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Human Immunodeficiency Virus *env* Expression Becomes Rev-Independent if the *env* Region Is Not Defined as an Intron

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The human immunodeficiency virus (HIV) Rev protein functions to facilitate export of intron-containing HIV mRNA from the nucleus to the cytoplasm. We have previously shown that splice site recognition plays an important role in Rev regulation of HIV *env* expression. Here we have further analyzed the effects of splice sites on HIV *env* expression and Rev regulation, using a simian virus 40 late replacement vector system. *env* expression from the vector became completely Rev-independent when an excisable intron was positioned upstream of the *env* region, provided that *env* was not recognized as an intron. Complete Rev regulation was restored either by the insertion of a 5′ splice site between the intron and the *env* open reading frame or by deletion of the 3′ splice site of the upstream intron. These results show that 5′ splice sites can function as *cis*-acting represssor sequence (CRS) elements to retain RNA in the nucleus in the absence of Rev. They also indicate that Rev regulation of HIV *env* expression is critically dependent on whether the *env* region is defined as an intron. This strengthens the hypothesis that Rev interacts with components of the splicing machinery to release splicing factors and enable export of the mRNA before splicing occurs.

Human immunodeficiency virus (HIV) is a complex retrovirus belonging to the lentivirus subfamily (14). Analysis of the RNA in HIV-infected cells has shown that a multitude of different HIV-specific mRNAs are expressed. To date, more than 30 different mRNAs have been identified (36–38). These are all derived from a genome-length primary transcript through alternative splicing. Many of the mRNAs, such as those used to express the regulatory proteins Tat and Rev, are multiply spliced. In contrast, the RNA used to express the Gag and Pol proteins is completely unspliced, whereas the RNAs for the other structural proteins are singly spliced (2, 30). Splicing in the HIV system thus has to be carefully regulated to maintain a proper balance between the different mRNAs.

As a rule, introns are removed from eukaryotic RNAs before the mRNA is exported out of the nucleus for translation. In a study by Chang and Sharp (4), which used a construct containing a rabbit β-globin intron, it was shown that mutation of either the 5′ or 3′ splice site inhibited splicing and led to the accumulation of intron-containing RNA in the nucleus. However, if both splice sites were mutated, the unspliced RNA was efficiently exported to the cytoplasm. These results suggested that splicing factors bound either to the 5′ or 3′ splice site functioned to retain incompletely spliced RNAs in the nucleus. Similar conclusions have also been reached from studies in yeast cells (24).

In the HIV system, all of the structural proteins are made from incompletely spliced RNAs which contain complete introns (30, 37). The HIV Rev protein appears to be a crucial part of the mechanism that allows these RNAs to be exported to the cytoplasm. Rev is a small phosphorylated protein (5, 20) encoded within the viral genome (10, 42), and several studies have shown that Rev promotes export from the nucleus of intron-containing HIV RNAs (9, 12, 17, 29). Rev binds to an element present in these RNAs known as the Rev-responsive element (RRE) (8, 16, 17, 29, 34, 46). A role for Rev in the export of RNA was further established by using the mutated β-globin constructs described above, which produced RNA that was retained in the nucleus (4). If the RRE was inserted into the intron of these constructs, unspliced RNA was exported to the cytoplasm in the presence of Rev.

In a previous study, we showed that Rev-dependent HIV *env* expression from a simian virus 40 (SV40) late replacement vector required a 5′ splice site positioned immediately upstream of the *env* open reading frame (ORF) (27). Experiments using mutated 5′ splice sites and U1 small nuclear RNAs (snRNAs) carrying compensatory mutations demonstrated that the *env* mRNA produced from this vector formed a complex with U1 snRNA, even though it remained completely unspliced. These experiments, together with the results of Chang and Sharp (4), suggested that Rev may interact directly with components of the splicing machinery to resolve splicing complexes and enable export from the nucleus of unspliced RNA.

In the experiments reported here, we have further analyzed the effect of splice sites on HIV *env* expression and Rev regulation. Our results show that *env* mRNA can be efficiently exported from the nucleus in the absence of Rev, provided that the *env* region is not defined as an intron.

MATERIALS AND METHODS

Cells and transfections. CMT3 cells (13) were maintained in Iscove's medium supplemented with 10% calf serum. Cells were transfected by using a modification of the DEAE-dextran method as previously described (18).

Plasmids. pβGenv was constructed by inserting an *Xba*I-*Xho*I fragment containing the HIV-1 *env* gene from plasmid pJTX (27) into the unique *Bgl*II site of the vector pBABY (32) after both fragment and vector had been repaired with T4 polymerase.

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pβGenv-3′Δ was constructed from pβGenv by deletion of a 141-bp *Apa*I-*Bal*I fragment containing the rabbit β-globin 3′ splice site. To make this plasmid, pβGenv was cleaved with *Apa*I and *Bal*I and repaired with T4 polymerase before religation.

pβGenv-5′ins was constructed similarly to pβGenv. In this construct, the fragment containing the HIV *env* region was created from a derivative of pJTX. In this derivative, an oligonucleotide containing a 24-bp sequence homologous to the 5′ splice site of the rabbit β-globin second intron had been inserted into the *Xba*I site just upstream of the *env* gene as previously described (27). The *env* region containing the upstream 5′ β-globin splice site was excised as a *Sal*I-*Xho*I fragment, which was subsequently repaired with T4 DNA polymerase and cloned into the repaired *Bgl*II site of pBABY.

pβGenv-tev 5′ mt is a mutant of pβGenv containing a 1-bp mutation (G to A) in the +1 position of the *tev* 5′ splice site. This changed a glycine to an aspartic acid residue within gp120 at the site of the mutation. It was constructed by the splicing-by-overlap-extension–PCR method (21, 22) using two mutagenic primers and two primers outside of *Kpn*I and *Bgl*II sites in pβGenv. The final PCR product was cleaved with *Kpn*I and *Bgl*II to yield a 690-bp fragment, which was exchanged for the corresponding wild-type fragment in pβGenv. The region between the *Kpn*I and *Bgl*II sites of the resulting plasmid was sequenced to verify that it contained only the desired mutation.

Plasmid pCMVrev was previously known as pRev1, and its construction has been previously described (41). pCMVrev$^-$ was created from this plasmid by cleavage with *Bam*HI at a unique site within the *rev* coding region, T4 DNA polymerase repair, and religation. pCMVrev$^-$ expresses a truncated nonfunctional Rev protein.

Protein analysis. Western blot (immunoblot) analysis was performed on extracts of cells harvested 65 to 70 h posttransfection as described previously (17, 18). The first antibody used was a gp120-specific polyclonal rabbit antibody made against a purified fragment of gp120 (amino acids 343 to 512) expressed in bacteria.

RNA extraction and analysis. For total poly(A)$^+$ RNA purification, each of three 150-mm-diameter plates of cells was transfected with 15 μg of each plasmid. Cells were harvested at 48 h posttransfection, and the plates were washed twice with 15 ml of cold phosphate-buffered saline (PBS). The cells were lysed directly on each plate in 3.3 ml of lysis buffer containing 0.2 M Tris-HCl (pH 7.5), 0.2 M NaCl, 1.5 mM $MgCl_2$, 2% sodium dodecyl sulfate (SDS), and 200 μg of proteinase K per ml. The cell lysates from the three plates were combined and passed through a 21-gauge needle until the viscosity resembled that of lysis buffer alone. The lysate was then incubated at 45°C for 1 to 2 h. For poly(A) selection, the final salt concentration of the lysate was increased to 0.5 M NaCl. Poly(A)$^+$ RNA was then isolated from the lysate by incubation at room temperature for 1 h with 30 mg of oligo(dT)-cellulose (Pharmacia) which had been preequilibrated in binding buffer (10 mM Tris-HCl [pH 7.5], 0.5 M NaCl). The oligo(dT)-cellulose was then washed three times with 10 ml of binding buffer, resuspended in a small volume of binding buffer, and transferred to an insert cup assembly containing a 0.45-μm-pore-size filter (Millipore). It was then spin filtered in an Eppendorf centrifuge and washed with multiple washes of 400 μl of binding buffer until the flowthrough liquid had an optical density at 260 nm (OD_{260}) of less than 0.05. Poly(A)$^+$ RNA was eluted from the oligo(dT)-cellulose sequentially with 200 and 150 μl of elution buffer (10 mM Tris-Hcl [pH 7.5]). The eluates were pooled, and the yield and purity of the RNA were determined by an OD_{260}/OD_{280} reading. Usually 3 to 5 μg of total poly(A)$^+$ RNA was used for Northern (RNA) blot hybridization analysis.

For cytoplasmic poly(A)$^+$ RNA purification, five 150-mm-diameter plates of cells were transfected with 15 μg of each plasmid. At 48 h posttransfection, after each plate was washed twice with 15 ml of cold PBS, the cells were scraped from the plates in 5 ml of cold PBS and pooled. The cells were then pelleted and resuspended in 3 ml of reticulocyte standard buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 1.5 mM $MgCl_2$); 5% Nonidet P-40 was added dropwise to a final concentration of 0.5%. The cell suspension was gently vortexed and incubated on ice for 5 min to lyse the cell membranes. Nuclei were isolated by centrifugation at 2,000 rpm in an IEC PR-7000 centrifuge for 5 min at 4°C and discarded. The supernatant was again cleared of any remaining nuclei by centrifugation at 2,000 rpm for 5 min at 4°C. An equal volume of 2× PK buffer (200 mM Tris-HCl [pH 7.5], 25 mM EDTA, 300 mM NaCl, 2% SDS, 400 μg of proteinase K per ml) was added to the resulting supernatant, which was incubated at 37°C for 30 min with gentle shaking. The lysate was extracted once with an equal volume of phenol–chloroform-isoamyl alcohol (24:1) and then twice with an equal volume of only chloroform-isoamyl alcohol (24:1). Sodium acetate (3 M, pH 5.2) was added to a final concentration of 0.3 M, and the RNA was precipitated with 2.5 volumes of absolute ethanol (−20°C). The ethanol precipitate was stored at −20°C. For poly(A) selection, the RNA was pelleted at 4,000 rpm in an IEC PR-7000 centrifuge at 4°C for 1 h. The pellet was gently resuspended in 10 mM Tris-HCl (pH 7.4)–10 mM EDTA–0.2% SDS and heated to 65°C for 5 min. The salt concentration was adjusted to 0.5 M with NaCl, and 30 mg of preequilibrated oligo(dT)-cellulose in 1 ml of binding buffer was added as described for total poly(A)$^+$ RNA isolation. The poly(A) selection was completed by the procedure described for total poly(A)$^+$ RNA preparation; 3 to 5 μg of cytoplasmic poly(A)$^+$ RNA was used for Northern blot hybridization analysis.

RNA electrophoresis on formaldehyde-containing 1% agarose gels and Northern blot analysis were performed as previously described (17).

RESULTS

A complete intron positioned upstream of the HIV *env* ORF makes *env* expression partially Rev-independent. As described in the introduction, our previous results suggested that Rev regulation may take place within the context of the cellular splicing machinery. In addition, our experiments showed that no stable *env* RNA was formed, with or without Rev, if the RNA expressed from our SV40 expression vector was not recognized by splicing factors. Removal of the 5′ splice site just upstream of the *env* ORF caused the RNA to become unstable (27). In view of these results, we decided to test the effect on RNA formation and Rev regulation of placing a complete rabbit β-globin intron upstream of the *env* ORF. We reasoned that the presence of this intron might stabilize the RNA and allow it to be spliced without removing any part of the *env* region. This would allow us to specifically study the effects of splice sites on Rev regulation separate from their effects on stability of the RNA.

The construction of the new plasmid was easily achieved by inserting the HIV fragment containing the *env* ORF, but lacking a 5′ splice site, into a unique *Bgl*II restriction enzyme site present in our expression vector, pBABY (32). This created plasmid pβGenv, which contained the *env* ORF positioned between the rabbit β-globin intron and poly(A) sequences. These sequences were immediately downstream from

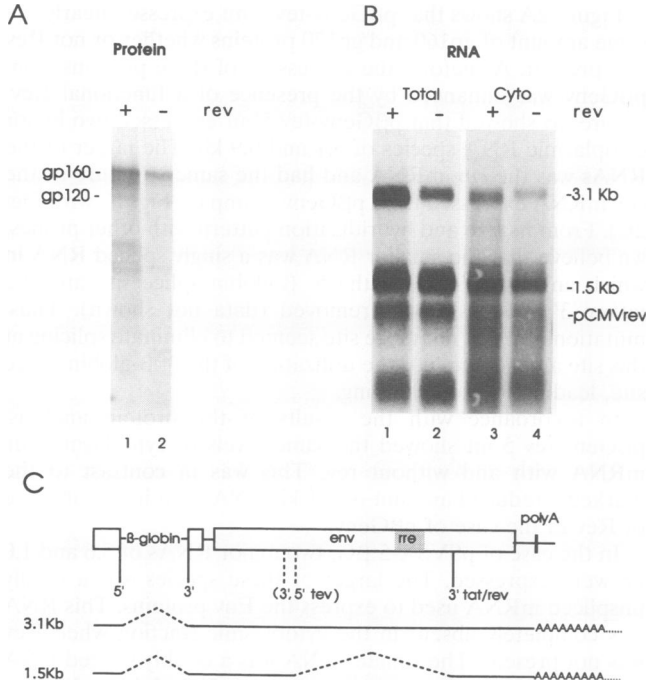

FIG. 1. Protein and RNA expression from plasmid pβGenv. (A) Western blot. Extracts from 5×10^5 CMT3 cells transfected with pβGenv and pCMVrev (lane 1) or pβGenv alone (lane 2) were subjected to electrophoresis on a 15.5% polyacrylamide gel, which was subsequently electroblotted to Immobilon-P. The Western blot was developed with a rabbit gp120-specific first antibody and an alkaline phosphatase-conjugated second antibody. (B) Northern blot. Three micrograms of poly(A)-selected total RNA (lanes 1 and 2) or poly(A)-selected cytoplasmic RNA (lanes 3 and 4) from cells cotransfected with pBGenv and pCMVrev$^-$ (lanes 2 and 4) or with pβGenv and pCMVrev (lanes 1 and 3) was subjected to electrophoresis on a formaldehyde-containing 1% agarose gel. The gel was subsequently blotted to Nitroplus 2000. The blot was probed with an oligonucleotide which was complementary to the RNA just upstream of the β-globin poly(A) site. (C) Schematic diagram of the region of pβGenv downstream from the SV40 late promoter. Vertical lines indicate the positions of 5′ and 3′ splice sites. Boxed areas indicate the β-globin and *env* ORFs. Also shown are the structures of the 3.1- and 1.5-kb RNAs found in cells transfected with this plasmid.

the SV40 late promoter. A schematic representation of this region of the plasmid, as well as all of the known splice sites, is shown in Fig. 1C. In addition to the β-globin 5′ and 3′ splice sites, the region contains the *tat/rev* 3′ splice site and the *tev* (or *tnv*) 3′ and 5′ splice sites. The *tev* sites are alternative splice sites within the HIV *env* region which have been shown to be used only in a small percentage of the RNA expressed in HIV-infected cells (3, 15, 35, 36).

To examine envelope protein expression, pβGenv was transfected into monkey CMT3 cells either alone or together with a plasmid which expressed a functional Rev protein (pCMVrev). The transfected cells were then subjected to Western blot analysis using an Env-specific serum. As can be seen (Fig. 1A), both gp160 and gp120 were expressed in the presence or absence of Rev. However, levels of expression were higher in the presence of Rev.

We next performed an analysis of the RNA expressed from pβGenv. To do this, cells were cotransfected with pβGenv and pCMVrev or its derivative, pCMVrev$^-$, which contains a frameshift mutation in the second coding exon of Rev. Both pCMVrev and pCMVrev$^-$ express a *rev* mRNA of about 950 bases, but pCMVrev$^-$ produces a nonfunctional truncated protein of 59 amino acids (data not shown). Poly(A) containing total or cytoplasmic RNA was isolated from the cells at 48 h after transfection. The RNA was then subjected to Northern blot analysis, using an oligonucleotide probe homologous to the rabbit β-globin sequences just upstream of the poly(A) site in pβGenv. This probe would be expected to hybridize to all of the mRNAs expressed from the late SV40 promoter of pβGenv as well as to the mRNA from pCMVrev and pCMVrev$^-$. The result of the Northern blot analysis is shown in Fig. 1B.

Two major species of mRNA, 3.1 and 1.5 kb in size, were observed in both the total and cytoplasmic RNA preparations. In addition, bands representing the 0.95-kb mRNA from pCMVrev and pCMVrev$^-$ were detected. Figure 1C shows the structures of the 3.1- and 1.5-kb RNAs. These structures were determined by further Northern blot analysis using additional oligonucleotide probes and by sequence analysis of PCR-amplified cDNAs (data not shown). As can be seen in Fig. 1C, both RNAs lacked the β-globin intron. No RNA containing this intron was detectable by Northern blot analysis even in the total RNA preparations, suggesting that the intron was rapidly and efficiently removed from the primary transcript whether or not Rev was present.

The 3.1-kb RNA contained a complete *env* ORF and was thus the mRNA used to express the Env protein. This RNA was present in approximately the same amounts in both preparations of total RNA. In contrast, its level was higher in the cytoplasmic preparations from cells which contained a functional Rev protein. However, a significant amount was still detectable even when a functional Rev protein was lacking. This result is consistent with the results of the protein analysis which showed a partial Rev independence. Thus, the singly spliced *env* mRNA appeared to be exported from the nucleus, albeit less efficiently, even in the absence of the Rev protein. The slightly larger size of this RNA species in the absence of Rev was due to a longer polyadenylate tract at the 3′ end of this RNA (data not shown).

The smaller 1.5-kb mRNA was determined to be a doubly spliced RNA in which an additional intron within the *env* region had been removed. The 5′ splice site used in this splicing event was the previously described 5′ *tev* splice site, whereas the 3′ splice site was the *tat/rev* 3′ splice site. This intron was thus identical to the third intron of the *tev* mRNA found in infected cells (3, 35). In total RNA preparations, this species appeared more prominent in the absence of the Rev protein. However, its levels in the cytoplasmic RNA preparations were similar whether or not Rev was present.

As a control for possible variation in transfection efficiency and loading, the Northern blots were stripped and reprobed with an oligonucleotide specific for the pCMVrev and pCMVrev$^-$ mRNAs. The observed levels of these RNAs were similar in each total and cytoplasmic lane (data not shown).

Mutation of the 5′ *tev* splice site makes *env* expression from pβGenv completely Rev-independent. Since *env* expression from pβGenv was more efficient in the presence of Rev, it seemed likely that some property of the 3.1-kb *env* mRNA kept it partially Rev-dependent. Some of this RNA was further spliced by using the *tev* 5′ splice site. This raised the possibility that binding of splicing factors to this splice site caused some of the RNA to be retained in the nucleus when Rev was not present. If so, we reasoned that mutation of the splice site might make *env* expression from pβGenv totally Rev-independent. To test this hypothesis, we used mutagenic oligonucle-

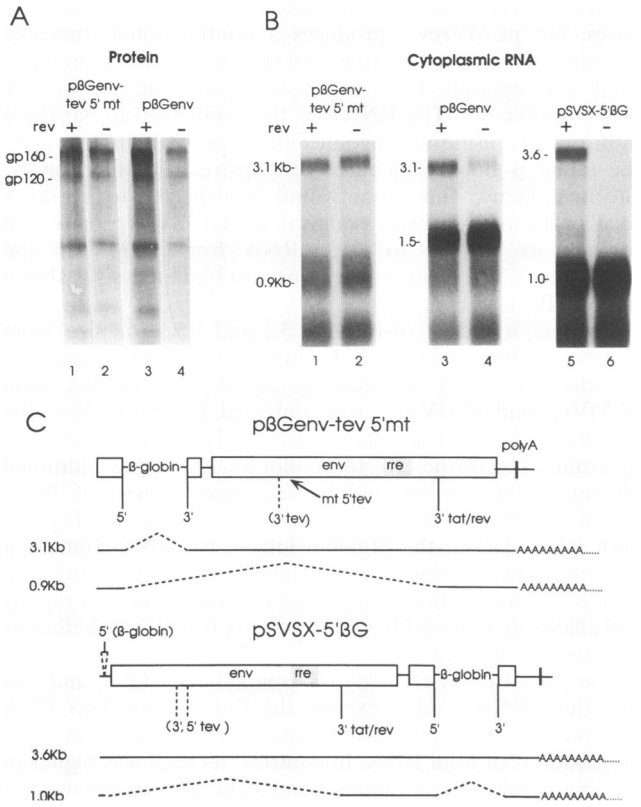

FIG. 2. Expression from plasmids pβGenv-tev-5′mt and pSVSX-5′βG. (A) Western blot. Extracts from 5×10^5 CMT3 cells transfected with pβGenv-tev 5′mt (lanes 1 and 2) or pβGenv (lanes 3 and 4), with (lanes 1 and 3) or without (lanes 2 and 4) pCMVrev, were subjected to electrophoresis on a 15.5% polyacrylamide gel, which was subsequently electroblotted to Immobilon-P. The Western blot was developed with a rabbit gp120-specific first antibody and an alkaline phosphatase-conjugated second antibody. (B) Northern blot. Three micrograms of poly(A)-selected cytoplasmic RNA from cells transfected with pβGenv-tev 5′mt (lanes 1 and 2), pβGenv (lanes 3 and 4), or pSVSX-5′βG, with pCMVrev (lanes 1, 3, and 5) or with pCMVrev$^-$ (lanes 2, 4, and 6), was subjected to electrophoresis on a formaldehyde-containing 1% agarose gel. The gel was subsequently blotted to Nitroplus 2000. The blot was probed with an oligonucleotide which was complementary to the RNA just upstream of the β-globin poly(A) site. (C) Schematic diagrams of the regions in pβGenv-tev 5′mt and pSVSX-5′βG downstream from the SV40 late promoter. Vertical lines indicate the positions of 5′ and 3′ splice sites. Boxed areas indicate the β-globin and *env* ORFs. Also shown are the structures of the 3.1- and 0.9-kb RNAs found in cells transfected with pβGenv-tev 5′mt and the 3.6- and 1.0-kb RNAs found in cells transfected with pSVSX-5′βG.

otides to mutate the +1 position of the 5′ *tev* splice site from a G to an A (see Materials and Methods).

The analyses of the proteins and cytoplasmic RNA that were expressed from this plasmid, with and without Rev, are shown in Fig. 2A and B. In this experiment, expression from pβGenv-tev 5′mt was directly compared with expression from pβGenv. As a control, we also examined the cytoplasmic RNA produced from pSVSX-5′βG, a plasmid that previously was shown to be completely Rev-dependent for *env* expression (26). pSVSX-5′βG is similar to our original Rev-dependent plasmid construction pSVSX1Δ1 (17). It contains just the 5′ β-globin splice site upstream of the *env* ORF rather than a complete β-globin intron. However, it contains a complete β-globin intron downstream from the *env* ORF (Fig. 2C).

Figure 2A shows that pβGenv-tev 5′mt expressed nearly the same amount of gp160 and gp120 proteins whether or not Rev was present. As before, the expression of these proteins from pβGenv was enhanced by the presence of a functional Rev. Figure 2B showed that pβGenv-tev 5′mt expressed two major cytoplasmic RNA species of 3.1 and 0.9 kb. The larger of the RNAs was the *env* mRNA and had the same structure as the *env* mRNA expressed from pβGenv (compare Fig. 1C with Fig. 2C). From its size and hybridization pattern with other probes, we believe that the smaller RNA was a singly spliced RNA in which an intron between the 5′ β-globin splice site and the *tat/rev* 3′ splice site was removed (data not shown). Thus, mutation of the 5′ *tev* splice site seemed to eliminate splicing at this site and also reduce the utilization of the 3′ β-globin splice site, leading to exon skipping.

In accordance with the results of the protein analysis, pβGenv-tev 5′mt showed the same levels of cytoplasmic *env* mRNA with and without rev. This was in contrast to the markedly reduced amount of 3.1-kb RNA seen in the absence of Rev in the case of pβGenv.

In the case of pSVSX-5′βG, two major RNAs of 3.6 and 1.0 kb were expressed. The larger of these species was a totally unspliced mRNA used to express the Env proteins. This RNA was completely absent in the cytoplasmic fraction when Rev was not present. The smaller RNA was a doubly spliced RNA in which an intron between the upstream 5′ β-globin splice site and the 3′ *tat/rev* splice site, as well as the downstream β-globin intron, was removed. Interestingly, no RNA which had only the downstream intron removed was observed. Thus, the downstream intron is recognized only once the upstream intron is removed, and nuclear export of the unspliced *env* mRNA is totally Rev-dependent. These data are consistent with our previously reported results for similar constructs (17, 27).

As before, as a control for possible variation in transfection efficiency and loading, the Northern blots were stripped and reprobed with an oligonucleotide specific for the pCMVrev and pCMVrev$^-$ mRNAs. The observed levels of these RNAs were similar in each of the total and cytoplasmic lanes (data not shown).

Deletion of the upstream 3′ splice site or insertion of an additional 5′ splice site restores Rev dependence. The results presented above suggested that Rev dependence of *env* expression was directly related to whether or not the *env* ORF was recognized as an intron. In addition, our previously published results for constructs analogous to pSVSX-5′βG showed that the *env* RNA interacted with U1 snRNA even though it remained unspliced (27). Thus, the complete Rev dependence seen in these constructs could be explained by the fact that the intron covering most of the *env* ORF was recognized 100% of the time by U1 snRNA and possibly other splicing factors. Reasoning along these same lines, the partial Rev dependence of pβGenv could be explained by the fact that the *tev* intron is an alternative intron which may be recognized by splicing factors only some of the time. Thus, abolition of this intron in pβGenv-tev 5′mt would be expected to result in the observed shift from partial Rev dependence to total Rev independence.

To further test the hypothesis that Rev dependence was determined by whether or not the *env* region was defined as an intron, we decided to insert an extra 5′ splice site between the rabbit β-globin intron and the *env* ORF of pβGenv. To do this, a small oligonucleotide homologous to the rabbit β-globin 5′ site was inserted upstream of the *env* ORF in pβGenv to create plasmid pβGenv-5′ins. This placed most of the *env* ORF within a new intron between the β-globin 5′ splice site and the *tat/rev* 3′ splice site. This intron would be expected to be recognized

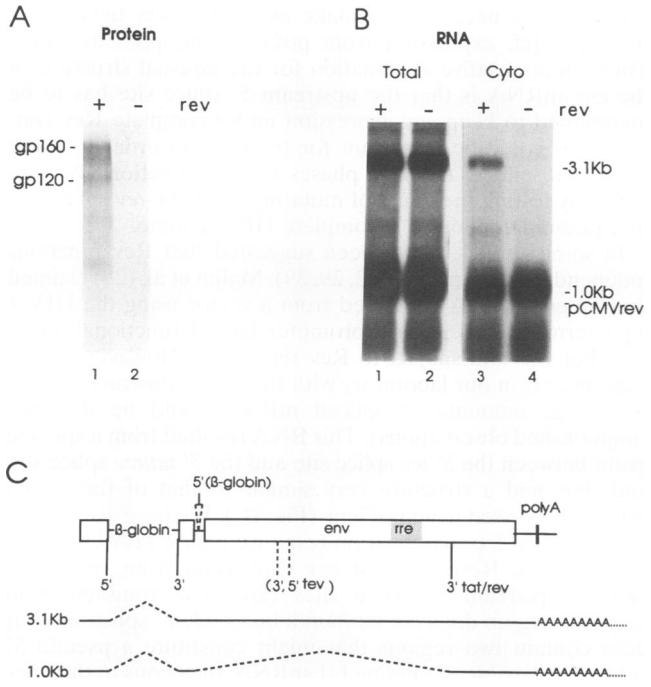

FIG. 3. Protein and RNA expression from plasmid pβGenv-5'ins. (A) Western blot. Extracts from 5×10^5 CMT3 cells transfected with pβGenv-5'ins and pCMVrev (lane 1) or pβGenv-5'ins alone (lane 2) were subjected to electrophoresis on a 15.5% polyacrylamide gel, which was subsequently electroblotted to Immobilon-P. The Western blot was developed with a rabbit gp120-specific first antibody and an alkaline phosphatase-conjugated second antibody. (B) Northern blot. Three micrograms of poly(A)-selected total RNA (lanes 1 and 2) or poly(A)-selected cytoplasmic RNA (lanes 3 and 4) from cells cotransfected with pβGenv-5'ins and pCMVrev$^-$ (lanes 2 and 4) or with pβGenv-5'ins and pCMVrev (lanes 1 and 3) was subjected to electrophoresis on a formaldehyde-containing 1% agarose gel. The gel was subsequently blotted to Nitroplus 2000. The blot was probed with an oligonucleotide which was complementary to the RNA just upstream of the β-globin poly(A) site. (C) Schematic diagram of the region of pβGenv-5'ins downstream from the SV40 late promoter. Vertical lines indicate the positions of 5' and 3' splice sites. Boxed areas indicate the β-globin and *env* ORFs. Also shown are the structures of the 3.1- and 1.0-kb RNAs found in cells transfected with this plasmid.

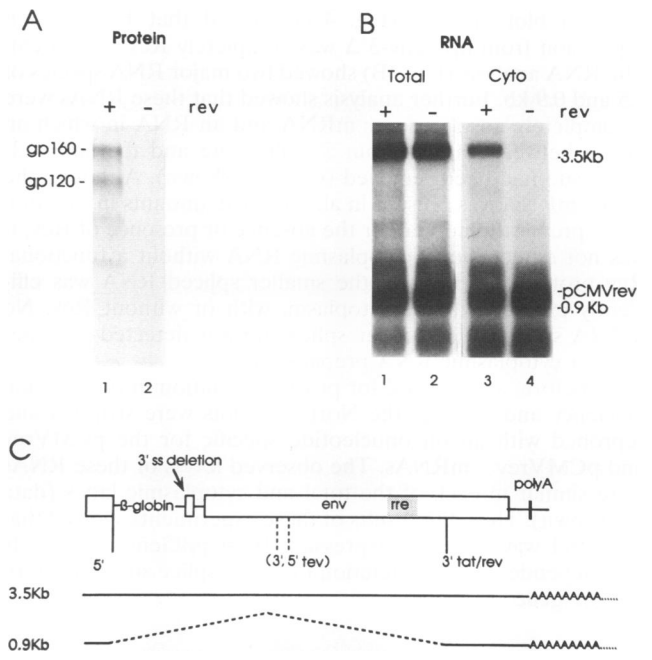

FIG. 4. Protein and RNA expression from plasmid pβGenv-3'Δ. (A) Western blot. Extracts from 5×10^5 CMT3 cells transfected with pβGenv-3'Δ and pCMVrev (lane 1) or pβGenv-3'Δ alone (lane 2) were subjected to electrophoresis on a 15.5% polyacrylamide gel, which was subsequently electroblotted to Immobilon-P. The Western blot was developed with a rabbit gp120-specific first antibody and an alkaline phosphatase-conjugated second antibody. (B) Northern blot. Three micrograms of poly(A)-selected total RNA (lanes 1 and 2) or poly(A)-selected cytoplasmic RNA (lanes 3 and 4) from cells cotransfected with pβGenv-3'Δ and pCMVrev$^-$ (lanes 2 and 4) or with pβGenv-3'Δ and pCMVrev (lanes 1 and 3) was subjected to electrophoresis on a formaldehyde-containing 1% agarose gel. The gel was subsequently blotted to Nitroplus 2000. The blot was probed with an oligonucleotide which was complementary to the RNA just upstream of the β-globin poly(A) site. (C) Schematic diagram of the region of pβGenv-3'Δ downstream from the SV40 late promoter. Vertical lines indicate the positions of 5' and 3' splice sites. Boxed areas indicate the β-globin and *env* ORFs. Also shown are the structures of the 3.5- and 0.9-kb RNAs found in cells transfected with this plasmid.

efficiently by splicing factors. A schematic representation of the relevant region of the plasmid is shown in Fig. 3C.

Analysis of protein and RNA expression from pβGenv-5'ins was carried out in experiments similar to those performed with the other plasmids. A Western blot of extracts of cells transfected with pβGenv-5'ins (Fig. 3A) showed that gp160 and gp120 expression was completely Rev-dependent. The RNA analysis showed that the *env* mRNA expressed from pβGenv-5'ins was 3.1 kb in size and was similar in structure to the *env* mRNA expressed from pβGenv. With this construct, however, the RNA was not present in the cytoplasm unless a functional Rev protein was present (Fig. 3B). A smaller mRNA of about 1 kb was efficiently transported out of the nucleus both in the absence and in the presence of Rev. Further analysis showed that this mRNA was a doubly spliced RNA in which both the rabbit β-globin intron and an intron between the inserted 5' β-globin splice site and the *tat/rev* 3' splice site had been removed (data not shown). No mRNA spliced at the 5' *tev* splice site was detected in either total or cytoplasmic RNA preparations.

As before, as a control for possible variation in transfection efficiency and loading, the Northern blots were stripped and reprobed with an oligonucleotide specific for the pCMVrev and pCMVrev$^-$ mRNAs. The observed levels of these RNAs were similar in each total and cytoplasmic lane (data not shown). The results of these experiments showed that the simple insertion of an extra 5' splice site in front of the *env* gene in pβGenv made *env* expression completely Rev-dependent.

We reasoned that a second way to place the *env* ORF of pβGenv within an efficiently recognized intron would be to remove the 3' rabbit β-globin splice site which was present immediately upstream from the ORF. This would position most of the *env* ORF within a new intron bounded by the β-globin 5' splice site and the *tat/rev* 3' splice site. To do this, a 141-bp *ApaI-BalI* fragment was deleted from pβGenv to create plasmid pβGenv-3'Δ. A schematic representation of this region of the plasmid is shown in Fig. 4C.

We then performed an analysis of the protein and RNA produced from this plasmid in the absence and presence of Rev. The results of these experiments are shown in Fig. 4. The

Western blot analysis (Fig. 4A) showed that Env protein expression from pβGenv-3′Δ was completely Rev-dependent. The RNA analysis (Fig. 4B) showed two major RNA species of 3.5 and 0.9 kb. Further analysis showed that these RNAs were a completely unspliced *env* mRNA and an RNA in which an intron between the β-globin 5′ splice site and the *tat/rev* 3′ splice site had been removed (data not shown). Although the 3.5-kb mRNA was present in about equal amounts in the total RNA preparation, in either the absence or presence of Rev, it was not detectable in cytoplasmic RNA without a functional Rev protein. In contrast, the smaller spliced RNA was efficiently exported to the cytoplasm, with or without Rev. No mRNA spliced at the 5′ *tev* splice site was detected in either total or cytoplasmic RNA preparations.

As before, as a control for possible variation in transfection efficiency and loading, the Northern blots were stripped and reprobed with an oligonucleotide specific for the pCMVrev and pCMVrev$^-$ mRNAs. The observed levels of these RNAs were similar in each of the total and cytoplasmic lanes (data not shown). Thus, the results of these experiments showed that a second way to make expression from pβGenv completely Rev-dependent was by deletion of the 3′ splice site in front of the *env* gene.

DISCUSSION

The results presented here show that Rev regulation of HIV *env* expression critically depends on the position and configuration of splice sites in the *env* RNA. Consequently, *env* expression could be made partially Rev-independent simply by insertion of a complete intron upstream of the *env* ORF. RNA expressed from this construct was rapidly spliced to remove the upstream intron, and in the absence of Rev, some of this singly spliced RNA was exported from the nucleus. However, a portion of the RNA was further spliced to remove an intron within the *env* region before transport ensued. When Rev was present, more singly spliced *env* RNA reached the cytoplasm and more envelope protein was expressed.

The 5′ splice site used in removal of the second intron, in the RNA expressed from pβGenv, was an alternative splice site used in virus-infected cells to make an RNA expressing the viral Tev protein (3, 35). If this splice site was mutated in the +1 intron position, splicing at this site was inhibited and *env* expression became totally Rev-independent. In contrast, complete Rev dependence was observed in another construct, in which an additional 5′ splice site was inserted upstream of the *env* ORF in pβGenv. The same phenotype could also be obtained by deletion of the upstream 3′ β-globin splice site in this plasmid. Thus, Rev dependence was determined by whether or not the *env* region was defined as an intron, suggesting that an important role of Rev is to enable the release from the splicing machinery of intron-containing RNAs. This hypothesis is further supported by the fact that all of the mRNAs that are regulated by Rev in HIV-infected cells contain complete introns (36–38).

By positioning the *env* region downstream of an intron in the RNA, we have mimicked the situation that exists in the virus. The *env* mRNA that is expressed in infected cells is a singly spliced mRNA from which an upstream intron containing the *gag/pol*, *vif*, and *vpr* ORFs has been removed (37). Interestingly, this mRNA still retains the *tat/rev* 5′ splice site and can be further spliced to generate a *nef* mRNA (36). In fact, the *env* mRNA is a bicistronic mRNA that contains a complete *vpu* ORF upstream of *env*, and it has been speculated that this reflects the need for a coordinated expression of *env* and *vpu* (37). Our results show that a 5′ splice site upstream of the *env* region seems necessary to make *env* expression tightly Rev regulated (cf. expression from pβGenv and pβGenv-5′ins). Thus, an alternative explanation for the unusual structure of the *env* mRNA is that the upstream 5′ splice site has to be maintained to keep *env* expression under complete Rev control. This could be important for the virus in order to avoid cytopathic effects in early phases of the infection. We are currently testing the effect of mutating the 5′ *tat/rev* splice site in a plasmid containing a complete HIV genome.

In some studies, it has been suggested that Rev functions independently of splicing (12, 29, 39). Malim et al. (29) claimed that an *env* mRNA expressed from a vector using the HIV-1 long terminal repeat as a promoter lacked functional splice sites but was still subject to Rev regulation. However, recent experiments in our laboratory with the same construct showed that large amounts of spliced mRNA could be detected (unpublished observations). This RNA resulted from a splicing event between the 5′ *tev* splice site and the 3′ *tat/rev* splice site and thus had a structure very similar to that of the 1.5-kb mRNA expressed from pβGenv (Fig. 1C). We have also shown that the HIV-1 transactivation response region (TAR) and *tat* can promote Rev-regulated *env* expression from constructs lacking upstream 5′ splice sites (26). The transactivation response region does not contain a bona fide 5′ splice site but does contain two regions that might constitute a pseudo 5′ splice site capable of binding U1 snRNA analogous to the ones that have been identified in the *Drosophila* system (40). Stephens and Cockett (43) reported that Env protein could be expressed in the absence of Rev by using a vector in which transcription was initiated from the immediate-early cytomegalovirus promoter. This could be explained by the fact that this construct contains a complete intron upstream of the *env* region, thus making it similar to pβGenv.

It has been hypothesized that Rev functions to counteract negative elements in the HIV RNA which might bind cellular factors that retain the RNA in the nucleus in the absence of Rev. Such elements have been identified by several groups and are known as *cis*-acting repressor sequences (CRS), instability sequence (INS) elements, or inhibitory region (IR) sequences (6, 9, 28, 34, 39). These sequences are found throughout the *gag/pol* intron as well as within the *env* intron. Although our experiments show that a 5′ splice site can efficiently function as a CRS element, some of the previously identified CRS elements do not seem to contain functional splice sites. However, such sequences could still function as pseudo 5′ or 3′ splice sites or bind other factors involved in splicing, such as heterogeneous nuclear ribonucleoproteins. It is also possible that CRS elements bind cellular factors not involved in splicing. Further experiments are clearly required to distinguish between these different possibilities.

In some studies, in which incompletely spliced HIV RNA was detected in the cytoplasm of cells in the absence of Rev, efficient protein expression still required the Rev protein (1, 7, 23). This led to the suggestion that Rev may also play a direct role in the translation of some HIV mRNAs. However, an alternative explanation might be that in these cases, Rev-independent transport either delivered these RNAs to the wrong compartment within the cytoplasm or left them bound to splicing factors or other cellular components capable of inhibiting translation.

Rev-like proteins have been found in several other lentiviruses (31, 44, 45). It is also known that the Rex protein of the human T-cell leukemia viruses can substitute for Rev and serve an analogous function (11, 19, 25, 33). Although all retroviruses face the same problem in that full-length intron-containing RNA has to be transported from the nucleus, no Rev/Rex-

like proteins have been found in the simpler retroviruses. If a cellular mechanism exists which prevents transport to the cytoplasm of incompletely spliced RNAs, this presents an interesting enigma. One possibility is that the RNA from these viruses contains *cis*-acting elements similar to the RRE that can be recognized by cellular factors. In fact, we have recently discovered that a small element from Mason-Pfizer monkey virus can efficiently substitute for Rev and the RRE in facilitating the transport of intron-containing HIV RNA from the nucleus to the cytoplasm (3a).

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REFERENCES

1. **Arrigo, S. J., and I. S. Chen.** 1991. Rev is necessary for translation but not cytoplasmic accumulation of HIV-1 vif, vpr, and env/vpu 2 RNAs. Genes Dev. **5**:808–819.
2. **Arya, S. K., C. Guo, S. F. Josephs, and F. Wong-Staal.** 1985. Trans-activator gene of human T-lymphotropic virus type III (HTLV-III). Science **229**:69–73.
3. **Benko, D. M., S. Schwartz, G. N. Pavlakis, and B. K. Felber.** 1990. A novel human immunodeficiency virus type 1 protein, Tev, shares sequences with Tat, Env, and Rev proteins. J. Virol. **64**:2505–2518.

3a. **Bray, M., S. Prasad, J. W. Dubay, E. Hunter, K.-T. Jeang, D. Rekosh, and M.-L. Hammarskjöld.** A small element from the Mason-Pfizer monkey virus genome makes HIV-1 expression and replication rev-independent. Proc. Natl. Acad. Sci. USA, in press.

4. **Chang, D. D., and P. A. Sharp.** 1989. Regulation by HIV Rev depends upon recognition of splice sites. Cell **59**:789–795.
5. **Cochrane, A., R. Kramer, S. Ruben, J. Levine, and C. A. Rosen.** 1989. The human immunodeficiency virus rev protein is a nuclear phosphoprotein. Virology **171**:264–266.
6. **Cochrane, A. W., K. S. Jones, S. Beidas, P. J. Dillon, A. M. Skalka, and C. A. Rosen.** 1991. Identification and characterization of intragenic sequences which repress human immunodeficiency virus structural gene expression. J. Virol. **65**:5305–5313.
7. **D'Agostino, D. M., B. K. Felber, J. E. Harrison, and G. N. Pavlakis.** 1992. The Rev protein of human immunodeficiency virus type 1 promotes polysomal association and translation of *gag/pol* and *vpu/env* mRNAs. Mol. Cell. Biol. **12**:1375–1386.
8. **Daly, T. J., K. S. Cook, G. S. Gray, T. E. Maione, and J. R. Rusche.** 1989. Specific binding of hiv-1 recombinant Rev protein to the Rev-responsive element in vitro. Nature (London) **342**:816–819.
9. **Emerman, M., R. Vazeux, and K. Peden.** 1989. The rev gene product of the human immunodeficiency virus affects envelope-specific RNA localization. Cell **57**:1155–1165.
10. **Feinberg, M. B., R. F. Jarrett, A. Aldovini, R. C. Gallo, and F. Wong-Staal.** 1986. HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. Cell **46**:807–817.
11. **Felber, B. K., D. Derse, A. Athanassopoulos, M. Campbell, and G. N. Pavlakis.** 1989. Cross-activation of the rex proteins of HTLV-I and BLV and of the rev protein of HIV-1 and nonreciprocal interactions with their RNA responsive elements. New Biol. **1**:318–330.
12. **Felber, B. K., M. Hadzopoulou-Cladaras, C. Cladaras, T. Copeland, and G. N. Pavlakis.** 1989. Rev protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA. Proc. Natl. Acad. Sci. USA **86**:1496–1499.
13. **Gerard, R. D., and Y. Gluzman.** 1985. New host cell system for regulated simian virus 40 DNA replication. Mol. Cell. Biol. **5**:3231–3240.
14. **Gonda, M. A., M. J. Braun, J. E. Clements, J. M. Pyper, F. Wong-Staal, R. C. Gallo, and R. V. Gilden.** 1986. Human T-cell lymphotropic virus type III shares sequence homology with a family of pathogenic lentiviruses. Proc. Natl. Acad. Sci. USA **83**:4007–4011.
15. **Gottlinger, H. G., T. Dorfman, E. A. Cohen, and W. A. Haseltine.** 1992. The role of the tnv protein and tnv RNA splicing signals in replication of HIV-1 IIIB isolates. Virology **189**:618–628.
16. **Hadzopoulou-Cladaras, M., B. K. Felber, C. Cladaras, A. Athanassopoulos, A. Tse, and G. N. Pavlakis.** 1989. The Rev (Trs/Art) protein of human immunodeficiency virus type 1 affects viral mRNA and protein expression via a *cis*-acting sequence in the *env* region. J. Virol. **63**:1265–1274.
17. **Hammarskjöld, M.-L., J. Heimer, B. Hammarskjöld, I. Sangwan, L. Albert, and D. Rekosh.** 1989. Regulation of human immunodeficiency virus *env* expression by the *rev* gene product. J. Virol. **63**:1959–1966.
18. **Hammarskjöld, M.-L., S.-C. Wang, and G. Klein.** 1986. High-level expression of the Epstein-Barr virus EBNA1 protein in CV1 cells and human lymphoid cells using a SV40 late replacement vector. Gene **43**:41–50.
19. **Hanly, S. M., L. T. Rimsky, M. H. Malim, J. H. Kim, J. Hauber, D. M. Duc, S. Y. Le, J. V. Maizel, B. R. Cullen, and W. C. Greene.** 1989. Comparative analysis of the HTLV-I Rex and HIV-1 Rev trans-regulatory proteins and their RNA response elements. Genes Dev. **3**:1534–1544.
20. **Hauber, J., M. Bouvier, M. H. Malim, and B. R. Cullen.** 1988. Phosphorylation of the *rev* gene product of human immunodeficiency virus type 1. J. Virol. **62**:4801–4804.
21. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77**:51–59.
22. **Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease.** 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene **77**:61–68.
23. **Lawrence, J. B., A. W. Cochrane, C. V. Johnson, A. Perkins, and C. A. Rosen.** 1991. The HIV-1 Rev protein: a model system for coupled RNA transport and translation. New Biol. **3**:1220–1232.
24. **Legrain, P., and M. Rosbash.** 1989. Some cis- and trans-acting mutants for splicing target pre-mRNA to the cytoplasm. Cell **57**:573–583.
25. **Lewis, N., J. Williams, D. Rekosh, and M.-L. Hammarskjöld.** 1990. Identification of a *cis*-acting element in human immunodeficiency virus type 2 (HIV-2) that is responsive to the HIV-1 Rev and human T-cell leukemia virus type I and II Rex proteins. J. Virol. **64**:1690–1697.
26. **Lu, X., M.-L. Hammarskjöld, and D. Rekosh.** 1991. A novel post-transcriptional role for HIV1 Tat and TAR, p. 355–367. *In* W. A. Haseltine and F. Wong-Staal (ed.), The genetic structure and regulation of HIV. Raven Press, New York.
27. **Lu, X. B., J. Heimer, D. Rekosh, and M. L. Hammarskjöld.** 1990. U1 small nuclear RNA plays a direct role in the formation of a rev-regulated human immunodeficiency virus env mRNA that remains unspliced. Proc. Natl. Acad. Sci. USA **87**:7598–7602.
28. **Maldarelli, F., M. A. Martin, and K. Strebel.** 1991. Identification of posttranscriptionally active inhibitory sequences in human immunodeficiency virus type 1 RNA: novel level of gene regulation. J. Virol. **65**:5732–5743.
29. **Malim, M. H., J. Hauber, S. V. Le, J. V. Maizel, and B. R. Cullen.** 1989. The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature (London) **338**:254–257.
30. **Muesing, M. A., D. H. Smith, C. D. Cabradilla, C. V. Benton, L. A. Lasky, and D. J. Capon.** 1985. Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus. Nature (London) **313**:450–458.
31. **Phillips, T. R., C. Lamont, D. A. Konings, B. L. Shacklett, C. A. Hamson, P. A. Luciw, and J. H. Elder.** 1992. Identification of the Rev transactivation and Rev-responsive elements of feline immunodeficiency virus. J. Virol. **66**:5464–5471.
32. **Rekosh, D., A. Nygren, P. Flodby, M. L. Hammarskjöld, and H. Wigzell.** 1988. Coexpression of human immunodeficiency virus envelope proteins and tat from a single simian virus 40 late replacement vector. Proc. Natl. Acad. Sci. USA **85**:334–338.
33. **Rimsky, L., J. Hauber, M. Dukovich, M. H. Malim, A. Langlois,**

B. R. Cullen, and W. C. Greene. 1988. Functional replacement of the HIV-1 rev protein by the HTLV-1 rex protein. Nature (London) **335:**738–740.

34. **Rosen, C. A., E. Terwilliger, A. Dayton, J. G. Sodroski, and W. A. Haseltine.** 1988. Intragenic cis-acting art gene-responsive sequences of the human immunodeficiency virus. Proc. Natl. Acad. Sci. USA **85:**2071–2075.
35. **Salfeld, J., H. G. Gottlinger, R. A. Sia, R. E. Park, J. G. Sodroski, and W. A. Haseltine.** 1990. A tripartite HIV-1 tat-env-rev fusion protein. EMBO J. **9:**965–970.
36. **Schwartz, S., B. K. Felber, D. M. Benko, E. M. Fenyo, and G. N. Pavlakis.** 1990. Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. J. Virol. **64:**2519–2529.
37. **Schwartz, S., B. K. Felber, E. M. Fenyo, and G. N. Pavlakis.** 1990. Env and Vpu proteins of human immunodeficiency virus type 1 are produced from multiple bicistronic mRNAs. J. Virol. **64:**5448–5456.
38. **Schwartz, S., B. K. Felber, and G. N. Pavlakis.** 1991. Expression of human immunodeficiency virus type 1 vif and vpr mRNAs is Rev-dependent and regulated by splicing. Virology **183:**677–686.
39. **Schwartz, S., B. K. Felber, and G. N. Pavlakis.** 1992. Distinct RNA sequences in the *gag* region of human immunodeficiency virus type 1 decrease RNA stability and inhibit expression in the absence of Rev protein. J. Virol. **66:**150–159.
40. **Siebel, C. W., L. D. Fresco, and D. C. Rio.** 1992. The mechanism of somatic inhibition of Drosophila P-element pre-mRNA splicing: multiprotein complexes at an exon pseudo-5′ splice site control U1 snRNP binding. Genes Dev. **6:**1386–1401.
41. **Smith, A. J., M. I. Cho, M. L. Hammarskjöld, and D. Rekosh.** 1990. Human immunodeficiency virus type 1 $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ expressed from a simian virus 40 late replacement vector are efficiently processed and assembled into viruslike particles. J. Virol. **64:**2743–2750.
42. **Sodroski, J., W. C. Goh, C. Rosen, A. Dayton, E. Terwilliger, and W. Haseltine.** 1986. A second post-transcriptional trans-activator gene required for HTLV-III replication. Nature (London) **321:**412–417.
43. **Stephens, P. E., and M. I. Cockett.** 1989. The construction of a highly efficient and versatile set of mammalian expression vectors. Nucleic Acids Res. **17:**7110.
44. **Stephens, R. M., D. Derse, and N. R. Rice.** 1990. Cloning and characterization of cDNAs encoding equine infectious anemia virus Tat and putative Rev proteins. J. Virol. **64:**3716–3725.
45. **Tiley, L. S., P. H. Brown, S. Y. Le, J. V. Maizel, J. E. Clements, and B. R. Cullen.** 1990. Visna virus encodes a post-transcriptional regulator of viral structural gene expression. Proc. Natl. Acad. Sci. USA **87:**7497–7501.
46. **Zapp, M. L., and M. R. Green.** 1989. Sequence-specific RNA binding by the HIV-1 Rev protein. Nature (London) **342:**714–716.