polylinker. To distinguish this construct from the parental pRSVNeo, it is referred to as pI (for intact)-RSVNeo. Two additional vectors were constructed from pI-RSVNeo. They lacked virus-specific sequences upstream of either the *Mst*II site located 39 bp upstream of the LTR (pM-RSVNeo) or the *Sph*I site in the LTR at position -141 from the transcription start site (pS-RSVNeo). The *Mst*II and *Sph*I sites used to generate these deletions were converted to *Bam*HI sites and religated into the Bluescript polylinker. In all cases, the orientation of inserts was such that the *Sst*I site in the Bluescript polylinker was upstream of the viral LTR.

(ii) **pRAV-0Neo.** Under certain conditions (20, 37), the ev-2 provirus can be transcriptionally activated to produce an infectious virus called RAV-0. Sequence comparisons have demonstrated that the LTRs of ev-2 and RAV-0 are identical (23, 41). A plasmid that contained the ev RAV-0 was obtained from P. Tschlis and used to construct pRAV-0Neo. This was accomplished in two steps by first converting the conserved *Mst*II site upstream of the 3' LTR to a *Bam*HI site and subsequently converting the *Bst*NI site in U5 to a *Hind*III site; this is the site in U5 that was used to construct the original pRSVNeo (16). This RAV-0 LTR fragment was then inserted in place of the RSV LTR in the RSV-Neo vector.

(iii) **pTKNeo.** The herpesvirus TK promoter was generated from a pTKCAT plasmid (30) obtained from H. Towle. The

promoter fragment in this plasmid was originally generated from LS mutant -115/-105 (32) and extended from a *Bam*HI linker located at -115 to -105 to a *Bgl*II site at +51. This region includes the two previously identified Sp1 binding sites at -100 and -50 (27), the inverted CCAAT box at -83 (17), and a TATA box. To generate pTKNeo, the *Bgl*II site at +51 was converted to a *Hind*III site and ligated to the Neo^r gene. After *Bam*HI digestion, the resultant TK-Neo insert was ligated to the *Bam*HI site of the Bluescript vector such that the *Sst*I site in the Bluescript polylinker was located upstream of the TK promoter.

(iv) **RSV enhancer fragment.** A 218-bp fragment generated by cleavage of the RSV LTR (from pRSVNeo) with *Mst*II and *Eco*RI was isolated, both ends were converted to *Sst*I sites, and the resultant fragment was inserted in both orientations into the unique *Sst*I site in the Bluescript polylinker of pS-RSVNeo, pRAV-0Neo, and pTKNeo. This fragment contains all sequences that are potentially components of the exogenous virus enhancer yet lacks promoter sequences essential for accurate transcription initiation (10).

(v) **ev U3 fragments.** Cloned copies of ev-1 and ev-2/RAV-0 were generously provided by A. Skalka and P. Tschlis, respectively. ev-3 was cloned from a chicken genomic library as previously described (8), and its sequence was determined by using the dideoxy chain termination method (Sequenase). U3-specific fragments from ev-1, ev-2/RAV-0, and ev-3 were generated by cleavage of cloned DNA with *Mst*II and *Acc*I (-51), and ends were converted to *Sst*I sites. The resultant fragments were then inserted into vectors as described for the RSV enhancer.

DNA manipulations were conducted following standard procedures. Supercoiled plasmid DNA used for transfections was purified by two rounds of ethidium bromide-cesium chloride gradient centrifugation. The contents and orientations of all inserts were verified by sequencing by the dideoxy chain termination method (Sequenase).

Transfection and RNA isolation. Approximately 8×10^5 QT6 cells (a chemically transformed quail fibroblast cell line [34]) were plated in 60-mm-diameter dishes in growth medium which consisted of Dulbecco modified Eagle medium (GIBCO) supplemented with 10% tryptose phosphate (Difco), 10% fetal calf serum (GIBCO), and 1% chicken serum (GIBCO). The following day, transfections were done essentially as described elsewhere (35). Briefly, growth medium was removed and replaced with the same medium containing 80 μ M chloroquine (Sigma), 1 mg of DEAE-dextran (Pharmacia) per ml, and 10 μ g of plasmid DNA per plate. The cells were then placed at 37°C for 30 min, after which 5 to 7 ml of growth medium was added. Cells were then incubated at 37°C for an additional 3 to 5 h and then the medium was removed and replaced with 5 ml of growth medium. Cells were then incubated for 48 to 72 h, after which RNA was harvested by acid guanidinium thiocyanate-phenol-chloroform extraction (4). RNA preparations were free of contaminating plasmid DNA as judged by Southern blot hybridization. Each transfection was repeated multiple times to test duplicate samples, different preparations of the same plasmid, and, in most cases, independently derived isolates of the same plasmid constructs.

Primer extension. To detect correctly initiated Neo^r gene-containing transcripts in transfected cells, primer extension analyses were conducted with a 22-base-long oligonucleotide primer specific for Neo^r sequences. This primer, 5'-GCAGCCCTTGCGCCCTGAGTGC-3', is complementary to sequences in Neo^r gene transcripts located between 14 and 35 nucleotides from the LTR-Neo^r gene junction. Tran-

FIG. 1. Sequence comparison of U3 regions from ev-1, ev-2/RAV-0, ev-3, and RSV. Sequences of the RSV, ev-1, and ev-2/RAV-0 U3 regions are from previously published sequences for each of these proviruses (22, 23, 41) and were independently confirmed. The ev-3 sequence was determined from a cloned 3' LTR obtained from a chicken genomic library (8). Dashes in the ev-2 and ev-3 sequences represent identity with ev-1, and designated nucleotides identify the sequence variations noted in the U3 regions of these proviruses. Capital letters represent nucleotides shared between the evs and RSV, and lowercase letters represent mismatches. Gaps were introduced to allow maximum alignment. Locations of the TATA box as well as several restriction enzyme sites used in construction of vectors are shown.

scripts correctly initiated from the RSV or RAV-0 LTR generate extension products 77 nucleotides in length, while those from the TK promoter are 99 nucleotides in length. A 20- μ g portion of total RNA from transfected and control cells was incubated at 65°C for 1 to 4 h with approximately 100,000 cpm (0.5 to 1 ng) of kinase-labeled primer in a reaction that contained 0.25 M KCl, 0.01 M Tris-HCl (pH 8.0), and 1 mM EDTA in a total reaction volume of 20 μ l. After being cooled on ice, 50 μ l of a solution was added to bring the reaction mixture to a final concentration of 10 mM MgCl₂; 10 mM Tris-HCl (pH 8.0); 5 mM dithiothreitol; 0.2 mM each dATP, dCTP, dGTP, and TTP; and 200 U of murine leukemia virus reverse transcriptase (BRL) per reaction, and the reaction mixture was further incubated for 30 min at 37°C. Reactions were stopped by the addition of EDTA to 0.2 M, and the mixtures were ethanol precipitated and washed once with 80% ethanol. The final pellet was suspended in a 90% formamide dye mix, heat denatured, and loaded onto a 6% polyacrylamide-urea gel. Dried gels were exposed to Kodak XRP-1 film with a Dupont intensifying screen at -70°C, and bands were quantitated with computer-assisted video densitometry (31).

Nucleotide sequence accession number. The GenBank nucleotide sequence accession number for the ev-3 U3 region is M60397.

RESULTS

U3 regions from avian evs can replace essential enhancer sequences in RSV LTR. Figure 1 compares the nucleotide sequence obtained for the ev-3 U3 region with those of ev-1 (22), ev-2 (23, 41), and RSV (from pRSVNeo) (42). As shown, ev-3 contains three changes relative to ev-2; one of these changes is common to ev-1. Figure 2 shows the plasmids and DNA fragments used to determine if the minor changes in the U3 region of ev-3 versus those of ev-1 and ev-2 might alter their relative enhancer activities (see Materials and Methods for details). The control plasmids, pI-RSVNeo and pM-RSVNeo, contain an RSV LTR driving expression of the neomycin resistance gene. The test plas-

mid used to compare enhancer activity of ev U3 regions, pS-RSVNeo, lacks all viral sequences upstream of the *Sph*I site in the RSV LTR at -141 nucleotides from the transcription start site. Previous reports (10, 28, 29, 36) have demonstrated that this deletion results in a 90 to 98% decrease in transcription from the LTR due to removal of essential enhancer sequences; this deletion leaves the viral promoter intact (10, 12, 13, 33, 36, 47). U3-specific fragments from ev-1, ev-2, and ev-3 that were inserted into pS-RSVNeo are shown in Fig. 2B; the RSV-derived enhancer was tested in parallel as a control. The U3 fragment from RSV includes all sequences that are potentially components of the exogenous virus enhancer yet lack promoter sequences essential for accurate transcription initiation (10, 28, 29, 36). The *Acc*I site was chosen as the 3' boundary of ev U3 fragments, as it is positioned similarly to the *Eco*RI site in RSV relative to the start site of transcription. The U3 fragments were introduced in both orientations upstream of the deleted LTR in pS-RSVNeo. Transcription from these plasmids was then monitored by primer extension after transfection into avian cells in culture as described in Materials and Methods.

As shown in Fig. 3, RNA isolated from cells transfected with a plasmid that contained the nondefective RSV LTR (pI-RSVNeo) gave rise to readily detectable levels of extension product of the expected length. The second control plasmid, pM-RSVNeo, gave results indistinguishable from those of the original construct (data not shown, but see Fig. 5). As previously described (10, 28, 29, 36), deletion of enhancer sequences upstream of the *Sph*I site at -141 (pS-RSVNeo) decreased transcript levels to between 2 and 10% of those seen with the intact LTR, while reintroduction of the RSV-derived enhancer into pS-RSVNeo in either orientation restored transcriptional activity to the viral promoter (Fig. 3). This result verified that the restriction enzyme sites used in plasmid constructions did not destroy sequences required for either promoter or enhancer function of the RSV LTR. Additional studies (data not shown) demonstrated that more extensive deletions into the RSV LTR (to either the *Pvu*I site at -112 or the *Eco*RI site at

FIG. 2. Plasmid constructs and U3 fragments. (A) Plasmid constructs. All RSV-Neo vectors were derived from pRSVNeo (16), which contains a 3' LTR from the Schmidt-Ruppin A strain of RSV (extending from a *PvuII* site in the viral *src* gene through a *BstNI* site in U5) that drives expression of the neomycin resistance gene. The *Neo^r* gene is followed by a splice site from the SV40 small-t-antigen gene and an SV40-derived poly(A) addition site (not shown). Diagram 1, pI-RSVNeo, which contains the entire insert from pRSVNeo cloned into the *Bam*HI site of the Bluescript (Stratagene) polylinker. Plasmids identical to pI-RSVNeo except for progressive deletions into the 5' viral sequences are pM-RSVNeo (diagram 2), which lacks viral sequences upstream of an *MstII* site located 39 bp upstream of the viral LTR, and pS-RSVNeo (diagram 3), which contains a deletion through the *SphI* site in the viral LTR at -141 from the transcription start site. The deletion in pS-RSVNeo removes essential enhancer sequences from the RSV LTR yet leaves the viral promoter intact. Diagram 4, pRAV-0Neo, constructed by replacing the RSV LTR in pM-RSVNeo with that from ev-2/RAV-0. The orientation of all inserts was such that the *SstI* site in the Bluescript polylinker was upstream of the promoter driving expression of the *Neo^r* gene. Abbreviations in parentheses below the line drawings denote restriction enzyme sites destroyed during cloning; sites indicated above the drawings at these positions were created by the addition of synthetic linkers. All other sites shown above the line are restriction enzymes sites in the original DNAs. A, *AccI*; B, *Bam*HI; Bs, *BstNI*; E, *EcoRI*; H, *HindIII*; M, *MstII*; N, *NdeI*; Sp, *SphI*; S, *SstI*. See Materials and Methods for details of construction. (B) U3 fragments. The RSV enhancer fragment was derived from pRSVNeo (16) and extended from the *MstII* site located 39 bp upstream of the viral LTR to an *EcoRI* site at -51 relative to the transcription start site. U3 fragments from ev-1, ev-2, and ev-3 extended from the same *MstII* site to an *AccI* site at -50. After digestion, the ends of all U3 fragments were converted to *SstI* sites by the addition of synthetic linkers and cloned into relevant test vectors as described in the text.

FIG. 3. Primer extension products generated by intact, enhancer-deleted, and reconstituted RSV LTRs. Indicated plasmids (10 μ g) were transfected into avian fibroblasts, total RNA was harvested after 48 h, and 20 μ g of each sample was then used in a primer extension assay using a *Neo^r* gene-specific primer (see Materials and Methods for details). Duplicate lanes represent independent preparations of each plasmid. The expected length of transcripts that correctly initiate at +1 in the RSV LTR is 77 nucleotides and is marked by an arrow. Lane M, *MspI* digest of pBR322 DNA used as marker. See Fig. 2 for a complete description of each plasmid. The RSV enhancer fragment inserted into the *SstI* site upstream of the deleted RSV LTR in pS-RSVNeo was either in the same orientation as in an intact LTR (+) or in the opposite orientation (-).

-50) were not complemented by reinsertion of the RSV enhancer fragment, suggesting, as proposed by others (10, 13, 33, 36, 47), that sequences downstream of the *SphI* site include essential promoter sequences that cannot be disrupted or supplied at alternate locations.

Results with the ev U3 fragments inserted into pS-RSV-Neo are included in Fig. 4. Insertion of U3 regions from ev-1, ev-2/RAV-0 (data not shown, but see Fig. 5), and ev-3 into pS-RSVNeo resulted in a dramatic increase in transcription from the RSV promoter. As with the reconstructed RSV LTR (Fig. 3), transcripts from the ev-RSV hybrid LTRs were all of the length expected for initiation at +1 within the deleted RSV LTR. There was no evidence of aberrant products that initiated in the ev U3 regions. Quantitation of transcript levels revealed that the hybrid ev-RSV LTRs were at least 20-fold more active than pS-RSVNeo and encoded transcripts to levels that were only about 2-fold lower than those seen with an intact RSV LTR. In addition, this increased transcription was independent of the orientation of the ev U3 fragments, indicating that it was due to an enhancerlike activity and was not the result of a direct promoter effect.

While it was possible that the experiments described above might have revealed the presence of an enhancer in the ev-3 U3 region, the finding that U3 regions from ev-1 and ev-2 also enhanced transcription from the truncated RSV LTR was unexpected. Previous studies have failed to detect an enhancer either in intact LTRs from these two proviruses (9-11, 36) or when these LTRs were tested in conjunction with several heterologous promoters (9, 46). Additional studies were therefore conducted to investigate the basis for this apparent discrepancy.

FIG. 4. Insertion of *ev* U3 regions into pS-RSVNeo resulted in orientation-independent enhancement of transcription from the RSV promoter. U3 regions (Fig. 1B) from the *ev*-1, *ev*-2 (data not shown), and *ev*-3 LTRs were inserted in both orientations upstream of the deleted RSV LTR in pS-RSVNeo. The resultant plasmids, along with relevant controls, were transfected into avian fibroblasts, and transcription was monitored as described in the legend to Fig. 3. Lane M, pBR322 *Msp*I-digested marker DNA. + and - are explained in the legend to Fig. 3.

Hybrid *ev*-RSV LTRs are more active transcriptionally than intact *ev* LTR. One explanation for the results described above was that the primer extension assay used in these studies was more sensitive than systems used previously to compare the transcriptional activity of *ev* and exogenous virus LTRs and that it therefore revealed enhancer activity in *ev* U3 regions not seen when other test systems were used. To evaluate this explanation, levels of transcripts encoded by intact *ev* and RSV LTRs as well as hybrid *ev*-RSV LTRs were quantitated and compared directly by this system. To this end, pRAV-0Neo, containing the *ev*-2/RAV-0 LTR linked to the Neo^r gene (Fig. 2), was constructed. As shown in Fig. 5, the intact *ev* LTR in pRAV-0Neo gave rise to very low levels of correctly initiated transcripts in this assay. Quantitation showed that, in agreement with previous reports (9–11, 36), the *ev*-2/RAV-0 LTR was 50- to 100-fold less active than the intact RSV LTR and was approximately equal in activity to pS-RSVNeo. Figure 4 also shows a direct comparison of transcript levels encoded by pRAV-0Neo and the *ev*-RSV hybrid LTRs that contained *ev* U3 regions from *ev*-1, *ev*-2, and *ev*-3 in the orientation opposite that of the deleted RSV LTR. Clearly, the hybrid LTRs were significantly more active than the intact *ev* LTR, generating from 30- to 50-fold more RNA. These data indicated that the enhancer activity of *ev* U3 regions in *ev*-RSV hybrid LTRs is not related to the sensitivity of the primer extension assay.

Transcription from *ev* LTR can be increased by RSV enhancer. The appearance of enhancer activity in *ev* U3 regions in the *ev*-RSV hybrid LTRs that is not seen in intact

FIG. 5. Absence of detectable enhancer activity in an intact *ev* LTR. Transcripts encoded by RSV, *ev*, and *ev*-RSV hybrid LTRs were quantitated by primer extension after transfection of the indicated plasmid DNAs into avian fibroblasts. Plasmids used contained intact RSV LTRs (pI-RSVNeo [lane 1] and pM-RSVNeo [lane 2]), the enhancer-deleted RSV LTR (pS-RSVNeo [lanes 3 and 4]), an intact *ev* LTR (pRAV-0Neo [lanes 5 and 6]), or *ev*-RSV hybrid LTRs (*ev*-1/RSV [lane 7], *ev*-2/RSV [lane 8], and *ev*-3/RSV [lane 9]). In the *ev*-RSV hybrid LTRs used here, *ev* U3 regions were in the orientation opposite to that of the deleted RSV LTR. Lane M, pBR322 *Msp*I-digested marker DNA.

ev LTRs might also have been due to the facts that *ev* LTRs contain a repressor that inhibits the activities of enhancers in *cis* and that this repressor was missing from the *ev* U3 fragments used to construct the hybrid LTRs. Alternatively, *ev* LTRs may contain promoters unable to respond to enhancers. To test these possibilities, the RSV enhancer was inserted upstream of the intact *ev* LTR in pRAV-0Neo to determine whether the *ev* promoter could respond to the presence of a known enhancer. Figure 6 shows primer extension products generated from two different plasmid isolates that contained the RSV enhancer upstream of the RAV-0 LTR and in the opposite transcriptional orientation. As shown, transcription from the RAV-0 LTR was increased from 20- to 50-fold when the RSV enhancer was placed in *cis*. All extension products were of the length expected for transcripts that initiated in the RAV-0 LTR. The *ev* promoter therefore is able to respond to the presence of a known enhancer, indicating, first, that it is not defective in this regard and, second, that *ev* LTRs do not contain sequences that repress the activity of at least the RSV enhancer.

***ev* U3 regions do not enhance transcription from the herpesvirus TK promoter.** To address directly whether the enhancer activity of *ev* U3 regions seen in the *ev*-RSV hybrid LTRs was due to the removal of a repressor that might normally be present in intact *ev* LTRs, the identical fragments from *ev*-2/RAV-0 and *ev*-3 that were used to construct the *ev*-RSV hybrid LTRs were placed upstream of the herpesvirus TK gene promoter. This promoter gives rise to relatively low transcript levels in the absence of an added

FIG. 6. Absence of detectable enhancer activity in intact ev LTRs is not due to defective promoters. The RSV *Mst*II-*Eco*RI enhancer fragment (Fig. 2) was inserted upstream of the intact ev LTR in pRAV-0Neo, and transcription from the ev promoter either in the presence (RSV U3) or absence (–) of this enhancer was compared by primer extension after transfection of the indicated plasmids into avian fibroblasts. Lane M, pBR322 *Msp*I-digested marker DNA.

enhancer but can be up-regulated after insertion of a wide variety of enhancer elements, including that from RSV (29). Therefore, the RSV enhancer was tested in parallel as a control. Utilization of both the ev-2/RAV-0 and the ev-3 U3 regions also provided a second system in which to analyze whether the minor nucleotide variations in these two LTRs might affect their relative enhancer activities and therefore their differential expression in vivo.

As shown in Fig. 7, the pTKNeo plasmid was virtually inactive in the absence of an added enhancer, giving rise to barely detectable levels of correctly initiated transcripts in the primer extension assay. As expected, insertion of the RSV enhancer upstream of the TK promoter in either orientation significantly increased levels of correctly initiated transcripts of 99 nucleotides in length. Figure 7 also shows the results obtained when the U3 regions from ev-2/RAV-0 and ev-3 were placed upstream of the TK promoter and in the same orientation. As shown, no enhancement of transcription was observed with either ev U3 region. Analogous results were obtained with additional isolates of these constructs and with constructs in which the ev U3 regions were inserted in the opposite orientation (data not shown).

The inability of either the ev-3 or the ev-2/RAV-0 U3 fragments to augment transcription from the TK promoter indicates that the enhancer activity of these same sequences in the ev-RSV hybrid LTR plasmids is not due to removal of sequences that normally inhibit the activity of an ev-associated enhancer in intact ev LTRs. In addition, the finding that the ev-3 U3 region was unable to enhance transcription from the TK promoter indicates that the minor nucleotide variations in the U3 region of this provirus relative to that of ev-2/RAV-0 do not result in the appearance of enhancer activity in the ev-3 LTR as monitored in this system.

FIG. 7. ev U3 fragments are unable to enhance transcription from the herpesvirus TK gene promoter. U3 fragments from RSV, ev-2/RAV-0, and ev-3 were inserted in the positive orientation upstream of the TK promoter in pTKNeo. Plasmids were transfected into avian cells, and primer extension analysis was conducted as described in Materials and Methods. Transcripts that correctly initiate at +1 in the TK promoter give rise to extension products of 99 nucleotides (arrow). Identical results were obtained when each of the U3 regions was inserted in the negative orientation (data not shown). Lane M, pBR322 *Msp*I-digested marker DNA.

DISCUSSION

Differential expression of avian evs in vivo is not explained by differences in U3-associated enhancer activity. The differential expression of avian endogenous proviruses in vivo has been correlated with the sites of provirus integration (7, 8, 21, 25, 26, 41) and with distinct patterns of proviral DNA methylation acquired during gametogenesis (19). Studies described in this report were initiated to investigate whether minor nucleotide variations in the LTRs of avian evs that are either expressed (ev-3) or silent (ev-1 and ev-2) in vivo might affect their relative enhancer function and thereby also contribute to the differential expression of these elements in vivo. The finding that U3 regions from both active (ev-3) and inactive (ev-2) endogenous proviruses failed to augment transcription from the TK promoter indicates that the minor nucleotide differences between the LTRs of these viruses do not affect enhancer function as assayed in this system. LTR-specific differences therefore are unlikely to contribute to the differential expression of these elements in vivo.

Enhancer-negative ev U3 regions can replace essential enhancer sequences in RSV LTR. In the course of these studies it was unexpectedly found that all ev U3 regions were able to replace the major enhancer domains of the RSV LTR in an orientation-independent manner. Additional studies demonstrated that the absence of such enhancer activity in intact ev LTRs was not explained by the fact that these LTRs contain defective promoters or repressor sequences that mask the activity of an ev U3-associated enhancer in its natural context. While other examples exist that show that enhancers function inefficiently in either a heterologous context or when moved to alternate positions relative to promoter elements (1), this is the only example I am aware of in which sequences that do not function as efficient enhancers in their natural context are active in another context.

It is interesting that evs have maintained sequences within the 5'-proximal portion of U3 that do not function efficiently

as enhancers in intact *ev* LTRs yet retain sufficient information to replace essential domains of the RSV enhancer. A comparison of the *ev* and RSV U3 regions (Fig. 1) shows that although *ev* U3 regions contain short stretches of similarity to the RSV enhancer, they also contain divergent regions and gaps in sequence. It will be interesting to determine whether the limited sequence similarities between the RSV enhancer and *ev* U3 regions allow *ev* U3 regions to interact with the same *trans*-acting proteins that bind the RSV enhancer or whether *ev* U3 regions bind a distinct set of proteins that serve an analogous function(s). It has been shown by others (40) that *ev* U3 regions bind the rat C/EBP protein at positions -77 to -57; the corresponding sequences within exogenous virus LTRs do not contain a C/EBP-binding site at the analogous position.

***ev* U3 enhancer activity is dependent on sequences in the deleted RSV LTR: implications for enhancer function.** The context-specific activation of the *ev* U3 enhancer seen here in *ev*-RSV hybrid LTRs suggests that this activation is dependent on sequences from RSV downstream of the *Sph*I site at -141. Other explanations, such as a fortuitous effect of plasmid or other insert sequences present in pS-RSVNeo, are not supported by the data obtained. For example, the pRAV-0Neo and pTKNeo plasmids, which contained plasmid sequences identical to those in *ev*-RSV hybrid plasmids, did not result in *ev* enhancer function. Sequences downstream of the *Sph*I site in the RSV LTR contain the viral promoter (10, 12, 13, 33, 36, 47) and may also contain one or more domains that comprise portions of the intact viral enhancer (see below). Given that *ev* U3 regions do not enhance transcription from their own promoters which can be activated by the RSV enhancer, it seems unlikely that the enhancer activity of *ev* U3 regions in the hybrid LTRs is dependent on the RSV promoter. However, it is possible that the RSV promoter, in contrast to those of *ev*s and several heterologous genes which do not respond to the *ev* enhancer, contains sequences that allow it to respond efficiently to a partial or low-level enhancer element present in *ev* U3 regions. This property may in part explain why the RSV LTR is utilized efficiently in a wide variety of avian and nonavian cell types which show differences in the presence or absence of specific RSV enhancer-binding proteins (3, 12, 14, 38-40, 43).

A second explanation for why *ev* U3 regions function as enhancers only in the hybrid LTRs is that sequences downstream of the *Sph*I site at -141 in the RSV LTR may contain one or more enhancer domains that complement a weak or partial enhancer in *ev* U3 regions. The existence of at least one such domain in this region is suggested by deletion studies of the RSV LTR (28, 36, 46) and by the finding that sequences from this region, when present in tandem copies, can augment transcription from promoters in *cis* (29). A candidate sequence for such an enhancer domain in this region is a recently identified potential serum response element (3, 12). This element specifically interacts with avian protein (EFIII; 3) and can, when present in multiple copies, augment transcription from promoters in *cis* (43a).

The possibility that one or more enhancer domains provided by the deleted RSV LTR can complement a weak or partial enhancer element in *ev* U3 regions is very interesting in terms of enhancer function. That is, detailed studies of several enhancers indicate that these elements share a modular organization, being composed of multiple interacting domains, each of which binds specific *trans*-acting proteins (reviewed in reference 1). In some cases, disruption of individual domains can have little effect on the activity of the

enhancer, while mutation of other regions can abrogate enhancer function. Multimerization of some enhancer domains can also generate a functional enhancer. Together, these data suggest that while the function(s) of some enhancer domains and their cognate proteins is unique and essential, the function(s) of others may be redundant. A dependence of *ev* U3 regions on residual enhancer domains in the truncated RSV LTR for enhancer activity indicates that individual components of an enhancer can be separated from each other spatially and rearranged with respect to orientation and still maintain activity. If proteins that bind to enhancers must interact with each other and/or with promoter-proximal elements, it is interesting to speculate how the requisite contacts can be made when the integrity of an enhancer is so compromised.

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