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U1 small nuclear RNA plays a direct role in the formation of a rev-regulated human immunodeficiency virus *env* mRNA that remains unspliced

(HIV envelope protein expression/RNA transport/RNA stability/splice site mutant suppression)

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ABSTRACT **rev-regulated expression of HIV-1 envelope proteins from a simian virus 40 late replacement vector was found to be dependent on the presence of a 5′ splice site in the *env* mRNA in spite of the fact that this mRNA remains unspliced. When the 5′ splice site upstream of the *env* open reading frame was deleted or mutated, expression of envelope protein was lost. RNA analysis of cells transfected with 5′ splice-site mutants showed a dramatic reduction in the steady-state levels of *env* mRNA whether or not rev was present. Envelope expression could be restored in one of the 5′ splice-site mutants by cotransfection with a plasmid expressing a suppressor U1 small nuclear RNA containing a compensatory mutation. These experiments show that U1 small nuclear RNA plays a direct and essential role in the formation of an unspliced RNA that is subject to regulation by rev.**

Retroviruses provide some of the best model systems for studying RNA processing and differential splicing (for a review, see ref. 1). The RNA genome of these viruses is reverse-transcribed into a proviral DNA copy, which is integrated into the host cell genome as a single transcription unit. This unit contains a promoter that, for all replication-competent retroviruses, is followed (5′ to 3′) by the genes *gag*, *pol*, and *env*. The primary transcription product includes all of the viral genes and functions as the mRNA for both gag and pol. The proteins encoded by *env* are made from a mRNA in which the *gag* and *pol* sequences have been removed by splicing. The *gag–pol* region is thus an intron in the processing of *env* mRNA. The situation is even more complex within the lentivirus subfamily of the retroviruses, of which human immunodeficiency virus (HIV) is the most prominent member (for reviews, see refs. 2–6) because the HIV genome also contains several genes that encode regulatory proteins. These genes are positioned in the 3′ half of the genome and are expressed from mRNAs that are doubly spliced to remove two introns comprising *gag/pol* and *env* sequences. As a consequence of this genetic organization, nonspliced RNA, as well as singly and doubly spliced RNA, has to be exported out of the nucleus to enable a balanced expression of the different viral proteins.

One of the HIV regulatory proteins, rev, has been clearly shown to be involved in differential RNA expression (7, 8); rev functions to facilitate transport of unspliced and singly spliced mRNAs from the nucleus to the cytoplasm (9–12). In the absence of rev, only completely spliced mRNAs can be detected in the cytoplasm of infected and transfected cells, whereas unspliced and singly spliced mRNAs accumulate in the nucleus. However, in the presence of rev, large amounts of these intron-containing mRNAs reach the cytoplasm. Thus, rev seems to be able to specifically recognize incompletely spliced RNA molecules to allow the export of these mRNAs from the nucleus. This recognition requires the presence in the RNA of a specific sequence called the rev-responsive element (RRE; refs. 9, 11–15), which has been mapped to a 234-base pair (bp) region within the *env* gene of HIV-1 (11) and to a 272-bp region within the *env* gene of HIV-2 (16). Recent results show that the rev protein binds specifically *in vitro* to RNA containing the RRE (17, 18).

It has been suggested that rev function might be dependent on the integrity of 5′ and 3′ splice sites in the RNA (19). This hypothesis originates from a study in which rev regulation of RNA from a β-globin construct containing an intron and the RRE was analyzed. Certain point mutations at the splice sites made nonspliced mRNA accumulate in the nucleus. These RNAs appeared in the cytoplasm only in the presence of rev. However, mutations at both splice sites eliminated the rev response and made unspliced RNAs appear in the cytoplasm even in the absence of rev. From these results it was concluded that rev action may require the RNA to enter into a presplicing complex.

We show here that rev-regulated expression of HIV-1 envelope proteins from a simian virus 40 (SV40)-based vector is dependent on the presence of a 5′ splice site in the *env* mRNA, in spite of the fact that this RNA is not spliced. If the 5′ splice site is mutated or deleted, *env* expression is lost, and only very small amounts of HIV *env* RNA are detected whether or not rev is present. The effect of one specific mutation in the 5′ splice site can be suppressed by cotransfection with a U1 small nuclear RNA (snRNA) mutant that restores base pairing at the splice site. These results provide direct genetic evidence that binding to U1 snRNA plays an essential role in the formation of an unspliced RNA that is subject to rev regulation.

MATERIALS AND METHODS

Cells and Transfections. CMT3 (20) cells were transfected by using DEAE-dextran as described (21).

Immunoblot Analysis. Blots were carried out by using Immobilon P membranes (Millipore) as described (9, 21). The blots were developed with an anti-HIV-1 glycoprotein gp160 (baculovirus-produced) goat serum (a gift from Jim Rusche).

RNA Purification and Blot Hybridization. Total and cytoplasmic RNA isolation and blot analysis were performed as described (9).

Nucleotide Numbering. The HIV-1 nucleotide numbering system used throughout corresponds to the Hxb2cg sequence present in GenBank (accession no. K03455).

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Abbreviations: HIV-1, human immunodeficiency virus 1; snRNA, small nuclear RNA; SV40, simian virus 40; EBV, Epstein–Barr virus; RRE, rev-responsive element.

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Polymerase Chain Reactions (PCR). The PCRs were carried out in a Perkin–Elmer/Cetus DNA thermal cycler by using the standard reaction mixtures suggested by the manufacturer. Each reaction included 100 ng of DNA template and 500 ng of each primer. A stepped cycle program was used [30 cycles of 1 min at 94°C, 2 min at the estimated melting temperature (t_m) − 5°C, and 2 min at 72°C]; t_m was estimated from the G+C content by the formula 4(G+C) +2 (A+T) = °C. Amplified fragments were purified by agarose electrophoresis.

Vector Constructions. The vectors pSVSX1, pSVSX1Δ1, and pRev1 have been described (9, 16, 22). pSVSX1Δ5 and pSVSX1Δ6 were constructed by inserting HIV sequences from nucleotide 6128 to 8896 and from nucleotide 6198 to 8896, respectively, into the *Xho* I site of the vector pBABY, the parent vector of pSVSX1 (22). Mutant pSVSX1 (+1T) was constructed by using the phage M13 mutagenesis method of Kunkel *et al.* (23) by mutating a *Hin*dIII–*Kpn* I fragment of pSVSX1 cloned in M13 that contained the *tat/rev* 5′ splice site. The mutated fragment was then used to replace the corresponding wild-type fragment in pSVSX1.

The mutant plasmids pSVSX1(+5C), pSVSX1(+5A), and pSVSX1(+5U) were made by the PCR method described by Ho *et al.* (24). DNA containing these mutations were obtained as 1123-bp-amplified fragments that were cleaved with *Sfi* I and *Asp*718I and used to replace the corresponding fragment in pSVSX1. The *Sfi*I–*Asp*718I fragments of the resulting constructs were sequenced to ensure that they contained only the desired mutations.

The plasmid 24/wtSD was constructed by using two partially overlapping 26-base single-stranded oligonucleotides that were homologous to "upper" or "lower" strand sequences surrounding the *tat/rev* 5′ splice site. The sequences of these oligonucleotides were 5′-GGGTCGACTATCAAAGCAGTAAGTAG-3′ and 5′-GGGTCTAGATGTACTACTTACTGCTT-3′, respectively. After annealing and repair with Klenow fragment, a double-stranded fragment was generated that contained a *Sal* I site at one end and an *Xba* I site at the other end. The fragment was then cleaved with *Sal* I and *Xba* I and used to replace the sequences between the *Sal* I site (HIV nucleotide 5785) and an engineered *Xba* I site (HIV nucleotide 6198) in the plasmid pJTX obtained from Eric Hunter. The *Sal* I–*Xho* I (HIV nucleotide 8896) fragment from this plasmid was then inserted into the unique *Xho* I site of the vector pBABY. The construction of 24/mtSD was identical except that the oligonucleotides used contained a single-base-pair mutation that changed the guanosine at the +1 position of the 5′ splice site to a thymidine.

To create 24/EBV a 88-bp *Sac* I–*Aha* II fragment from the *EBNA1* gene of Epstein–Barr virus (EBV) containing a known 5′ splice site (nucleotide 109,856 in the EBV genome), was initially inserted into the polylinker region of pUC18 and excised as a *Sal* I–*Xba* I fragment. As described for 24/wtSD, the fragment was then inserted into pJTX, whereafter the *Sal* I–*Xho* I fragment was cloned into pBABY.

RESULTS

***env* Expression in a SV40 Vector System Requires Upstream Sequences.** We have previously shown that large amounts of HIV envelope proteins can be produced from the SV40 late replacement vector pSVSX1 (22). The HIV sequences in pSVSX1 include the genes for tat and rev (in addition to the *env* gene), and the vector expresses both of these regulatory proteins (Fig. 1 *Upper*). The *rev* and *tat* genes each contain two coding exons that are separated by an intron that contains a major portion of the *env* open reading frame. We have shown that rev is necessary for envelope protein expression in this system, whereas tat is not required (9).

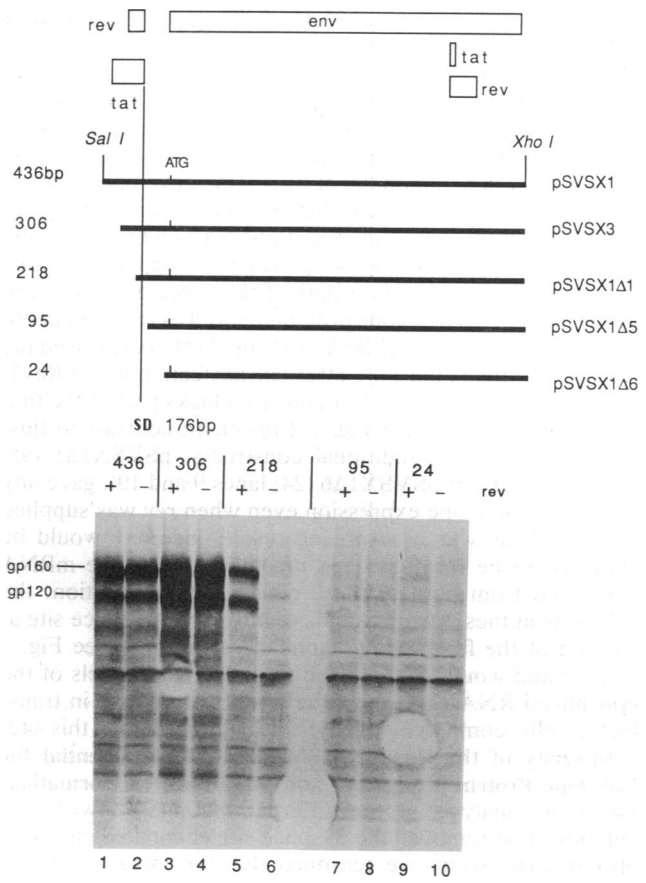

FIG. 1. Effect of deletions removing sequences upstream of the HIV *env* open reading frame on envelope protein expression. (*Upper*) This diagram shows the HIV sequences in pSVSX1 and the deletions that were made to create the indicated plasmids. The position of the open reading frames for *tat*, *rev*, and *env* are shown at the top. The numbers on the left indicate the remaining nucleotides upstream of the first ATG in the *env* open reading frame. SD denotes the position of the *tat/rev* 5′ splice site. (*Lower*) Western blot analysis of lysates from cells transfected with the different deletion mutants alone or in cotransfections with pRev1. Lysates of 2×10^5 transfected cells were separated by SDS/PAGE, and the proteins were transferred to Immobilon P. The blot was developed with a goat serum directed against gp160 expressed from a baculovirus vector.

Transcription of the HIV sequences in pSVSX1 is directed by the SV40 late promoter and terminates in rabbit β-globin sequences 3′ of the HIV insert. The β-globin sequences present in the vector include the second intron as well as the polyadenylylation site. Thus, the primary transcript from pSVSX1 contains two complete introns. In our previous studies (9), we have shown that the mRNA for the envelope proteins remains completely unspliced and retains the β-globin as well as the HIV intron sequences. In contrast to this, the mRNA encoding the tat and rev proteins is a doubly spliced RNA in which both of these introns have been removed. In the absence of the rev protein, the nonspliced *env* mRNA accumulates in the nucleus, whereas the doubly spliced RNA is transported to the cytoplasm.

In the unspliced mRNA expressed from pSVSX1, *env* is the fourth open reading frame downstream of the first coding exons of *tat* and *rev* (Fig. 1 *Upper*) and a major part of the coding sequences for the *vpu* gene (not shown in Fig. 1 *Upper*). Thus, we expected that removal of sequences upstream of the *env* open reading frame would lead to higher levels of envelope proteins, provided that *rev* was supplied in trans from a separate vector. To directly test this, we made a series of constructions that removed increasing amounts of

the sequences between the SV40 late promoter and the start of the *env* open reading frame (Fig. 1 *Upper*).

The deletion mutants and the original plasmid pSVSX1 were transfected into CMT3 cells either alone or together with a plasmid known to express the rev protein (pRev1 [ref. 16]). Cell lysates were subjected to immunoblot analysis (Fig. 1 *Lower*). Large amounts of the HIV envelope proteins (gp160 and gp120) were produced in cells transfected with pSVSX1 (436; lanes 1 and 2) and pSVSX3 (306; lanes 3 and 4) irrespective of whether or not *rev* was supplied in trans. This was expected because both of these vectors have been shown to express envelope proteins as well as rev protein (9). The vector pSVSX1Δ1 (218; lanes 5 and 6) also expressed the envelope proteins but only after cotransfection with pRev1. This was predicted since this construct lacks part of the first coding exon of *rev* (see Fig. 1 *Upper*). In contrast to this, neither of the two additional constructs, pSVSX1Δ5 (95; lanes 7 and 8) and pSVSX1Δ6 (24; lanes 9 and 10), gave any detectable envelope expression even when *rev* was supplied in trans. This was a surprising result since *env* would be expected to be the first open reading frame in the mRNA expressed from both of these constructs. In addition, the deletions in these two plasmids removed the 5′ splice site at the end of the first coding exons of *rev* and *tat* (see Fig. 1 *Upper*) and would be expected to yield higher levels of the nonspliced RNA expressing the envelope proteins in transfected cells, compared with the constructs retaining this site.

Integrity of the Upstream 5′ Splice Site Is Essential for Envelope Protein Expression and Stable mRNA Formation. Since the analysis of the different mutants showed that deletions that removed the 5′ splice site eliminated envelope protein expression, we reasoned that the integrity of this splice site might be essential. The sequence surrounding the 5′ splice site is GCA ↓ GTAAGT, with the six bases on the intron side fitting the consensus sequence. To directly test our hypothesis, we made a specific point mutation in pSVSX1 that changed the strictly conserved guanosine residue at the +1 position to a thymidine. The resulting plasmid, pSVSX1(+1T), was transfected into CMT3 cells alone or together with pRev1. pSVSX1 and pSVSX1Δ2, a plasmid in which a major part of the second coding exon of *rev* has been removed (9), were used as controls. Lysates of cells were subjected to immunoblot analysis (Fig. 2 *Left*). Envelope protein expression could not be detected in the cells transfected with pSVSX1(+1T), whether or not *rev* was supplied in trans (SD mt; lanes 1 and 2). In contrast to this, cells transfected with pSVSX1 with or without pRev1 (lanes 3 and 4) contained large amounts of envelope proteins, whereas cells transfected with pSVSX1Δ2 (lanes 5 and 6) expressed detectable amounts of the envelope proteins only after cotransfection with pRev1. The result of this experiment supports the hypothesis that the integrity of the 5′ splice site is essential for envelope protein expression.

We next analyzed the levels of HIV-specific RNA in cells transfected with pSVSX1(+1T) and pSVSX1Δ2 with or without pRev1. Total RNA from these cells was subjected to blot analysis with a labeled oligonucleotide probe complementary to sequences within the first coding exon of *tat* (Fig. 2 *Right*). This probe will not detect the RNA expressed from pRev1 but will detect all of the different HIV mRNA species expressed from pSVSX1Δ2 (9). Two different autoradiographic exposures of the filter are shown (overnight and 4 days). As described previously, two different size classes of HIV mRNA were detected in cells transfected with pSVSX1Δ2 whether extra *rev* was provided (lanes 4 and 8) or not (lanes 3 and 7). These represent unspliced mRNA encoding the envelope proteins and mRNA that has been doubly spliced to remove the intron within the HIV sequences as well as the β-globin intron. In contrast, only a weak band was present in cells transfected with pSVSX1(+1T) with or without *rev* at the position expected for unspliced RNA (SD mt; lanes 1, 2, 5, and 6), and no spliced RNA was observed. The additional bands seen in the high molecular weight range of the gel in the longer exposure probably represent residual plasmid DNA in the RNA preparations. The experiments presented in Fig. 2 *Right* have been repeated several times with the same results. Furthermore, no HIV-specific RNA was detected when poly(A)$^+$ cytoplasmic RNA from cells transfected with pSVSX1(+1T) was analyzed, whereas the nuclear fraction showed a small amount of unspliced HIV-specific RNA, consistent with the results shown in Fig. 2 *Right* (data not shown). An analysis of total and nuclear RNA using the construct pSVSX1Δ6, in which the 5′ splice site was deleted, gave results similar to those obtained with pSVSX1(+1T) (data not shown).

Taken together, the results of the RNA analysis showed that the integrity of the *tat/rev* 5′ splice site was essential for the accumulation of high steady-state levels of unspliced HIV *env* mRNA in transfected cells. When the splice site was mutated or deleted, low levels of nuclear unspliced RNA were observed, but this RNA was not exported from the nucleus whether or not rev was present.

Envelope Protein Expression Can Be Restored by the Insertion of a Heterologous 5′ Splice Site Upstream of the *env* Gene. We next studied the effect of the introduction of a heterologous 5′ splice site into the deletion mutant pSVSX1Δ6 (see Fig. 1 *Upper*). To this end, a 88-bp fragment from the *EBNA1* gene of EBV was inserted into the *Xba* I site 24 bp upstream of the *env* ATG to create the plasmid 24/EBV. This fragment contains a known 5′ splice site with the sequence AGG ↓ GTGAGG (the underlined bases fit the consensus sequence for 5′ splice sites). Cells were transfected with

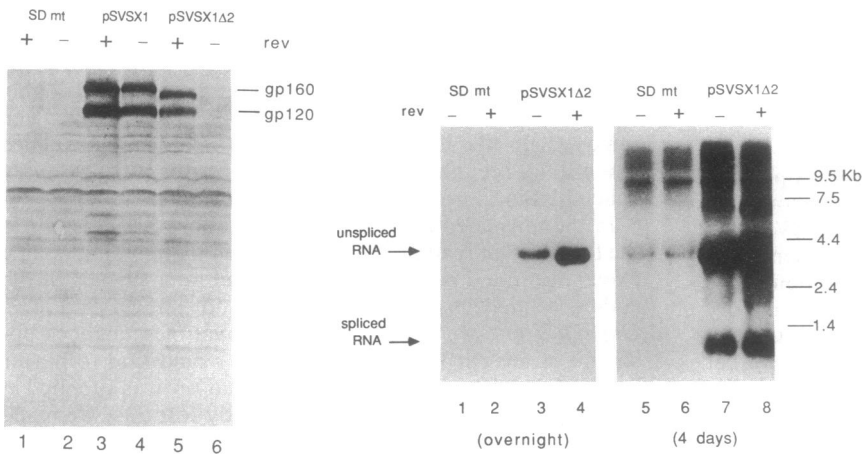

FIG. 2. Effects of a guanosine-1 → thymidine mutation in the *tat/rev* 5′ splice site on HIV env protein and mRNA expression. (*Left*) Western blot showing envelope protein expression in cells transfected with the indicated vectors (+ or − pRev1). Blotting was performed as described in the legend to Fig. 1. (*Right*) Northern blot of total RNA from cells transfected with the indicated vectors (+ or − pRev1). The RNA was separated on a formaldehyde/1% agarose gel. Each lane contained 40 μg of RNA. The probe used was a ^{32}P-5′-end-labeled oligonucleotide from the first coding exon of *tat*. Two different autoradiographic exposures are shown; overnight (16 hr) and 4 days (96 hr). The blots were exposed at −70°C with an intensifying screen. The positions of RNA size markers are shown.

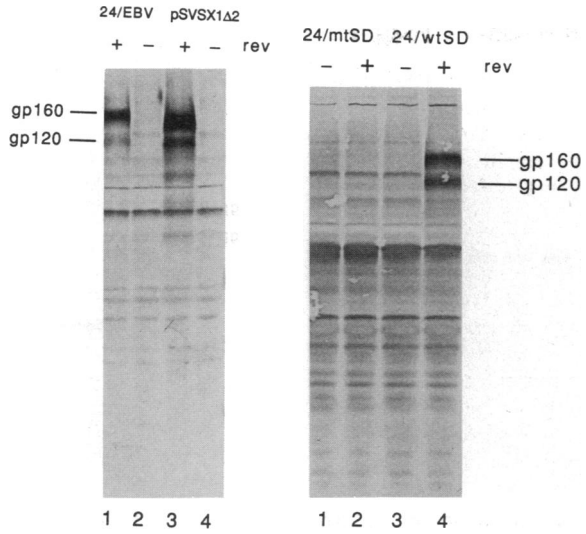

FIG. 3. Insertion of a fragment containing a heterologous 5′ splice site or a synthetic oligonucleotide containing the *tat/rev* 5′ splice site into pSVSX1Δ2 restores envelope protein expression. (*Left*) Western blot of cells transfected with the indicated vectors (+ or − pRev1). (*Right*) Western blot of cells transfected with the indicated vectors (+ or − pRev1). The cells were harvested at 60 hr post-transfection, and the analysis was performed on lysates of 2×10^5 cells as described in the legend to Fig. 1.

24/EBV alone or together with the plasmid pRev1 and subjected to immunoblot analysis. As a control, pSVSX1Δ2 was used. In the cotransfection with 24/EBV and pRev1, large amounts of the envelope proteins were expressed (Fig. 3 *Left*, lane 1) at levels comparable to those seen in cells cotransfected with pSVSX1Δ2 and pRev1 (lane 3). As expected, no *env* expression was detected in the absence of *rev* (lanes 2 and 4). The results of this experiment support the hypothesis that a 5′ splice site is essential for *env* expression in this system and show that a heterologous 5′ splice site can efficiently substitute for the original one.

Effects of the Insertion of Synthetic Oligonucleotides Containing Wild-Type or Mutated 5′ Splice Sites. The effect of inserting short synthetic oligonucleotides containing either the HIV wild-type or a +1T-mutated 5′ splice site into the plasmid pSVSX1Δ6 (see Fig. 1 *Upper*) was also studied. To do this the plasmids 24/wtSD and 24/mtSD were created. These plasmids contained an insertion of 24 bp at the *Xba* I site 24 bases upstream of the *env* ATG in pSVSX1Δ6. The sequence of the inserted fragment was 5′-CTATCAAAGCA-GTAAGTAGTACAT-3′ in 24/wtSD and 5′-CTATCAAAG-CATTAAGTAGTACAT-3′ in 24/mtSD.

Immunoblot analysis (Fig. 3 *Right*) showed that large amounts of envelope proteins were expressed in cells transfected with the 24/wtSD plasmid when *rev* was supplied in trans (lane 4). In contrast to this, no envelope protein expression was detected in cells from a cotransfection using the 24/mtSD plasmid (lane 2). In the absence of *rev*, no envelope proteins were detected with either of the plasmids (lanes 1 and 3). This experiment showed that a small fragment containing the *tat/rev* 5′ splice site was sufficient to restore envelope expression. Consistent with the results presented in Fig. 2 *Left*, the guanosine-1 → thymidine mutation failed to give any detectable envelope protein expression, supporting the hypothesis that the integrity of the splice site is essential.

***env* Expression Can Be Restored in a 5′ Splice-Site Mutant Plasmid by Cotransfection with a Construct Expressing a Suppressor U1 snRNA.** Since an intact 5′ splice site was important for *env* expression and U1 snRNA has been shown to bind to 5′ splice sites prior to splicing, we hypothesized that U1 snRNA might be directly involved in the formation of stable *env* mRNA. Previous experiments have shown that splicing can be efficiently restored in RNA containing mutated 5′ splice sites if the mutants are cotransfected with plasmids expressing mutant U1 snRNAs (25–27), if these contain compensatory mutations that restore base pairing at the splice site. We decided to use a similar strategy to try to demonstrate a possible involvement of U1 snRNA in the processing of the unspliced *env* mRNA. To this end we made three different site-specific mutations in pSVSX1 to change the guanosine at the +5 position of the *tat/rev* 5′ splice site upstream of the *env* gene to either a uridine, adenosine, or cytidine and tested suppression of these by using the corresponding U1 snRNA mutants in which the cytidine at position +4 from the cap site is replaced by adenosine (pUCBU1-4A), uridine (pUCBU1-4U), or guanosine (pUCBU1-4G) (25). The sequence of the wild-type and mutant splice site and the expected base pairing between these sites and the wild-type and mutant +4 suppressor U1 snRNAs are shown in Fig. 4 *Left*.

CMT3 cells were transfected with the different +5 mutants [pSVSX1(+5U), -(+5A), and -(+5C)] either alone or together with their respective suppressor mutants with or without pRev1, and lysates of the cells were subjected to immunoblot analysis (Fig. 4 *Center*). None of the +5 mutants expressed any detectable envelope protein when transfected alone (lanes 4, 7, and 10). In contrast to this, a substantial amount of envelope protein was seen when the pSVSX1(+5C) mutant was transfected together with the 4G U1 suppressor mutant (pUCBU1-4G) and pRev1 (lane 8). A small amount of envelope protein was also detected in the cotransfection of the +5C mutant and pUCBU1-4G without added rev, indicating that some of the mRNA was spliced at the correct position (lane 9). This has been confirmed by RNA analysis (data not shown). No detectable suppression was obtained with the 4A (pUCBU1-4A) and 4U (pUCBU1-4U) suppressors (lanes 2, 3, 6, and 7). This could possibly be due to lower stability of the complexes formed compared with the wild-type interaction (see Fig. 4 *Left*). Control experiments in which the different +5 mutants were cotransfected with pRev1 and "noncomplementary" U1 suppressors or with pRev1 alone failed to give any detectable *env* expression (data not shown).

We also transfected cells with the pSVSX1(+5C) mutant and pRev1 and analyzed *env* expression in these cells compared with cells cotransfected with the +5C mutant and pUCBU1-4G with or without pRev1 (Fig. 4 *Right*). No *env* expression was detected when extra *rev* was provided in the absence of the suppressor (lane 3). As before, a low level of envelope protein gp160 was obtained with suppressor alone (lane 2), whereas high-level expression was observed in the presence of both suppressor and pRev1 (lane 1).

The results of these experiments verify the importance of the integrity of the 5′ splice site and clearly show that a U1 snRNA–5′ splice site interaction is essential for env expression despite the fact that the *env* mRNA remains unspliced.

DISCUSSION

In the present study we provide evidence that U1 snRNA plays an essential role in the formation of an unspliced HIV *env* mRNA. It is clear that complex formation with U1 snRNA does not commit this RNA to splicing, since in the presence of the rev protein, unspliced RNA is transported to the cytoplasm and used to express the HIV envelope proteins. Our data are consistent with the notion that splice-site recognition may be important in rev regulation (19), since binding of the *env* mRNA to U1 snRNA is obviously a prerequisite for rev function in our system. Although it cannot be ruled out that U1 plays only a transient role, it seems possible that rev recognizes the env mRNA in a snRNP complex.

Both of the rev-regulated mRNAs that have been identified in HIV-infected cells, the 9-kilobase (kb) full-length mRNA

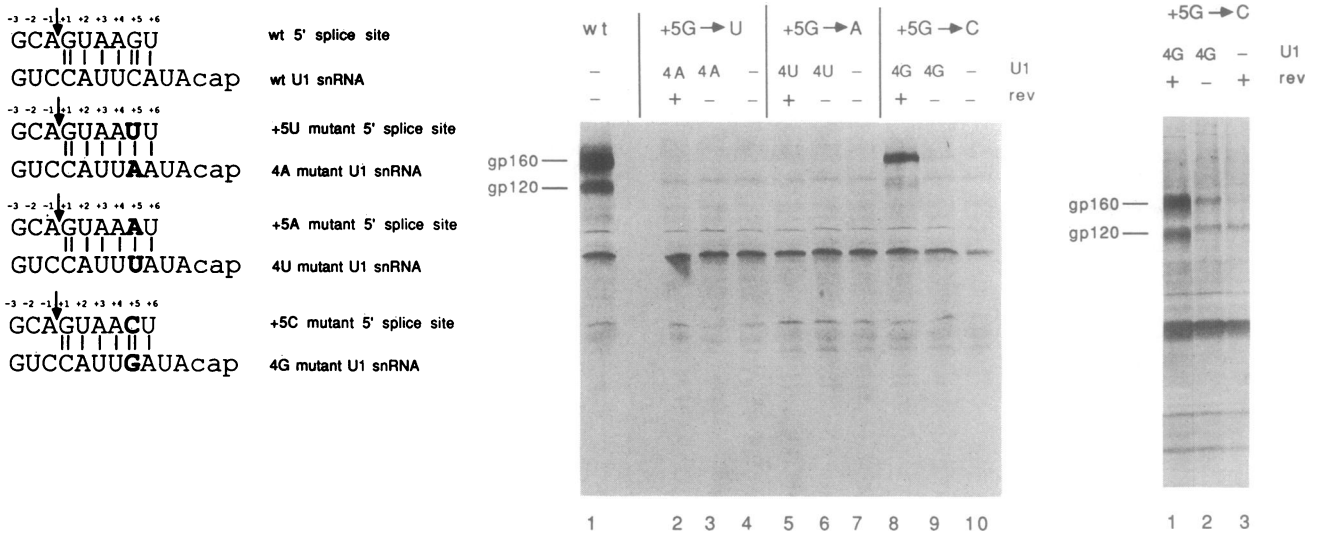

FIG. 4. Cotransfection of mutant pSVSX1(+5C) with a plasmid expressing a suppressor U1 snRNA restores envelope protein expression from pSVSX1(+5C). (*Left*) A schematic representation of the expected base-pairing between RNA from the three different +5 mutants in the *tat/rev* 5′ splice site and the RNA from the corresponding U1 snRNA suppressor mutants. The expected wild-type interaction is also shown. The arrows indicate the exon/intron junction. (*Center*) Western blot of lysates of 2×10^5 cells transfected with the +5 5′ splice site mutants [pSVSX1(+5U), -(+5A), and -(+5C)] alone or in cotransfections with the indicated U1 snRNA suppressor mutants in the absence or presence of pRev1. (*Right*) Western blot of lysates of 2×10^5 cells transfected with pSVSX1(+5C) cotransfected with pRev1 and/or the pUCBU1-4G mutant suppressor. Blotting was performed as described in the legend to Fig. 1.

used to express the gag/pol proteins and the 4.3-kb mRNA believed to be the *env* mRNA, contain 5′ splice sites close to their 5′ ends. Nevertheless, it has been argued that rev-dependent expression of gag/pol and env proteins from some expression vectors is independent of the presence of functional splice sites (11, 12, 15, 28). However, it should be noted that none of the plasmids used in these studies were specifically examined for activation of cryptic 5′ splice sites.

Studies using different vectors have shown that the presence in the expressed RNA of an excisable intron is often a prerequisite for efficient expression of products from an inserted gene (29, 30). In the case of the SV40 late genes, it was recently shown that transcripts lacking introns were defective in both stability in the nucleus and transport to the cytoplasm (29). These results led the authors to hypothesize that most transcripts may require processing via a pathway that couples stabilization of the primary transcripts within the nucleus to excision of intervening sequences and transport to the cytoplasm. Since several viral and cellular genes have been shown to completely lack excisable introns, it is clear that splicing cannot be a universal requirement for the formation of stable mRNAs and for their transport to the cytoplasm. From our results it seems possible that some of these genes might still contain sequences that mediate base pairing to U1 snRNA enabling the formation of stable RNA.

It is clear from our results that the presence of the *tat/rev* 5′ splice site is essential to obtain high steady-state levels of *env* mRNA whether or not *rev* is present. It is possible that complexing to U1 snRNA either directly protects the RNA from nucleases or enables it to be transported to a privileged compartment within the nucleus. Alternatively it cannot be excluded that binding to U1 snRNA could be required at the transcriptional level, although this seems less likely. Further experiments will be necessary to discriminate between these different possibilities.

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