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The Human Immunodeficiency Virus Type 1 Rev Protein and the Rev-Responsive Element Counteract the Effect of an Inhibitory 5′ Splice Site in a 3′ Untranslated Region

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A 5′ splice site located in a 3′ untranslated region (3′UTR) has been shown previously to inhibit gene expression. Natural examples of inhibitory 5′ splice sites have been identified in the late 3′UTRs of papillomaviruses and are thought to inhibit viral late gene expression at early stages of the viral life cycle. In this study, we demonstrate that the interaction of the human immunodeficiency virus type 1 Rev protein with the Rev-responsive element (RRE) overcomes the inhibitory effects of a 5′ splice site located within a 3′UTR. This was studied by using both a bovine papillomavirus type 1 L1 cDNA expression vector and a chloramphenicol acetyltransferase expression vector containing a 5′ splice site in the 3′UTR. In both systems, coexpression of Rev enhanced cytoplasmic expression from vectors containing the RRE even when the RRE and the inhibitory 5′ splice site were separated by up to 1,000 nucleotides. In addition, multiple copies of a 5′ splice site in a 3′UTR were shown to act synergistically, and this effect could also be moderated by the interaction of Rev and the RRE. These studies provide additional evidence that at least one mechanism of Rev action is through interactions with the splicing machinery. We have previously shown that base pairing between the U1 small nuclear RNA and a 3′UTR 5′ splice site is required for inhibition of gene expression. However, experiments by J. Kjems and P. A. Sharp (J. Virol. 67:4769–4776, 1993) have suggested that Rev acts on spliceosome assembly at a stage after binding of the U1 small nuclear ribonucleoprotein to the 5′ splice site. This finding suggests that binding of additional small nuclear ribonucleoproteins, as well as other splicing factors, may be necessary for the inhibitory action of a 3′UTR 5′ splice site. These data also suggest that expression of the papillomavirus late genes in terminally differentiated keratinocytes can be regulated by a viral or cellular Rev-like activity.

Human immunodeficiency virus type 1 (HIV-1) encodes a 19-kDa nuclear phosphoprotein named Rev (15, 27). Rev forms a multimeric complex with an RNA structure called the Rev-responsive element (RRE) (24, 25, 40, 49). This interaction facilitates the expression of unspliced and singly spliced viral RNAs (19, 34, 39, 60). The mechanism by which the Rev-RRE interaction affects HIV-1 gene expression is currently under investigation. Several lines of evidence suggest that Rev affects posttranscriptional processes (18, 19, 25, 40). Various investigators studying HIV-1 have suggested that suboptimal or unpaired splice sites render an RNA containing the RRE susceptible to Rev regulation (11, 26, 35). 5′ splice sites in particular may function to retain RNA in the nucleus, an effect which can be overcome when the Rev protein is supplied in *trans* and the RRE is present in *cis* (11, 26, 35). Genetic suppression experiments have demonstrated that base pairing of U1 small nuclear RNA to a 5′ splice site just upstream of the *env* open reading frame is essential for *rev*-responsive expression (35). Human T-cell leukemia virus types 1 and 2 use a similar mechanism to regulate RNA expression (29, 30), and the activity of their regulatory protein, Rex, is dependent on the presence of the Rex-responsive element and a 5′ splice site (55). The characterization of spliceosome complexes has suggested that Rev does not block the binding of U1 small nuclear ribonucleoprotein (snRNP) to the 5′ splice site but rather blocks the entry of the U4, U5, and U6 snRNPs in spliceosome assembly (33). Additional evidence that Rev interacts with the splicing machinery comes from the observation that Rev colocalizes to nuclear speckles, which are thought to be active sites of transcription and RNA processing (31). In toto, these studies suggest that Rev may facilitate transport of unspliced mRNAs into the cytoplasm by interference with spliceosome assembly.

Some investigators stress that other RNA sequences, distinct from splice sites, may prevent the expression of the HIV-1 unspliced and singly spliced RNAs (14, 17, 19, 24, 34, 45, 54). These non-splice site inhibitory elements (called variously CRS, INS, or IR elements) are proposed to bind to host cell factors, leading to the instability, nuclear retention, or inefficient translation of RNAs which contain them. When Rev is bound to the RRE, expression of RNAs containing these inhibitory elements can occur. Several such elements have been characterized in the *gag*, *pol*, and *env* genes and are active in the absence of splicing or functional splice sites (14, 17, 45, 52, 54). Several studies have also identified inhibitory elements in the HIV-1 RRE itself (10, 45). It seems likely, then, that the Rev-RRE interaction can counteract many different types of inhibitory RNA sequences, perhaps through different mechanisms (54).

Like human retrovirus mRNAs, bovine papillomavirus type 1 (BPV-1) mRNAs show a complex pattern of splicing and polyadenylation (2, 3). Expression of the BPV-1 late genes is restricted to terminally differentiated keratinocytes (7, 58). Expression of these genes in nonpermissive cells is limited both by the inactivity of the late promoter and by posttranscriptional

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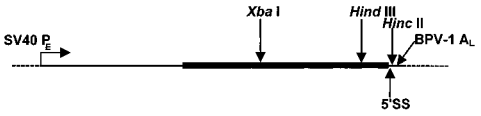

FIG. 1. Map of the L1 cDNA expression vector. The L1 cDNA expression vectors are driven by the SV40 early promoter (P_E) and contain all three exons of the L1 mRNA, including the entire 1,485-bp coding sequence of the L1 protein (heavy bar). The RRE was inserted in sense and antisense orientations into the *Xba*I, *Hin*dIII, and *Hin*cII sites (BPV-1 nt 6133, 6959, and 7145, respectively). The 3′UTR is short (82 nt) and contains the BPV-1 late polyadenylation signal (A_L) at BPV-1 nt 7156 to 7161. The BPV-1 3′UTR 5′ splice site (5′SS) is immediately upstream of the *Hin*cII site at BPV-1 nt 7136 to 7144.

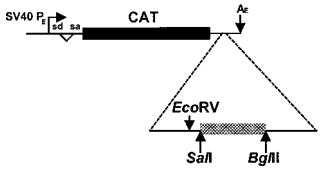

FIG. 2. Map of the CAT expression vector. The CAT expression vector (p2518 [4]) used in these studies is driven by the SV40 early promoter (P_E) and contains a 97-bp intron (sd, splice donor; sa, splice acceptor) upstream of the CAT coding sequence (heavy bar) as shown. The 231-bp 3′UTR contains the SV40 early polyadenylation signal (A_E). An expanded view of the polylinker region within the 3′UTR is shown. The BPV-1 3′UTR fragments were inserted between the *Sal*I and *Bgl*II sites as indicated by the hatched bar. The RRE was inserted in a sense orientation into either the *Eco*RV or *Bgl*II cloning sites indicated with an arrow. A 624-bp fragment of β-galactosidase coding sequence inserted into the *Sal*I site was used to increase the distance between the 3′UTR inhibitory element and an RRE cloned into the *Eco*RV site.

mechanisms (7, 21, 22). Significant transcription of the late region can be seen in nonpermissive cells although no cytoplasmic late mRNA is expressed (6). This finding suggests that posttranscriptional mechanisms are important for regulation of BPV-1 gene expression.

One posttranscriptional regulatory element which inhibits the cytoplasmic expression of BPV-1 late mRNAs in nonpermissive cells is a 5′ splice site located in the late 3′ untranslated region (3′UTR) (21, 22). No mRNAs which use this potential splice site have been identified, suggesting that the only function of this sequence is the inhibition of late gene expression in nonpermissive cells (22). The exact mechanism by which this 5′ splice site exerts its effect is unknown. However, it does not appear to affect cytoplasmic mRNA stability and presumably acts on a nuclear process (21). This notion is consistent with the observation that the inhibitory function of this 5′ splice site requires base pairing between the 5′ splice site and the 5′ end of the U1 small nuclear RNA (22).

This study was designed to identify posttranscriptional regulatory elements in BPV-1 which are functionally homologous to the inhibitory elements in HIV-1 that respond to the interaction of Rev and the RRE. Using expression vectors, we have demonstrated that the interaction of the HIV-1 Rev protein with the RRE is sufficient to overcome the inhibitory effects of the BPV-1 late 3′UTR 5′ splice site. These findings support the hypotheses that at least some Rev functions require interactions with the splicing machinery. These data also suggest that expression of the BPV-1 late genes in terminally differentiated keratinocytes may be regulated by a viral or cellular Rev-like activity.

MATERIALS AND METHODS

Expression vectors. Nucleic acid manipulations, bacterial transformations, recombinant screening, and plasmid preparation were performed by standard methods (41).

The BPV-1 L1 expression vector p2743 contains a full-length L1 cDNA (starting at BPV-1 nucleotide [nt] 7251) (5, 7) which was cloned into pUC18 downstream of the *Hin*dIII-to-*Xho*I simian virus 40 (SV40) early promoter fragment from the vector pL1 (48). BPV-1 genomic sequences were substituted for cDNA sequences downstream of the *Xba*I site in exon 3 and extended downstream of the late poly(A) cleavage site (nt 7175) to nt 7449. Plasmid p2744 is identical to p2743 except for a late 3′UTR deletion (BPV-1 nt 7097 to 7145) accompanied by insertion of linker sequences (GCTTGG). Detailed maps and sequences for the L1 expression vectors are available upon request.

The RRE was obtained from plasmid KS330 (courtesy of G. Pavlakis), which contained nt 7266 to 7595 of infectious HIV-1 proviral clone HXB2 cloned into the polylinker of pBluescript KS(−) (54). An *Eco*RI-to-*Bam*HI fragment from KS330 containing the RRE was blunt ended by Klenow enzyme and cloned in sense and antisense orientations into the *Xba*I, *Hin*dIII, and *Hin*dII sites of p2743, which also were blunted with Klenow enzyme, creating plasmids p2724, p2721, and p2723 and plasmids p2725, p2720, and p2722 (Fig. 1). The RRE fragment was also cloned in a sense orientation into the *Xba*I site of p2744, creating plasmid p2740.

A high-expressing SV40-based chloramphenicol acetyltransferase (CAT) vector pOBCAT20 (p2518; CCB186) which has a polylinker in the 3′UTR was used to test the effect of Rev on the function of the BPV-1 late 3′UTR element (4) (Fig. 2). Plasmids p2621 and p2628 contain the BPV-1 3′UTR negative element cloned into the *Sal*I and *Bgl*II sites in the 3′UTR of p2518 as described previously (21, 22) (Fig. 2). p2621 contains a 53-bp BPV-1 fragment from nt 7094 to 7146. p2628 contains a 9-bp fragment (BPV-1 nt 7136 to 7144) which is identical to the 5′ splice site consensus sequence. Two complementary synthetic oligonucleotides containing two copies of a 5′ splice site separated by 8 nt of spacer sequence (ucgacAAGGTAAGTagtctgacAAGGTAAGTa) were cloned into the *Sal*I and *Bgl*II sites of p2518, creating plasmid p2738.

The RRE fragment from plasmid KS330 was blunted by Klenow enzyme and cloned in both the sense and antisense orientations into either the *Eco*RV site upstream or a *Bgl*II site (blunted with Klenow enzyme) downstream of the BPV-1 negative element within the 3′UTR of plasmid p2621 (creating plasmids p2726 and p2730) and plasmid p2628 (creating plasmids p2727 and p2729) (Fig. 2). The RRE was also cloned into the *Eco*RV site of the parent vectors p2518 to create a plasmid that does not contain a 3′UTR negative element, creating plasmid p2731. The *Hpa*I fragment of the β-galactosidase expression vector pCH110 (Pharmacia), containing 624 nt of β-galactosidase coding sequence, was inserted in a sense orientation into a *Sal*I site (blunted with Klenow enzyme) between the insertion site of the BPV-1 3′UTR negative element and the upstream RRE insertion site in plasmid p2727 (creating plasmid p2733) and plasmid p2726 (creating plasmid p2732) (Fig. 2). The β-galactosidase *Hpa*I fragment was also inserted into the blunted *Sal*I site of the versions of these plasmids without the RRE, creating plasmids p2736 and p2737. The β-galactosidase *Hpa*I fragment was cloned into the blunted *Sal*I site in the parental vector p2518, which does not contain a 3′ inhibitory element (creating p2735), and upstream of the RRE in the version of the parental plasmid which contained the RRE (creating p2734).

The *rev* expression vector pHCMVsrev was provided by George Pavlakis (8). pCMV(−) (p2745) was created from this plasmid by cleavage with *Hin*dIII and *Sma*I, Klenow repair, and religation.

A human α-globin expression vector (pSVα1) was provided by Michael Greenberg (56).

Cells. The HeLa cell line HLtat (53) was maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, antibiotics, and glutamine.

CAT enzyme analyses. Plasmid DNA was prepared by anion-exchange resin purification (Qiagen, Chatsworth, Calif.). Five micrograms of CAT vector was cotransfected with 2.5 μg of pCH110, 10 μg of sheared herring sperm DNA (Promega), and 2.5 μg of pHCMVsrev or pCMV(−) into 10-cm plates of HLtat cells by the calcium phosphate precipitation technique as previously described (21). Cells were harvested at 48 h, and extracts were prepared by freeze-thawing. CAT activity was assessed by thin-layer chromatography using standard methods. The acetylated and unacetylated forms of chloramphenicol were quantitated by radioanalytic imaging (AMBIS) and expressed as percent acetylation. β-Galactosidase activity was measured and used to correct CAT activity for differences in transfection efficiency as previously described (4).

RNA analyses. Plasmid DNA was prepared by cesium chloride banding (41). Ten micrograms of L1 expression vector was cotransfected with 2.5 μg of pSVα1 and 2.5 μg of pHCMVsrev or pCMV(−) and 10 μg of carrier DNA as described above. For each plasmid, two 10-cm-diameter plates of HLtat cells were transfected. After 48 h of incubation, duplicate plates were pooled for RNA preparation.

Cytoplasmic RNA was isolated by Nonidet P-40 lysis buffer according to standard procedures (41). The RNA was quantitated on a Beckman spectrophotometer. The poly(A)$^+$ and poly(A)$^-$ RNA fractions were separated by using polystyrene latex particles covalently linked to oligo(dT) (Qiagen). Northern (RNA) blot analysis of 10 μg of poly(A)$^-$ RNA or poly(A)$^+$ RNA from 250 μg of total RNA was accomplished as reported previously (21); 250 μg represents approximately 30% of the total mRNA sample. mRNA was detected by random-primer-labeled (Amersham) PCR-amplified fragments. The fragments used for

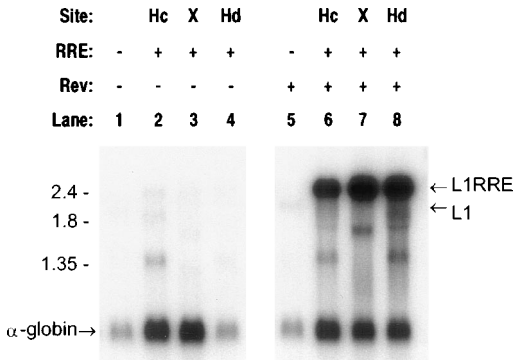

FIG. 3. Enhancement of BPV-1 L1 mRNA expression by the HIV-1 Rev protein in *trans* and the RRE in *cis*. L1 cDNA expression vectors with the RRE inserted in the sense orientation into the *Hin*cII (Hc), *Xba*I (X), and *Hin*dIII (Hd) sites were cotransfected into HLtat cells with pSVα1 and either plasmid pHCMVsrev or plasmid pCMV(−). Cytoplasmic RNA was harvested 48 h later as described in Materials and Methods. For each sample, poly(A) RNA was isolated from 250 μg of total RNA and subjected to Northern blot analysis. L1 mRNA and α-globin mRNA were detected by the appropriate PCR-amplified DNA fragments which were random primer labeled. The α-globin, L1, and L1 mRNAs containing the RRE (L1RRE) are indicated with arrows. The positions of RNA size markers are indicated on the left in kilobases.

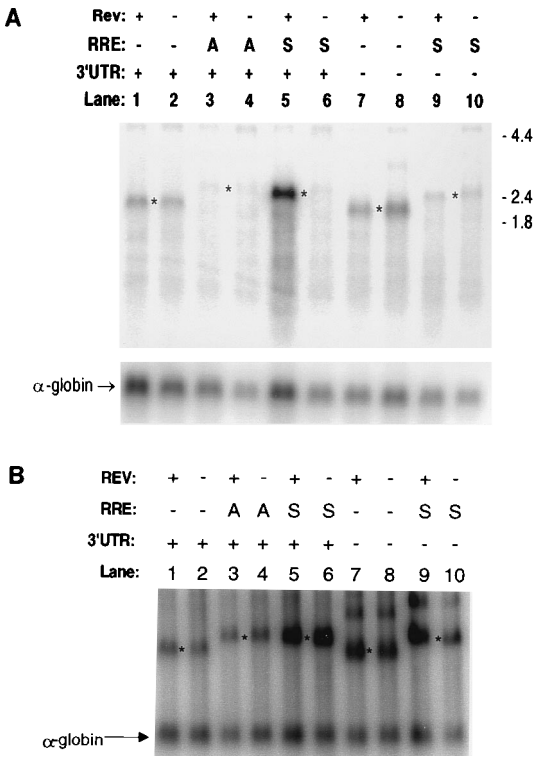

FIG. 4. Enhancement of BPV-1 L1 mRNA expression by the HIV-1 Rev protein requires sequences in the BPV-1 late 3′UTR. (A) The L1 cDNA expression vectors with the RRE inserted into the *Xba*I site in either a sense (S) or antisense (A) orientation were cotransfected into HLtat cells with pSVα1 and either plasmid pHCMVsrev or plasmid pCMV(−). The plasmids in lanes 7 to 10 had a 53-bp deletion in the 3′UTR which removed the 3′UTR inhibitory element. Cytoplasmic RNA was harvested 48 h posttransfection as described in Materials and Methods and subjected to poly(A) selection and Northern blot analysis as described for Fig. 3. The positions of RNA size markers are indicated on the right in kilobases. The asterisks between pairs of lanes indicate the bands quantitated in Table 1. (B) The L1 cDNA expression vectors with the RRE inserted into the *Xba*I site in a sense orientation were cotransfected as for panel A. Nuclear RNA was harvested as described in Materials and Methods. Northern analysis of the entire pool of polyadenylated nuclear RNA is shown in lanes 1 to 10. The positions of the L1 and L1/RRE RNAs are indicated with asterisks between pairs of lanes.

mRNA detection were as follows: for L1, BPV-1 nt 4983 to 7096; for CAT, pOBCAT20 nt 599 to 1233; and for α-globin, nt 10530 to 11197. L1 or CAT expression levels were quantitated and normalized to α-globin expression levels by PhosphorImager (Molecular Dynamics) analysis.

Nuclear RNA was prepared by using TRIzol (Bethesda Research Laboratories) as instructed by the manufacturer. Pelleted nuclei were washed in Nonidet P-40 buffer, then lysed in TRIzol, extracted, and precipitated with isopropanol. The entire sample of nuclear RNA was poly(A) selected and subjected to Northern analysis as above.

Primer extension analyses were carried out on CAT mRNA as reported previously (5). A synthetic oligonucleotide (20-mer) with the 5′ nucleotide 308 bp downstream from the SV40 early mRNA start site was used as a primer. The oligonucleotide was labeled with polynucleotide kinase (Pharmacia) at the 5′ terminus with ^{32}P; 7.5 μg of total RNA was annealed to the primer. cDNA synthesis was initiated by the addition of 200 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) at 37°C for 1 h. The cDNA products were analyzed on a 6% polyacrylamide gel along with a dideoxy sequencing ladder generated from the CAT plasmid, using the 5′-labeled oligonucleotide and a 100-bp ladder (Life Sciences) labeled with [γ-^{32}P]ATP and polynucleotide kinase (Pharmacia).

Image processing. Halftone images were prepared directly from PhosphorImager scans or from scanned autoradiograms by using Aldus Photostyler and Micrografx Designer and printed on a Tektronix Phaser IIsdx dye sublimation printer.

RESULTS

Identification of a Rev/RRE-responsive negative regulatory element in the BPV-1 late region. Our initial experiments were designed to determine if the BPV-1 L1 mRNA contains Rev/RRE-responsive inhibitory elements. An L1 expression vector (p2743) in which a BPV-1 L1 cDNA is transcribed by the SV40 early promoter was used in these studies (Fig. 1). The 3′ end of the cDNA was replaced by BPV-1 genomic sequences so that an intact late 3′UTR and late poly(A) site were present. The HIV-1 RRE was cloned into three sites in this vector: the *Xba*I and *Hin*dIII sites within coding sequences and the *Hin*cII site in the 3′UTR (Figure 1). The Rev responsiveness of the resulting vectors was assayed in HLtat cells by cotransfection with either pHCMVsrev or pCMV(−). Expression of cytoplasmic L1 mRNA from the resulting plasmids was assayed by Northern blotting. Cytoplasmic levels of L1 mRNA increased up to 50-fold when the RRE was cloned in a sense orientation in any of these three locations and the transfection included the *rev* expression vector (Fig. 3; compare lanes 6 to 8 with lanes 2 to 4). Rev had no effect on L1 mRNA expression when the RRE was either absent (Fig. 3, lane 5) or cloned in an antisense orientation (Fig. 4A, lane 3). Furthermore, the location of the RRE in the L1 mRNA had little influence on the degree of activation of L1 mRNA expression by Rev. These results suggest that the L1 mRNA contains Rev/RRE-responsive inhibitory elements.

Previous studies from this laboratory have shown that a consensus 5′ splice site in the BPV-1 late 3′UTR acts post-transcriptionally to repress L1 mRNA expression (22). Since 5′ splice sites have been shown to be involved in the Rev response, we investigated the possibility that the late 3′UTR element is the Rev/RRE-responsive inhibitory element in the L1 mRNA. Deletion of a 53-nt fragment containing the late 3′UTR 5′ splice site from the L1 expression vector increased cytoplasmic levels of L1 mRNA up to sixfold in transfection assays as seen previously (Fig. 4A, Table 1, and data not shown) (22). Coexpression of Rev had no effect on cytoplasmic expression of the L1 mRNA lacking the 3′UTR negative element regardless of whether the RNA also contained the RRE inserted at the *Xba*I site (Fig. 4A, lanes 9 and 10; Table 1). In contrast, a 15-fold increase in cytoplasmic levels of wild-type L1 mRNA containing the RRE was seen in the presence of

TABLE 1. Enhancement of cytoplasmic BPV-1 L1 mRNA expression by the HIV-1 Rev protein

3′UTR[a]	RRE[b]	Normalized L1 mRNA[c]					
		Cytoplasmic			Nuclear		
		− Rev	+ Rev	+Rev/−Rev[d]	− Rev	+ Rev	+Rev/−Rev
+		1.0	0.9	0.9	1.0	1.0	1.0
+	AS	0.6	0.2	0.3	1.2	1.4	1.2
+	S	0.6	8.7	14.9	2.7	3.2	1.2
−		3.5	2.9	0.8	2.5	2.5	1.0
−	S	1.4	1.1	0.8	3.4	2.6	0.8

[a] +, the wild-type sequence of the BPV-1 late 3′UTR; −, a 53-nt deletion containing the late 3′UTR element.

[b] The HIV-1 RRE was cloned in the sense (S) or antisense (AS) orientation into the *Xba*I site of the L1 cDNA expression vector shown in Fig. 1.

[c] L1 mRNA levels were quantitated from the PhosphorImager scan shown in Fig. 4A (cytoplasmic) and Fig. 4B (nuclear) and normalized to levels of α-globin mRNA expressed from a cotransfected expression vector pSVα1. These values were further normalized to L1 mRNA levels expressed in the absence of Rev. Cytoplasmic and nuclear values were normalized independently. Cotransfections were carried out with either the vector pHCMVsrev (+Rev) or a similar vector from which the *rev* coding sequences were deleted [pCMV(−)] (−Rev).

[d] Calculated by dividing the amount of L1 mRNA synthesized in the presence of Rev by the amount synthesized in the absence of Rev.

TABLE 2. Effect of HIV-1 Rev on inhibition of CAT expression by the BPV-1 late 3′UTR element

Line[a]	Length (nt) of 3′UTR fragment[b]	RRE[c]	*n*[d]	Relative CAT activity[e]		+Rev/−Rev[f]
				−Rev	+Rev	
1		−	12	100 ± 0.0	122.2 ± 70.5	1.2
2		+	12	23.0 ± 5.7	145.9 ± 67.6	6.3
3	53	−	10	8.7 ± 2.2	10.5 ± 3.8	1.2
4	53	+ US	10	9.8 ± 2.9	129.7 ± 47.3	13.2
5	53	+ DS	4	5.4 ± 1.1	99.5 ± 25.4	18.4
6	9	−	12	0.4 ± 0.4	0.8 ± 0.9	2.0
7	9	+ US	12	2.1 ± 0.9	99.4 ± 34.0	47.3
8	9	+ DS	4	1.1 ± 0.4	58.2 ± 22.3	52.9

[a] Plasmids used in this experiment were as follows: line 1, p2518; line 2, p2731; line 3, p2621; line 4, p2726; line 5, p2730; line 6, p2628; line 7, p2727; and line 8, p2729.

[b] The 53- and 9-nt fragments containing the BPV-1 late 3′UTR inhibitory element are described in Materials and Methods. The 9-nt fragment is a consensus 5′ splice site.

[c] The HIV-1 RRE was cloned either immediately upstream (US) or downstream (DS) of the BPV-1 late 3′UTR element (see Fig. 2).

[d] *n*, number of times the plasmids were assayed.

[e] The CAT expression vectors were cotransfected into HLtat cells with a β-galactosidase control plasmid (pCH110) and a CMV expression plasmid. The percentage of chloramphenicol acetylated was corrected for differences in transfection efficiency by using β-galactosidase activity and normalized to the activity of the parent CAT vector (p2518) in the absence of Rev. The results of multiple experiments were averaged, and the standard deviations were calculated. CAT plasmids were cotransfected with either pHCMVsrev (+Rev) or a CMV vector from which *rev* was deleted (−Rev).

[f] Fold activation of CAT expression by Rev.

Rev (Fig. 4A, lanes 5 and 6; Table 1). These results suggest that the 5′ splice site in the late 3′UTR is responsive to the Rev-RRE interaction. In fact, sequences in the late 3′UTR appear to be the only Rev/RRE-responsive inhibitory element in the L1 mRNA since an L1 expression vector lacking this region is no longer Rev responsive. Rev is able to counteract the effect of the late 3′UTR even when the RRE is located 1,000 nt upstream at the *Xba*I site, suggesting that Rev does not function by steric interference with binding of factors to the late 3′UTR.

Nuclear polyadenylated L1 mRNAs were also examined to determine if L1 mRNAs are retained in the nucleus in the absence of Rev and the RRE (Fig. 4B; Table 1). The entire nuclear polyadenylated RNA sample was assayed on the Northern blot shown in Fig. 4B, compared with 30% of the total cytoplasmic pool of polyadenylated mRNA shown in Fig. 4A. Quantitation of the mRNAs indicated that nuclear levels of polyadenylated L1 mRNA in the absence of both Rev and the RRE were at least 5- to 10-fold lower than cytoplasmic mRNA levels. This result suggests either that the wild-type L1 mRNA is not selectively retained in the nucleus or that nuclear L1 RNA is unstable. Deletion of the 3′UTR 5′ splice site produced only a modest 2.5-fold increase in the levels of L1 mRNA in the nucleus (Fig. 4B, lanes 7 and 8; Table 1). Furthermore, the nuclear pools of L1 mRNA containing an RRE in the sense orientation do not appear to change significantly in the presence of Rev (Fig. 4B, lanes 5 and 6; Table 1). In contrast, cytoplasmic levels of L1 mRNAs containing an RRE increased at least 15-fold in the presence of Rev (Fig. 4A, lanes 5 and 6; Table 1). Northern analysis of poly(A)$^-$ nuclear RNA gave a smear pattern when hybridized with the L1 probe as would be expected since poly(A)$^-$ RNA has no defined 3′ end. No significant effect of Rev and the RRE on poly(A)$^-$ nuclear RNA could be demonstrated (data not shown).

Rev and the RRE counteract the inhibitory activity of the BPV-1 late 3′UTR element in a heterologous CAT expression system. To confirm the identification of the BPV-1 late 3′UTR element as a Rev/RRE-responsive inhibitory element, we tested the interaction of Rev and the 3′UTR negative element in a heterologous CAT expression system (Fig. 2). In these experiments, 53- or 9-bp fragments containing the BPV-1 late 3′UTR inhibitory element were cloned into the 3′UTR of a CAT expression vector as described previously (21, 22). The RRE was then cloned upstream and downstream of the BPV-1 late 3′UTR fragments. In addition, the RRE was also cloned into the 3′UTR of the parental CAT vector. The vectors were then assayed by cotransfection into HLtat cells with either pHCMVsrev or pCMV(−). As seen previously, insertion of fragments containing the BPV-1 late 3′UTR element into the 3′UTR of a CAT expression vector decreased CAT expression (22) compared with the parent CAT vector that does not contain the negative element (Table 2). The effect was an 11-fold reduction of CAT activity when 53 nt of BPV-1 (nt 7094 to 7146) containing the 5′ splice site was present (Table 2, line 3). Over 100-fold repression was seen when the 9-nt consensus 5′ splice site was present (line 6). These results are consistent with those published previously (21, 22). Coexpression of Rev in the absence of an RRE had no significant effect on the expression of these plasmids (lines 1, 3, and 6). This observation is to be expected since Rev function requires binding to the RRE. Interestingly, the presence of the RRE in the 3′UTR of the parent vector (line 2) also repressed CAT expression (approximately fourfold) in the absence of Rev. Coexpression of Rev, however, reversed this effect (line 2). This is not surprising since a CRS/INS element which overlaps the RRE has been identified previously (10, 45).

In the absence of Rev, the RRE had little effect on expression from plasmids containing the 53-bp BPV-1 sequence (Table 2, lines 4 and 5). In contrast, the RRE, when cloned upstream of the 9-bp fragment, caused an approximately fourfold reproducible increase in CAT activity in the absence of Rev (line 7). In all cases, when the RRE was present in *cis* and Rev was supplied in *trans*, CAT activity increased to near the level of the parental control plasmid (lines 2, 4, 5, 7, and 8). In the case of the CAT plasmids containing the 53-bp BPV-1 late

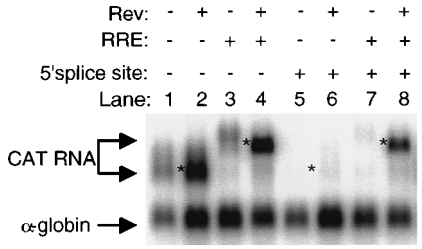

FIG. 5. The suppressive effect of a 5′ splice site in the 3′UTR of CAT mRNA can be overcome by expression of the HIV-1 Rev protein. CAT expression vectors were cotransfected into HLtat cells with pSVα1 and either plasmid pHCMVsrev or plasmid pCMV(−). Cytoplasmic RNA was harvested 48 h post-transfection as described in Materials and Methods and subjected to poly(A) selection and Northern blot analysis as described for Fig. 3. The RRE and/or a 5′ splice site was cloned into the 3′UTR as described for Fig. 2. The asterisks between pairs of lanes indicate the bands quantitated in Table 3.

3′UTR fragment and the RRE, Rev induced CAT activity 13- to 18-fold. In the case of the CAT plasmid containing the 9-bp consensus 5′ splice site and the RRE, Rev induced activity 47- to 53-fold. These data confirm that the BPV-1 late 3′UTR contains a Rev/RRE-responsive inhibitory element and further map this element to the consensus 5′ splice site.

Rev has been shown to promote the polysomal loading and translation of HIV-1 mRNAs (17, 50). To assess whether the effect on CAT activity occurred at the RNA or protein level, we carried out Northern analysis of cytoplasmic polyadenylated CAT mRNA (Fig. 5; Table 3). The presence of the 9-bp consensus 5′ splice site fragment caused a 37-fold reduction in cytoplasmic CAT polyadenylated mRNA (Fig. 5, lanes 1 and 5; Table 3). However, the RRE in *cis* and Rev in *trans* increased cytoplasmic CAT mRNA levels 20-fold to near the level expressed from the parental control plasmid (Fig. 5, lanes 1, 7 and 8; Table 3). As seen in the enzymatic assays, the presence of the RRE in the 3′UTR of the parent vector decreased CAT mRNA levels approximately four- to fivefold in the absence of Rev (Fig. 5, lanes 1 and 3; Table 3), and coexpression of Rev reversed this effect (Fig. 5, lanes 3 and 4; Table 3).

These data indicate that the increase in CAT activity in the presence of Rev in *trans* and the RRE in *cis* are due predominantly to effects of Rev on cytoplasmic mRNA levels. However, the increases in CAT enzymatic activity were greater than the increases in CAT mRNA (50-fold compared with 20-fold),

TABLE 3. Enhancement of cytoplasmic CAT mRNA expression by the HIV-1 Rev protein

3′UTR fragment[a]	RRE[b]	Normalized CAT mRNA[c]		+Rev/−Rev[d]
		− Rev	+ Rev	
−	−	100.0 ± 0	111.0 ± 6.7	1.1
−	+	21.0 ± 3.9	118.5 ± 29.5	5.6
+	−	2.7 ± 0.57	2.0 ± 0.43	0.7
+	+	3.3 ± 0.6	67.5 ± 10.86	20.5

[a] + or − indicates the presence or absence of the 9-nt consensus 5′ splice site in the 3′UTR.

[b] The HIV-1 RRE was cloned upstream of the BPV-1 late 3′UTR element (see Fig. 2).

[c] CAT mRNA levels were quantitated from the PhosphorImager scans of two identical transfections (one is shown in Fig. 5) and normalized to levels of α-globin mRNA expressed from a cotransfected expression vector pSVα1. Co-transfections were carried out with either the vector pHCMVsrev (+Rev) or a similar vector from which the *rev* coding sequences were deleted [pCMV(−)] (−Rev).

[d] Calculated by dividing the amount of CAT mRNA synthesized in the presence of Rev by the amount in the absence of Rev.

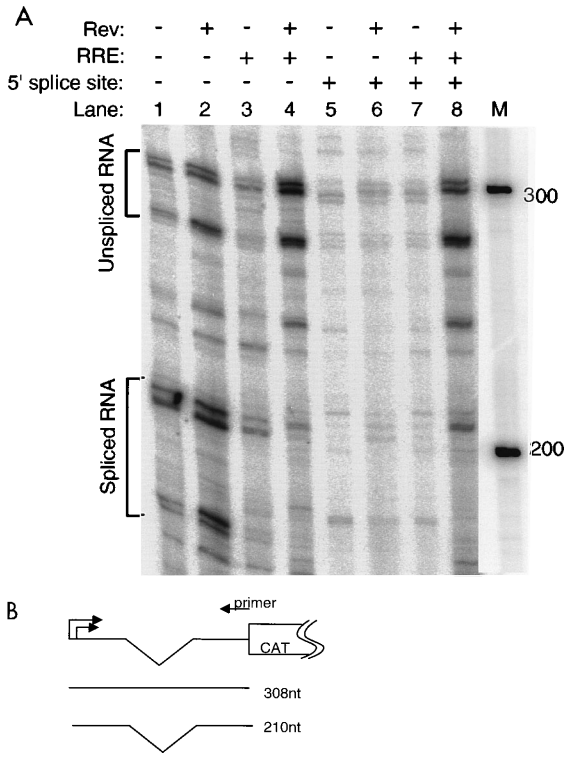

FIG. 6. The Rev-RRE interaction leads to an increase of unspliced CAT mRNAs in the cytoplasm. (A) Primer extension analysis was performed on RNAs generated from CAT expression vectors transfected into HLtat cells as described in Materials and Methods. The known RNA start sites predicted from previous characterization of other SV40 vectors are indicated with brackets at the left. The 100-bp ladder is shown in lane M and labeled on the right. (B) Schematic of the CAT vectors. Some heterogeneity of the SV40 early promoter start sites is expected and is indicated by the multiple arrows at the left. The position of the primer used for primer extension analysis and the expected sizes of the cDNAs generated from spliced and unspliced mRNAs are shown.

suggesting that Rev may also affect translation of CAT mRNAs. Rev has been shown to increase the cytoplasmic accumulation of unspliced mRNAs. An increase in the efficiency of translation of unspliced CAT mRNA compared with spliced CAT mRNA could have accounted for this difference in magnitude of the Rev effect. We therefore performed primer extension analysis on the cytoplasmic CAT mRNAs to quantitate spliced and unspliced CAT mRNAs (Fig. 6). Two sets of cDNAs were generated by primer extension of mRNAs expressed from the parental plasmid and correspond to spliced and unspliced RNAs with 5′ ends mapping to the heterogeneous SV40 early mRNA start sites (Fig. 6, lanes 1 and 2). In the absence of Rev and the RRE, the parental plasmid expressed spliced CAT mRNAs more abundantly than unspliced CAT mRNAs in the cytoplasm (Fig. 6, lanes 1). In contrast, unspliced CAT mRNAs predominated in the cytoplasm in the presence of Rev and the RRE (lane 4). The normalized levels of both CAT mRNA and protein expressed from the parental plasmid or expressed in the presence of the RRE and Rev are comparable (compare Tables 2 and 3), suggesting that the translational efficiency of unspliced CAT mRNA is similar to that of spliced CAT mRNA. Consistent with the results of the Northern blots (Fig. 5; Table 3), only very low levels of CAT mRNAs were produced from vectors containing the 9-bp consensus 5′ splice site with or without the RRE (Fig. 6, lanes 5 to 7). However, in the presence of both Rev and the RRE, CAT

TABLE 4. Effect of distance between the RRE and the BPV-1 late 3′UTR element on Rev function

Line[a]	3′UTR fragment[b]	RRE[c]	XTN[d]	n[e]	Relative CAT activity[f]		+Rev/ −Rev[g]
					−Rev	+Rev	
1	−	−	−	12	100.0 ± 0	122.2 ± 70.5	1.2
2	−	−	+	2	19.8 ± 3.6	27.4 ± 4.0	1.4
3	−	+	+	2	14.3 ± 2.1	41.8 ± 5.8	2.9
4	+	−	−	12	0.4 ± 0.4	0.8 ± 0.9	2.0
5	+	−	+	2	0.3 ± 0.1	0.3 ± 0	1.0
6	+	+	+	2	1.7 ± 0.1	23.0 ± 3.6	13.5

[a] Plasmids used in these experiments were as follows: line 1, p2518; line 2, p2735; line 3, p2734; line 4, p2628, line 5, p2736; and line 6, p2733.

[b] + or − indicates the presence or absence, respectively, of the 9-nt consensus 5′ splice site in the 3′UTR.

[c] The HIV-1 RRE was cloned upstream of the BPV-1 late 3′UTR element (see Fig. 2).

[d] A 624-nt fragment of β-galactosidase coding sequence (XTN) was cloned between the RRE and the BPV-1 late 3′UTR inhibitory element as described in Material and Methods (see Fig. 2).

[e] n, number of times the plasmids were assayed.

[f] The CAT expression vectors were cotransfected into HLtat cells with a β-galactosidase control plasmid (pCH110) and a CMV expression plasmid. The percentage of chloramphenicol acetylated was corrected for differences in transfection efficiency by using β-galactosidase activity and normalized to the activity of the parent CAT vector (p2518) in the absence of Rev. The results of multiple experiments were averaged, and the standard deviations were calculated. CAT plasmids were cotransfected with either pHCMVsrev (+Rev) or a CMV vector from which *rev* was deleted (−Rev).

[g] Fold activation of CAT expression by Rev.

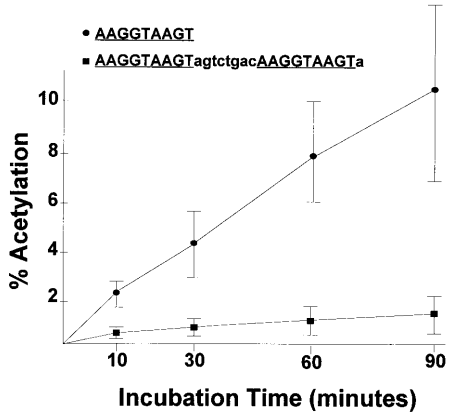

FIG. 7. CAT activity expressed from CAT vectors containing one or two 5′ splice sites. HLtat cells were transfected with the CAT vectors p2628 and p2738. These vectors contain one and two copies, respectively, of a consensus 5′ splice site within the 3′UTR. The sequences of the 5′ splice site fragments inserted into the 3′UTR are shown. Cells were harvested and extracts were prepared as described in Materials and Methods. For each time point, 10% of the extract was assayed. Incubations were halted at the time points indicated. The percentage of chloramphenicol acetylated was corrected for differences in transfection efficiency by using β-galactosidase activity.

mRNA levels increased dramatically and again unspliced mRNAs predominated (lane 8).

Separation of the inhibitory 3′UTR 5′ splice site and the RRE by 624 nt does not abolish Rev function. In the previous set of experiments, the RRE was cloned immediately upstream or downstream of the BPV-1 late 3′UTR inhibitory element. In this configuration, the effect of the Rev-RRE interaction could be due to steric interference between Rev and factors binding to the 3′UTR 5′ splice site. To assess this possibility, a 624-bp *Hpa*I fragment of β-galactosidase was cloned between the 3′UTR 5′ splice site and the RRE in the 3′UTR of the CAT expression vectors. The plasmids with the 624-bp spacer fragment were cotransfected with either pHCMVsrev or pCMV(−). The 624-nt extension significantly decreased the activity of the parent plasmid, which contains no BPV-1 3′UTR fragment (Table 4, line 2). When this plasmid contained the RRE in addition, cotransfection with Rev increased CAT activity 2.9-fold but did not return CAT activity to the level of the parental vector without the extension (Table 4, line 3; Table 2, line 2). This result shows that the 624-bp β-galactosidase fragment contains sequences which inhibit expression by a mechanism not responsive to the Rev-RRE interaction. When the 624-bp extension and the RRE were present in the plasmid containing the 9-nt BPV-1 3′UTR fragment, coexpression of Rev restored the level of CAT activity to approximately 50% of that seen with the plasmid containing only the 624-bp β-galactosidase fragment and the RRE (Table 4, lines 3 and 6). Similarly, Rev functioned in the L1 cDNA expression system even when the RRE was cloned approximately 1,000 bp upstream of the 3′UTR 5′ splice site (Fig. 3 and 4A; Table 1). These data indicate that the interaction of Rev and the RRE can counteract the effect of the 3′ negative element even when the two elements are widely spaced. This observation suggests that Rev does not function only by steric interference with binding of factors to the 5′ splice site.

Two copies of the 5′ splice site act synergistically to inhibit CAT expression and this inhibition can be partially reversed by the Rev-RRE complex. Previous mapping of an inhibitory element in the HPV-16 late 3′UTR identified four overlapping weak 5′ splice sites, no one of which could account for the total inhibitory activity (22). This finding suggested that multiple 5′ splice sites could act synergistically to inhibit gene expression. To see if this was the case, an oligonucleotide containing two sense copies of a consensus 5′ splice site separated by 8 bp was cloned into the 3′UTR of the CAT expression vector. CAT expression from the resultant plasmid was minimal and increased little with prolonged incubation times (Fig. 7). In contrast, the CAT activity expressed from the plasmid containing only one 5′ splice site was at least eightfold higher (Fig. 7). The overall inhibition of CAT activity by two 5′ splice sites was approximately 5,000-fold (Table 5). These results suggest that multiple 5′ splice sites act synergistically. Coexpression of Rev increased CAT expression from the plasmid containing the RRE upstream of two consensus 5′ splice sites to approximately 25% of the level from a plasmid containing just the RRE (Table 5, lines 2 and 6). The Rev-RRE interaction caused an approximately 1,200-fold increase in expression from the plasmid with two 5′ splice sites.

DISCUSSION

Although the mechanisms of Rev function are still far from understood, there is a compelling body of evidence that at least one mode of Rev action involves interactions between Rev and components of the splicing machinery. This theory is supported by the data in this report, which show that cytoplasmic expression from vectors containing an inhibitory 5′ splice site within their 3′UTRs could be increased as much as 50-fold by the HIV-1 Rev protein in *trans* and the RRE in *cis* (Fig. 3). HIV-1 has suboptimal splice sites as well as splicing inhibitory elements which are responsible for incomplete splicing, leading to accumulation of unspliced and singly spliced mRNAs (1, 59). It has been suggested that the presence of suboptimal splice sites in these mRNAs is responsible for retention on spliceosomes in the nucleus (12). A 5′ splice site upstream of

TABLE 5. Effect of Rev on inhibition of CAT expression by two 5′ splice sites

Line[a]	5′ splice sites[b]	RRE[c]	n[d]	Relative CAT activity[e]		+Rev/−Rev[f]
				−Rev	+Rev	
1		−	12	100 ± 0	122.2 ± 70.5	1.2
2		+	12	23.0 ± 5.7	145.9 ± 67.6	6.3
3	1	−	12	0.4 ± 0.4	0.8 ± 0.9	2.0
4	1	+	12	2.1 ± 0.9	99.4 ± 34.0	47.3
5	2	−	4	0.02 ± 0.01	0.03 ± 0.02	1.5
6	2	+	4	0.03 ± 0.02	36.0 ± 10.2	1,200

[a] Plasmids used in these experiments were as follows: line 1, p2518; line 2, p2731; line 3, p2628; line 4, p2727; line 5, p2738; and line 6, p2739.

[b] Fragments containing one or two consensus 5′ splice sites (shown in Fig. 7) were cloned into the 3′UTR of the CAT expression vector p2518 (Fig. 2).

[c] The HIV-1 RRE was cloned immediately upstream of the fragment containing the 5′ splice sites (see Fig. 2).

[d] n, number of times the plasmids were assayed.

[e] The CAT expression vectors were cotransfected into HLtat cells with a β-galactosidase control plasmid (pCH110) and a CMV expression plasmid. The percentage of chloramphenicol acetylated was corrected for differences in transfection efficiency by using β-galactosidase activity and normalized to the activity of the parent CAT vector (p2518) in the absence of Rev. The results of multiple experiments were averaged, and the standard deviations were calculated. CAT plasmids were cotransfected with either pHCMVsrev (+Rev) or a CMV vector from which *rev* was deleted (−Rev).

[f] Fold activation of CAT expression by Rev.

the *env* ORF has been shown to be required for expression and Rev regulation of unspliced *env* mRNA (26, 35). Furthermore, the U1 snRNP, which normally recognizes 5′ splice sites at early stages of splicing, was shown to be required for Rev regulation of *env* mRNA (35). These experiments clearly show that 5′ splice sites can function as inhibitory elements which respond to the interaction of Rev and the RRE. Furthermore, experiments by Chang and Sharp (11) showed that mutation of either the 5′ or 3′ splice site of the β-globin gene led to nuclear retention and that this effect could be overcome by cloning the RRE into intron 2 and expression of Rev in *trans*. The data presented in this report also suggest that Rev inhibits splicing in vivo. Approximately equal levels of CAT mRNA accumulated in the cytoplasm in the presence of Rev in *trans* and the RRE in *cis* as in the absence of Rev and the RRE, but the mRNAs were predominantly unspliced, compared with the predominantly spliced mRNAs seen in the absence of Rev and the RRE (Fig. 6; Table 3). Taken together, these data suggest an inhibition of splicing as opposed to an alteration of transport of unspliced mRNAs. Rev is also able to inhibit the splicing of an RRE containing pre-mRNA in vitro (32). Furthermore, a basic peptide containing the RNA-binding domain of Rev is even more active at inhibiting in vitro splicing and blocks spliceosome assembly at a stage after binding of the U1 snRNP (32, 33). Although this peptide is lacking the activation domain of Rev, there is evidence that the Rev RNA-binding domain is required for more than binding the RRE. This domain is required in vivo for Rev function even when RNA binding is provided by a heterologous RNA-binding domain from the coat protein of the bacteriophage MS2 (42, 62). The basic RNA-binding domain has also been shown to interact with the murine protein YL2, which is the homolog of the human p32 protein (37). p32 binds to the splicing factor ASF/SF2, which has been shown to bind to 5′ splice sites, committing the pre-mRNA to splicing (20). Overexpression of YL2 potentiates the effect of Rev (37). This finding suggests that Rev may influence commitment complex formation. Additional evidence that Rev interacts with the splicing machinery comes from the observation that Rev colocalizes to nuclear speckles, which are thought to be active sites of transcription and RNA processing (31). These studies, together with the data presented in this report, suggest that the Rev-RRE interaction interferes with the assembly of spliceosome components on an inhibitory 5′ splice site located in a 3′UTR. Furthermore, although the U1 snRNP has been shown to be required in vivo for the inhibition of gene expression by a 3′UTR 5′ splice site (22), the in vitro studies of Kjems and Sharp (33) suggest that the binding of additional snRNPs may be necessary for the inhibition of gene expression.

The goal of this study was to identify common posttranscriptional regulatory mechanisms used by viruses. In particular, we have focused on the papillomaviruses because posttranscriptional regulation must play an important role in the regulation of papillomavirus gene expression. The complex posttranscriptional regulation of papillomavirus gene expression may allow full activation of viral early transcription before high-level production of viral antigen, providing a means of escaping the host immune response. BPV-1 capsid proteins are produced only in the most superficial and terminally differentiated keratinocytes (58). Several posttranscriptional mechanisms contribute to the early-to-late shift in viral gene expression and include the regulation of both splicing and polyadenylation. In previous studies, we have identified a consensus 5′ splice site in the BPV-1 late 3′UTR which posttranscriptionally inhibits viral late gene expression in nonpermissive cells (21, 22). Here we demonstrated that the inhibitory action of the 3′UTR 5′ splice site can be overcome by the interaction of the HIV-1 Rev protein and the RRE (Fig. 3 and 4A; Table 1). Furthermore, this 5′ splice site appears to be the only Rev/RRE-responsive negative posttranscriptional regulatory element present within the L1 mRNA since deletion of late 3′UTR sequences from an RRE-containing L1 cDNA expression vector eliminated Rev responsiveness (Fig. 4A; Table 1). It has been suggested that the inhibitory effect of the late 3′UTR element may be regulated by titration out of inhibitory factors due to the abundance of late pre-mRNA following vegetative viral DNA replication and activation of a strong late promoter (7, 22). Alternatively, the data presented here demonstrate that the inhibitory activity of the 3′UTR 5′ splice site may be regulated in differentiated keratinocytes by a viral or cellular factor which acts similarly to Rev by binding to some responsive sequence on the L1 pre-mRNA. Further studies are in progress to investigate these possibilities.

The demonstration that the activity of a papillomavirus posttranscriptional regulatory element can be regulated by an HIV-1 protein suggests that there are similar posttranscriptional regulatory mechanisms used by the two viruses. However, to the best of our knowledge, the data presented in this report provide the first evidence of a Rev/RRE-responsive inhibitory 5′ splice site located within a 3′UTR. The mechanism by which a 5′ splice site in this location affects RNA expression could be very different from that by which splice sites in other locations affect expression. However, any effect on expression which involves binding of splicing factors could be disrupted by Rev if Rev acts by stripping splicing factors from the pre-mRNA or preventing their binding in the first place.

The presence of inhibitory 5′ splice sites in papillomavirus late 3′UTRs is not restricted to BPV-1. Previous analysis of the HPV-16 late 3′UTR revealed an inhibitory element containing four overlapping weak 5′ splice sites, suggesting that multiple 5′ splice sites may have additive or synergistic effects. Here we extended this observation by demonstrating that the two consensus 5′ splice sites can act synergistically to inhibit gene expression (Fig. 7). Furthermore, this effect could be largely

overcome by Rev and the RRE, giving increases in expression of approximately 1,200-fold (Table 5).

Analysis of the effect of Rev on CAT expression from vectors containing both the 5′ splice site and the RRE in the 3′UTR showed that both CAT protein and mRNA levels were raised to levels comparable to those obtained with the parent vector containing no 5′ splice site (Tables 2 and 3). This finding suggests that Rev simply blocks the effect of the inhibitory 5′ splice site. The interaction of at least one large splicing factor, the U1 snRNP, has been shown to be required for the function of the inhibitory 5′ splice site (22). The proximity of the two elements in the CAT mRNA suggests that Rev could function by simple steric hindrance between Rev bound at the RRE and factors bound at the 5′ splice site. This mechanism is unlikely, however, since insertion of a 624-nt spacer between the RRE and the 5′ splice site did not eliminate Rev activation of CAT expression (Table 4, line 6). In addition, Rev activation of L1 mRNA expression appears to be equally effective whether the RRE is located 1,000 nt upstream of the inhibitory 5′ splice site or immediately adjacent to the 5′ splice site (Fig. 3; compare lanes 6 and 7). These results suggest a nonsteric interaction between Rev and splicing factors. An interesting discrepancy between the CAT and the L1 expression systems was noted. Rev in *trans* and the RRE in *cis* enhanced L1 mRNA expression as much as 50-fold, even though the 3′UTR 5′ splice site inhibits L1 mRNA expression only about 6-fold (Fig. 3; see reference 21). This enhancement of expression over the level of repression of the 3′UTR 5′ splice site was not seen in the CAT system. Expression from CAT plasmids containing the 3′UTR 5′ splice site could only be restored to the level of the parental plasmid with no 5′ splice site. The reason for this discrepancy between the results obtained with L1 cDNA expression vectors and CAT expression vectors is not understood. However, it is unlikely to be due to the presence of an additional *cis* element in the L1 mRNA since experiments in which the 5′ splice site was deleted from the L1 mRNA showed that no other Rev/RRE-responsive elements are present. One possibly relevant difference between these two expression systems is that the L1 mRNAs are unspliced whereas the CAT mRNAs are spliced.

Several mechanisms have been proposed for the inhibitory action of a 3′UTR 5′ splice site, all of which involve binding of splicing factors (22). These factors could interact directly with the polyadenylation machinery and inhibit polyadenylation. The U1 snRNP-A protein, for example, has been shown to have both positive and negative influences on polyadenylation, depending on how it is bound to the pre-mRNA (9, 23, 38, 61). Alternatively, a 5′ splice site can indirectly inhibit polyadenylation through interference with 3′-terminal exon definition. Recognition of a 3′-terminal exon as a unit appears to enhance the efficiency of polyadenylation (13, 28, 36, 44, 47). The coupling between splicing and polyadenylation is mediated by weak interactions between the U1 snRNP and regions of the pre-mRNA upstream of the polyadenylation site which have limited complementarity with the 5′ end of the U1 small nuclear RNA (63). Strong interactions between the U1 snRNP and a 5′ splice site located in a 3′UTR, however, have been shown to depress polyadenylation both in vivo and in vitro, accompanied by depression of binding of the polyadenylation factor cleavage stimulation factor (CstF) (46). Either mechanism could be disrupted by Rev if Rev acts by stripping these factors from the pre-mRNA or preventing their binding in the first place.

Another possibility is that a 5′ splice site located in the 3′UTR blocks nucleocytoplasmic transport by trapping the mRNA on nonproductive spliceosomes. This is similar to the nuclear retention of intron containing β-globin pre-mRNAs seen when single splice sites are mutated (11). Again, Rev could facilitate nucleocytoplasmic transport by either preventing spliceosomal components from binding to the pre-mRNA or stripping these factors off the pre-mRNA. It has also been proposed that Rev plays a more active role in nucleocytoplasmic transport by shunting the pre-mRNA into a transport pathway which is independent of the splicing machinery (12, 19, 54). This mechanism is suggested by the fact that Rev shuttles between the nucleus and cytoplasm (43). It is generally felt that splicing and polyadenylation are cotranscriptional events. If an alternative transport pathway does exist, then the pre-mRNA must be polyadenylated before entering this pathway since unspliced mRNAs expressed in the presence of Rev are still polyadenylated (Fig. 3 and 4). The steady-state levels of polyadenylated cytoplasmic and nuclear L1 mRNA were assayed in transfection studies to determine if BPV-1 L1 mRNAs are retained in the nucleus. Results from multiple transfections indicated that in the absence of the RRE, cytoplasmic levels of polyadenylated L1 mRNA were at least 5- to 10-fold higher than nuclear levels, suggesting that wild-type L1 mRNAs are not preferentially retained in the nucleus (Fig. 4B). We cannot rule out, however, that L1 mRNA is retained in the nucleus and rapidly degraded.

It has been suggested that recognition of the HIV-1 *env* region as an intron is necessary for regulation of the *env* gene by Rev (26). In our study, the BPV-1 L1 cDNA was fully Rev responsive despite a lack of introns, although cryptic 3′ splice sites could theoretically exist in the expression vector, creating potential introns. However, no mRNAs which utilize the 5′ splice site present in the late 3′UTR of BPV-1 have ever been identified (22). Other studies have shown that expression of the HIV-1 *gag* (8, 17, 19) and *env* (24, 40, 45) genes are Rev responsive in the absence of functional splice sites. Although these studies have not ruled out the presence of pseudo-splice sites or the binding of other factors involved in splicing, it seems that Rev has other effects than inhibiting active splicing of RNA.

The Rev protein has also been shown to regulate the stability of HIV-1 RNAs (19, 54). As mentioned above, the BPV-1 L1 mRNA does not accumulate in the nucleus, suggesting that this transcript is unstable. We do not see a significant increase in the nuclear pool of either polyadenylated or nonpolyadenylated L1 RNA in the presence of Rev and the RRE. However, we cannot state with certainty that nuclear stability is not increased since a concomitant increase in the rate of nucleocytoplasmic transport could deplete the nucleus of these RNAs. Cytoplasmic L1 mRNAs have a relatively long half-life (5.5 h) which is not significantly affected by the 3′UTR 5′ splice site (21). Therefore, it is unlikely that Rev acts primarily by increasing cytoplasmic L1 mRNA stability.

We observed in the CAT system that the magnitude of the effect of the 3′UTR 5′ splice site was greater at the protein level (>100-fold) than at the mRNA level (37-fold) (Tables 2 and 3). Rev restored the expression of both CAT protein (47- to 50-fold effect) and mRNA (20-fold effect) close to the levels of the parent plasmid. One possible explanation for the lesser effect on RNA may be that CAT enzyme assays can be more accurately quantitated over a wide range of activities whereas quantitation of very low levels of signal from the Northern blots is less accurate. The observation that interaction of Rev and the RRE dramatically increases the fraction of unspliced CAT mRNA in the cytoplasm (Fig. 6) raised the possibility that this difference is due to differences in translational efficiency of spliced and unspliced CAT mRNAs. However, the levels of both CAT mRNA and protein expressed in the pres-

ence of Rev from the CAT vector containing an RRE and the levels of CAT mRNA and protein expressed from the control vector in the absence of Rev were comparable even though these plasmids express predominantly unspliced and spliced mRNAs, respectively. These data suggest that spliced and unspliced CAT mRNAs are translated with comparable efficiencies (Tables 2 and 3). Rev has been shown to promote the polysomal loading and translation of HIV-1 mRNAs (17, 50). If Rev acts on many steps along the pathway of expression, effects at the protein level might be expected to be greater than those at the RNA level. The possibility therefore exists that the 3′UTR 5′ splice site also has a small effect on polysomal loading and/or translation of mRNAs and that this effect is also reversed by the interaction of Rev and the RRE.

Several studies have identified CRS/INS elements in the HIV-1 RRE (10, 45). Our data are consistent with these studies since the presence of only the RRE in the 3′UTR of the CAT vector consistently inhibited expression of both CAT mRNA and protein approximately four- to fivefold (Tables 2 and 3). This inhibition was also overcome by Rev as would be expected for inhibition by a CRS/INS element. Surprisingly, a 5′ splice site plus an RRE was no more inhibitory than a 5′ splice site alone (Table 2, lines 6 to 8), and in fact the RRE in the absence of Rev increased expression from vectors approximately two- to fivefold. While two 5′ splice sites act synergistically to inhibit expression (Fig. 7; Table 5), there is not a synergistic inhibition of expression with an RRE and a 5′ splice site, suggesting that the CRS element in the RRE acts by a different mechanism from a 5′ splice site.

The data presented here may be useful in the design of vectors for the gene therapy of AIDS. As shown above, we have been able to inhibit CAT expression at least 5,000-fold by insertion of two 5′ splice sites into the 3′UTR of a CAT expression vector. Addition of the RRE to these vectors and coexpression of Rev-induced CAT expression 1,200-fold. We are currently investigating whether the expression of other genes can be regulated in a similar manner. If so, this system could be useful for limiting expression of other genes to HIV-1-infected cells. Several strategies for the gene therapy of AIDS have been proposed, including the expression of various toxins and transdominant mutant HIV gene products in HIV-1-infected cells which would either kill infected cells or limit replication and spread of the virus (16, 51, 57). Expression of toxin genes would have to be very tightly restricted to HIV infected cells to prevent general cytotoxicity. Likewise, constitutive expression of HIV transdominant mutant proteins could lead to an immune response against the cells expressing the proteins.

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