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Regulation of Human Immunodeficiency Virus *env* Expression by the *rev* Gene Product

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A single simian virus 40 late replacement vector which expresses both the *rev* and envelope (*env*) genes of human immunodeficiency virus was used to examine the mechanism underlying the dependence of *env* gene expression on the rev protein. When *rev* was deleted from the vector, no envelope protein expression could be detected in transfected cells, and the levels of cytoplasmic *env* mRNA were dramatically reduced. In contrast to this, the levels of *env* RNA in total cellular RNA preparations were similar with or without *rev* coexpression, and analysis of nuclear RNA showed that the levels of nuclear *env* RNA were increased in the absence of *rev*. These results suggest that *rev* functions to regulate nuclear export of *env* mRNA. It was possible to restore *env* expression from the vector lacking *rev* by supplying *rev* in *trans*, provided that a *cis*-acting sequence was also present. This sequence was mapped to a 854-base-pair region within the *env* open reading frame, and it was shown that the sequence could be moved but that it worked only in its original orientation.

The human immunodeficiency virus (HIV) genome contains at least six novel genes (*vif*, *vpr*, *tat*, *rev*, *vpu*, and *nef*) in addition to the three genes common to all retroviruses (*gag*, *pol*, and *env*) (11). Several of these genes have been implicated in the regulation of virus growth. The product of the *tat* gene has been shown to dramatically increase overall viral gene expression (1, 25, 31, 32), and another regulatory gene, *rev* (formerly called *art* or *trs*), encodes a protein that selectively enhances the expression of *gag* and *env* (9, 30). In contrast, the product of *nef* (*3'orf*) may function to reduce virus yields (13, 18). It is likely that a complex interplay between negative and positive regulators determines the course of HIV infection and leads to the unusual ability of this virus to switch between a latent and a productive state.

The *tat* and *rev* gene products are encoded in the same region of the HIV genome (28, 30). Each gene contains two coding exons. Both exons of *tat* partially overlap those of *rev*. In addition, the second exons completely overlap the coding region for the envelope protein. Several groups have studied the function of the *tat* gene and have shown that the tat protein is a transactivator which acts in consort with a target region located downstream of the promoter in the viral long terminal repeat (15, 21, 22, 25, 26, 33). Mutations in this gene make clones of proviral DNA noninfectious (5, 10).

The *rev* gene, like *tat*, has been shown to be essential for the production of virus. Two groups, using infectious proviral clones to study its function, demonstrated that mutations in this gene led to severely reduced levels of proteins from *gag* and *env* (9, 30). However, different underlying mechanisms for this effect were suggested. Sodroski et al. (30) reported normal levels of all species of mRNA even in the absence of *rev* and thus hypothesized that *rev* might affect posttranscriptional utilization of *gag* and *env* mRNA, possibly by derepressing *cis*-acting negative regulatory sequences present in the mRNAs. They thus proposed the name *art* (antirepression transactivator) for this gene. In contrast to this, Feinberg et al. (9) reported that in the absence of *rev*, the levels of the large mRNAs encoding the structural proteins were reduced, whereas the levels of doubly spliced small mRNAs encoding nonstructural proteins were increased. They thus suggested that the *rev* gene might directly regulate splicing and proposed the name *trs* (transregulator of splicing). Subsequently, Knight et al. (16), utilizing a vector in which the envelope gene was placed under the control of the HIV long terminal repeat, proposed that *rev* may regulate *env* gene expression by directly relieving a translational block. This hypothesis was based on the fact that envelope protein was not detected in transfected cells unless the *rev* and *tat* genes were also supplied, despite the fact that high levels of *env* RNA were detected even in the absence of *rev*. Because of these controversies, *art*/*trs* has been renamed *rev* (regulator of expression of virion proteins) (11).

Recently, Rosen et al. (27) described experiments using chimeric constructions which fuse the coding regions of chloramphenicol acetyltransferase (*CAT*) or human growth hormone (*HGH*) genes to different portions of the HIV envelope gene. The data obtained from these experiments supported the notion that there were sequences located within the coding region of the envelope gene that exerted a negative effect on expression. This negative effect could be overcome by providing *rev* in *trans*. In addition, deletion analysis suggested that there might be a specific region within the *env*-coding region that was required for the *rev* response. The mechanism underlying this regulation was not further elucidated since no RNA analysis was performed.

We have recently described efficient envelope protein and *tat* expression from a single simian virus 40 (SV40) late replacement vector, pSVSX1 (24). In this vector, a fragment of HIV proviral DNA containing the entire *tat*, *rev*, *vpu*, and *env* genes was positioned between the SV40 late promoter and a segment of the rabbit β-globin gene which includes an intron and a polyadenylation signal (Fig. 1). Levels of *env* expression were shown to be very high in this system due to the strong SV40 late promoter and amplification of the vector by replication. We thus reasoned that the vector might provide a convenient model to specifically study *rev* regulation of the *env* gene, providing that it also produced rev and that *env* expression could be shown to be *rev* dependent. Although produced by the vector, *tat* was not

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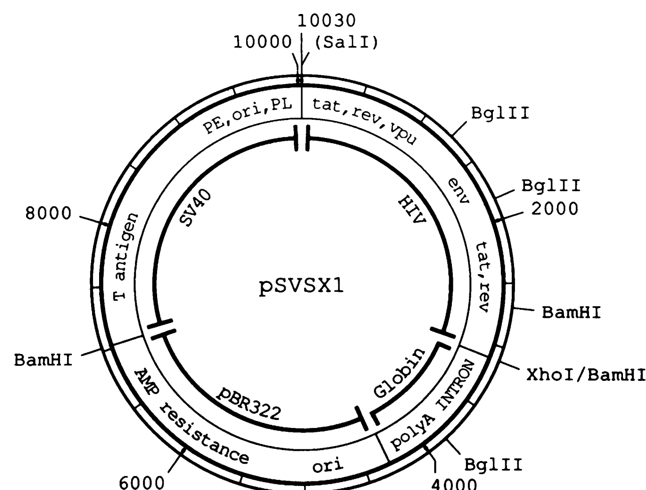

FIG. 1. Expression vector pSVSX1. The vector is composed of sequences derived from HIV-1 (BH-10 clone), rabbit β-globin, pBR322, and SV40. Its construction has been previously described (24). The site marked *XhoI*/*BamHI* indicates two adjacent restriction sites. The map was drawn by computer using the programs of the University of Wisconsin Genetics Computer Group (6).

expected to have an effect on *env* expression since the target region was not present.

In the work presented here, we demonstrate that *env* expression from pSVSX1 requires coexpression of *rev*. This has enabled us to perform an analysis of *rev* gene function. Our results show that when *rev* was deleted from the vector, steady-state levels of *env* mRNA in the cytoplasm were greatly reduced and no env protein could be detected unless *rev* was provided in *trans*. In contrast, there was little effect on the levels of total *env* RNA, and *env* RNA accumulated in the nucleus. These results strongly suggest that *rev* exerts its effect by regulating nuclear export of *env* mRNA. Furthermore, we have verified the existence of a *rev*-responsive *cis*-acting element in the envelope-coding region and demonstrated that this element must be present in order to obtain significant levels of *env* mRNA in the cytoplasm. We have also shown that the element can be moved but that its effect is orientation dependent.

MATERIALS AND METHODS

Cells and transfections. CMT3 COS cells (12) were obtained from Y. Gluzman. These cells were transfected by a modification of the DEAE-dextran method as previously described (14). For the RNA and protein analysis, the cells were harvested at 48 and 65 h posttransfection, respectively.

Vector constructions. Construction of the vector pSVSX1 was described previously (24). Deletion mutants of pSVSX1 were made by using existing restriction enzyme sites as outlined in Fig. 2.

Western (immuno-) blot analysis. The Western blots were performed as previously described (14) except that the proteins were transferred to Immobilon P membranes (Millipore Corp.) under the conditions recommended by the manufacturer. The sera used for developing the blots were either human sera from HIV-positive individuals or a goat serum directed against gp160 produced by using a baculovirus vector (a kind gift from Scott Putney and Jim Rusche). The reactive proteins were visualized with the Protoblot system (Promega Biotec).

Preparation of RNA from transfected cells. Nuclear and total RNA was extracted by the guanidinium-isothiocyanate method (2). Cytoplasmic RNA was extracted, and poly(A) was selected essentially as described by Favaloro et al. (7).

Northern (RNA) blot analysis. Poly$(A)^+$ cytoplasmic RNA (3 or 5 μg per lane), nuclear RNA (40 μg per lane), or total RNA (25 μg per lane) was separated on 1% formaldehyde-agarose gels (23). The RNA was then transferred to Nitroplus 2000 membranes (MSI) and hybridized to end-labeled oligonucleotide probes or to restriction fragments labeled by the random primer method (8).

RESULTS

Transactivation of envelope protein expression by *rev*. The vector pSVSX1 contains the *SalI*-*XhoI* fragment (nucleotides 5785 to 8896) of the BH10 clone of HIV-1 DNA (29). This fragment encodes at least three small proteins (tat, rev, and vpu) in addition to the envelope proteins (Fig. 1 and 2) (3, 9, 20, 30). In our previous work, immunological detection methods were used to show that large amounts of envelope proteins were produced by this vector, and transactivation experiments allowed us to conclude that the vector also expressed a functional *tat* gene product. To analyze whether the *tat* gene product affected envelope expression, we made a deletion in pSVSX1 which removed part of the first coding exon of *tat* but left the *rev*, *vpu*, and *env* open reading frames intact. This vector was called pSVSX3 and produced as much envelope protein as pSVSX1 (data not shown), clearly demonstrating that *tat* expression is not necessary for efficient envelope expression in this system. Both pSVSX1 and pSVSX3 also produced a 20-kilodalton protein detectable on Western blots and by immunoprecipitation with a rev-specific antiserum (data not shown).

Other deletions in pSVSX1 were then made which were designed to assess the role of *rev* on *env* expression in this system (Fig. 2). Three of these affected the coding exons of *rev* and would not be expected to produce any envelope proteins if rev protein was required. pSVSX1Δ1 lacked part of the first coding exon of *rev* but contained a normal envelope gene, and if *env* expression occurred, normal-sized gp160, gp120, and gp41 would be produced. pSVSX1Δ2 contained a deletion in the second coding exon of *rev*. If *env* expression occurred, it should make normal-sized gp120 but truncated forms of gp160 and gp41. pSVSX1Δ4 lacked the entire second coding exon of *rev*. If *env* expression occurred, it was expected to produce a secreted form of gp120, since, in addition to the indicated deletion, it contained a stop codon (TAG) in the *env* open reading frame created by inserting an *XbaI* linker at the *StyI* site (nucleotide 7719). The deletion in pSVSX1Δ3 did not alter the *rev*-coding exons but caused a frame shift which introduced a stop codon in the envelope reading frame immediately 3′ of the deletion. This vector was expected to make a 60- to 70-kilodalton envelope protein. It produced a rev protein which was detectable on Western blots with a rev-specific antiserum (data not shown).

When expression from these mutants in transfected COS cells was analyzed on Western blots with an *env*-specific serum, we failed to detect any envelope protein from either pSVSX1Δ2, pSVSX1Δ4, or pSVSX1Δ1 (Fig. 3, lanes 3, 4, and 5). Likewise, no envelope protein could be found in the medium from these cells (data not shown). However,

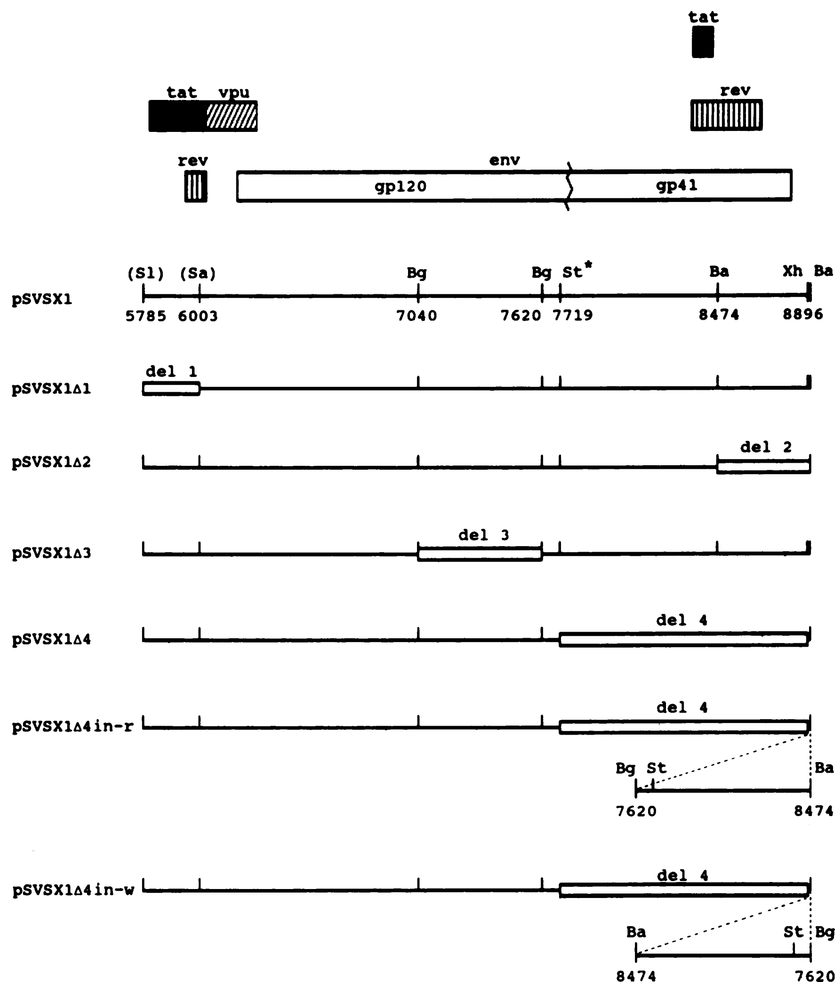

FIG. 2. Deletion mutants of pSVSX1. A diagram of the HIV-1 (BH-10 clone) insert contained within pSVSX1 is shown. The top part of the figure shows the positions of open reading frames encoding known proteins. The cleavage site between gp120 and gp41 is indicated. The nucleotide numbering system utilized corresponds to the HIV-1 reference genome (Hxb2cg) contained in GenBank. Restriction enzyme sites are abbreviated as follows: Sl, *Sal*I; Sa, *Sac*I; Bg, *Bgl*II; St, *Sty*I; Ba, *Bam*HI; Xh, *Xho*I. Only one *Sty*I site (*) of the four present in the HIV segment is shown. This site has been changed by addition of an *Xba*I linker in pSVSX1Δ4 and its derivatives. pSVSX1Δ1 was constructed from the BH-8 clone of HIV-1 by using the indicated *Sac*I site, which is not present in the BH-10 clone. The *Bam*HI site at the extreme right of the figure is located in the adjacent vector sequences. The *Sal*I site is no longer present in pSVSX1 as a result of cloning into an *Xho*I site. Deletions of DNA relative to pSVSX1 are shown (▭), as are insertions of DNA (– – –).

pSVSX1Δ3 could be shown to produce an envelope protein of the expected size (gp70) (Fig. 4, lane 7). This band is not visible in Fig. 3, which shows only the gp160/gp120 region of the gel.

These results suggested that *rev* was necessary for envelope protein expression, since part of this gene was deleted in pSVSX1Δ1, pSVSX1Δ2, and pSVSX1Δ4, whereas it was intact in pSVSX1Δ3. We therefore performed an experiment in which pSVSX1Δ1, pSVSX1Δ2, or pSVSX1Δ4 was cotransfected into COS cells together with pSVSX1Δ3. Since pSVSX1Δ3 contains an intact *rev* gene, it would be expected to supply *rev* in *trans*. The results of this experiment are presented in Fig. 3, lanes 7 to 9. In this figure, envelope-specific bands of the sizes expected from pSVSX1Δ1 (lane 9) and pSVSX1Δ2 (lane 7) can be seen in the cotransfections. This demonstrates that providing *rev* in *trans* is sufficient to restore envelope protein expression from these two vectors. In contrast, pSVSX1Δ4 (lane 8) still failed to express any detectable envelope protein, suggesting that this construct might lack *cis*-acting sequences which are present in the other vectors and which are necessary for the *rev* response. The same results were obtained with an SV40 late replacement vector containing a *rev* cDNA (pSVrev) to supply *rev* in *trans* (data not shown).

A *cis*-acting sequence is required for the function of *rev*. A comparison of the different deletions (Fig. 2) shows that pSVSX1Δ4 lacks a region of HIV DNA present in all of the other constructions. This region maps between HIV nucleotides 7719 and 8474 and is totally contained within a slightly larger *Bgl*II-*Bam*HI fragment (nucleotides 7620 to 8474). To test the hypothesis that *rev* may act in consort with a *cis*-acting sequence within this fragment, the fragment was inserted into pSVSX1Δ4 immediately 3′ of the deletion. This created the plasmids pSVSX1Δ4in-r and pSVSX1Δ4in-w (Fig. 2).

These constructs, as well as their parent vector pSVSX1Δ4, were then transfected into COS cells, either individually or together with pSVSX1Δ3, to supply *rev* in *trans*. The results of this experiment are shown in Fig. 4. Envelope protein could not be detected in the transfections

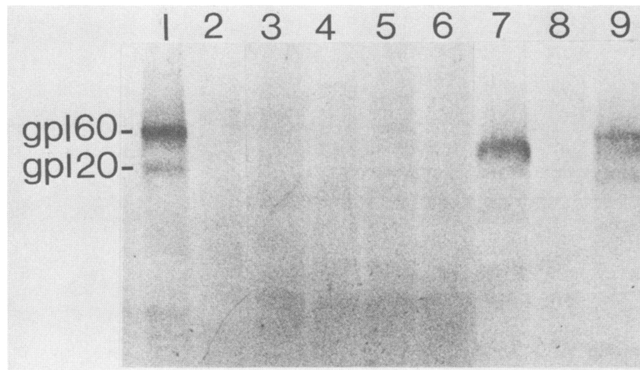

FIG. 3. Western blot analysis of proteins from transfections and cotransfections with pSVSX1 and various deletion mutants. Proteins from transfected cells were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel. The blot was developed with serum from a goat immunized with gp160. Cultures were transfected individually with pSVSX1 (lane 1), vector control (lane 2), pSVSX1Δ2 (lane 3), pSVSX1Δ4 (lane 4), pSVSX1Δ1 (lane 5), or pSVSX1Δ3 (lane 6), or were cotransfected with pSVSX1Δ3 plus either pSVSX1Δ2 (lane 7), pSVSX1Δ4 (lane 8), or pSVSX1Δ1 (lane 9).

of any of the individual vectors except pSVSX1Δ3 (lanes 1, 3, 5, and 7). In the cotransfections, however, a protein of the expected size (gp120) was produced with pSVSX1Δ4in-r (lane 4) but not with either pSVSX1Δ4 or pSVSX1Δ4in-w (lanes 2 and 6). The band of gp70 from pSVSX1Δ3 was also detected in all of the cotransfections. Identical results were obtained when concentrated medium from the transfected cell cultures was analyzed (data not shown). These results indicate that there is indeed a *cis*-acting sequence mapping between HIV nucleotides 7620 and 8474 which, in addition

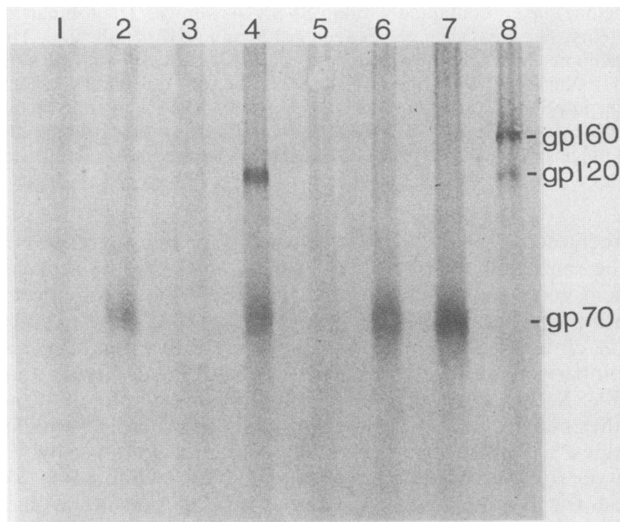

FIG. 4. Western blot analysis of proteins from transfections and cotransfections with pSVSX1 and various derivatives. Proteins from transfected cells were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel. The blot was developed with the goat serum used in Fig. 3. Lanes 1, 3, and 5 show individual transfections with pSVSX1Δ4, pSVSX1Δ4in-r, and pSVSX1Δ4in-w, respectively. Lanes 2, 4, and 6 show cotransfections with the same vectors and pSVSX1Δ3. Lanes 7 and 8 show cells transfected individually with pSVSX1Δ3 and pSVSX1, respectively.

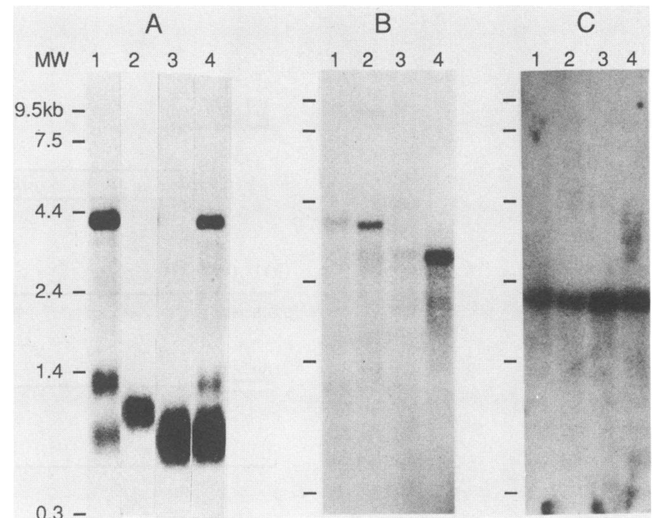

FIG. 5. Northern blot analysis of poly(A)$^+$ cytoplasmic RNA from transfections and cotransfections with pSVSX1 and various deletion mutants. Each lane contains 5 μg of RNA from transfected cells, which was separated on formaldehyde-containing agarose gels. (A) RNA from cells transfected with pSVSX1 (lane 1), pSVSX1Δ2 (lane 2), pSVSX1Δ4 (lane 3), and pSVSX1 plus pSVSX1Δ4 (lane 4). The blot was probed with a ^{32}P-labeled oligonucleotide from the first coding exon of *tat*. (B) RNA from cells transfected with pSVSX1Δ1 (lane 1) and pSVSX1Δ2 (lane 3) alone or together with pSVSX1Δ3 (lanes 2 and 4, respectively). The blot was probed with the *Bgl*II fragment of HIV DNA mapping between nucleotides 7040 and 7620 (Fig. 2). The probe was ^{32}P-labeled by random priming. The region constituting the probe is lacking from pSVSX1Δ3. (C) Blot in panel B stripped and probed with a ^{32}P-labeled cDNA copy of chicken actin mRNA.

to the rev protein, is required for envelope protein expression. Since pSVSX1Δ4in-r can produce envelope protein when rev is supplied but pSVSX1Δ4in-w fails to do so, we can conclude that this sequence functions only in its original orientation.

***rev* regulates the levels of steady-state cytoplasmic *env* mRNA.** To further analyze the mechanism underlying *rev* regulation of *env* expression, we isolated poly(A)-containing cytoplasmic RNA from cells transfected with either pSVSX1 or the different deletion mutants. RNA was also isolated from cells cotransfected with each of these mutants and pSVSX1 or pSVSX1Δ3 to provide *rev* in *trans*. Equal amounts of RNA from each transfection were then subjected to Northern blot analysis with various probes. Autoradiograms of these blots are shown in Fig. 5.

The blot in Fig. 5A was probed with an oligonucleotide mapping within the first coding exon of *tat*. Figure 5A, lane 1, contains RNA isolated from cells transfected with pSVSX1 alone. Three bands with molecular sizes of 4.1, 1.1, and 0.6 kilobases (kb) were detected. The same pattern of hybridization was seen when this blot was probed with the entire HIV insert, whereas probing with a *Bgl*II fragment from the *env* open reading frame (HIV nucleotides 7040 to 7620; Fig. 2) detected only the 4.1-kb band (data not shown).

The 4.1-kb band is of the size predicted for a totally unspliced mRNA starting at the SV40 late promoter and terminating close to the β-globin poly(A) addition signal. This band must represent the envelope protein mRNA since no other high-molecular-weight species could be detected with any of the probes. The fact that even a probe from the first open reading frame of *tat* hybridized to this RNA is of

considerable interest, since it suggests that the envelope protein is produced from a mRNA in which *env* is the fourth open reading frame. Consistent with this, primer extension sequence analysis of mRNA with an oligonucleotide mapping at the start of the *env* open reading frame failed to detect a splice in front of the envelope gene (data not shown).

The lower-molecular-weight RNAs represent spliced species that probably utilize the known splice donors and acceptors. The 1.1-kb species is consistent in size and hybridization pattern with an RNA spliced at both the *tat*/*rev* donor-acceptor and the β-globin donor-acceptor and probably is the mRNA that produces tat and rev. The 0.6-kb species is consistent in size and hybridization pattern with an RNA in which all of the sequences between the *tat*/*rev* splice donor and the β-globin splice acceptor have been removed. Such an RNA would be expected to express a protein corresponding to the first coding exon of *tat*. Probing the blot with an oligonucleotide specific for the β-globin intron confirmed that the intron is removed from the smaller species but is still present in the 4.1-kb transcript (data not shown).

When RNA was isolated from cells transfected with either pSVSX1Δ1, pSVSX1Δ2, or pSVSX1Δ4, only very faint bands were seen at the positions expected for their respective *env* mRNAs, in contrast to the abundant *env* RNA observed in cells transfected with pSVSX1. For pSVSX1Δ2, the *tat* oligonucleotide probe detected a strong band at 0.7 kb and an extremely faint band at approximately 3.2 kb (Fig. 5, lane 2; the 3.2-kb band is not visible in the photograph). For pSVSX1Δ4 (Fig. 5A, lane 3), this probe detected a strongly hybridizing broad band at 0.6 to 0.7 kb and a very faint band at approximately 3.0 kb. For pSVSX1Δ1 (Fig. 5B, lane 1), the *Bgl*II fragment specific for *env* mRNA detected only a weak band at 3.9 kb. When the pSVSX1Δ2 RNA preparation was probed with this fragment, only the faint 3.2-kb band was visible (Fig. 5B, lane 3). Thus, the lack of envelope protein expression from all of these constructions seems to be a result of severely reduced levels of poly(A)-containing cytoplasmic *env* mRNA.

In accordance with the protein data, supplying *rev* in *trans* dramatically increased the levels of cytoplasmic *env* mRNA in the case of pSVSX1Δ1 (Fig. 5B, lane 2) and pSVSX1Δ2 (Fig. 5B, lane 4) but not in the case of pSVSX1Δ4 (Fig. 5A, lane 4). In these experiments, pSVSX1Δ3 and pSVSX1 were the vectors which were used to supply *rev*. RNA originating from pSVSX1Δ3 was not detected in Fig. 5B, since the *Bgl*II fragment used to probe this blot is not present in this vector (Fig. 2). RNA originating from pSVSX1 was seen in Fig. 5A, lane 4, but its 4.1-kb *env* mRNA was readily distinguishable from the barely visible 3.0-kb *env* mRNA of pSVSX1Δ4. Figure 5C shows the blot in Fig. 5B reprobed with a chicken actin cDNA to demonstrate that equal amounts of RNA were present in each lane.

It should be noted that the 3.2-kb mRNA from pSVSX1Δ2 is 900 bp smaller than the 4.1-kb species seen with pSVSX1 despite the fact that pSVSX1Δ2 contains only a 400-bp DNA deletion. The reason for this is that this RNA lacks the β-globin intron (573 bp) still present in the mRNA from pSVSX1. In this experiment, only a very faint band of nonspliced RNA (3.7 kb) was present (not visible in the photograph). In other experiments, somewhat more unspliced *env* mRNA could be detected, but the spliced form always predominated (e.g., see Fig. 6, lane 4). With pSVSX1Δ1, which contains a 200-bp deletion in the DNA, the *env* mRNA is 3.9 kb, consistent with the β-globin intron still being present in the RNA. It thus appears that sequences mapping within the DNA deleted from pSVSX1Δ2 affect utilization of the β-globin splice donor-acceptor. Further experiments will be needed to determine the significance of this observation.

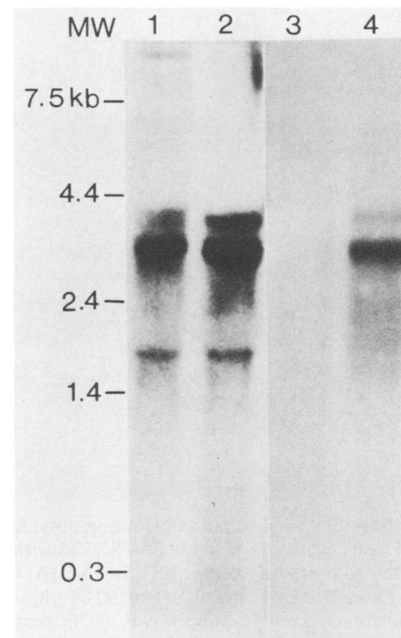

FIG. 6. Northern blot analysis of total and poly(A)$^+$ cytoplasmic RNA from cells transfected with pSVSX1Δ2 either alone or together with the *rev*-supplying vector pSVSX1Δ3. Total RNA (25 μg) from cells transfected with pSVSX1Δ2 (lane 1) or pSVSX1Δ2 plus pSVSX1Δ3 (lane 2) or with cytoplasmic poly(A)$^+$ RNA (3 μg) from cells transfected with the same vectors (lanes 3 and 4, respectively) was separated on a formaldehyde-containing agarose gel. The blot was probed with the *Bgl*II fragment used in Fig. 5B. Lanes 1 and 2 are from a fluorograph of the blot exposed for 17 h at −70°C. Lanes 3 and 4 are from an autoradiograph exposed for 48 h.

***env* RNA accumulates in the nucleus in the absence of *rev* expression.** To determine whether the decreased steady-state levels of cytoplasmic poly(A)$^+$ *env* mRNA in pSVSX1Δ2-transfected cells were paralleled by a decrease in the overall levels of *env*-containing RNA, total RNA from transfected cells was prepared and analyzed. As a control, cytoplasmic poly(A)$^+$ RNA was extracted from the same cells. In these experiments, pSVSX1Δ2 was transfected either alone or together with pSVSX1Δ3 to supply *rev* in *trans*. The RNA was then subjected to Northern blot analysis, using the *env*-specific *Bgl*II fragment as a probe. As in the experiments above, this probe could not detect RNA from pSVSX1Δ3, since the *Bgl*II fragment was deleted in that vector. Figure 6 shows the results of this analysis. Lanes 1 and 2 show the hybridization to total RNA from cells transfected with pSVSX1Δ2 either alone (lane 1) or together with pSVSX1Δ3 (lane 2). The hybridization to poly(A)$^+$ cytoplasmic RNA from the same transfections is shown in lane 3 (pSVSX1Δ2) and lane 4 (pSVSX1Δ2 and pSVSX1Δ3).

Once again, as in the experiment described above, the cytoplasmic RNA preparation from cells transfected with pSVSX1Δ2 alone contained greatly diminished levels of *env* mRNA compared with the preparation from the cotransfected cells. However, significant levels of *env* RNA could be detected in each of the two total RNA preparations. The cotransfection appeared to increase total *env* RNA levels only slightly, suggesting that significant levels of *env* RNA

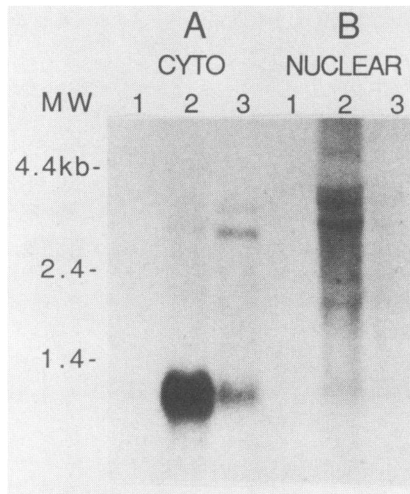

FIG. 7. Northern blot analysis of nuclear and poly(A)$^+$ cytoplasmic RNA from cells transfected with pSVSX1Δ2 either alone or together with the *rev*-supplying vector pSVSX3Δ3. (A) Cytoplasmic poly(A)$^+$ RNA (3 μg per lane). (B) Nuclear RNA (40 μg per lane). pSVSX3Δ3, the vector used to supply *rev*, lacks part of the first coding exon of *tat* complementary to the oligonucleotide used as a hybridization probe. RNA isolated from cells transfected with pSVSX3Δ3 (lanes 1), pSVSX1Δ2 (lanes 2), and pSVSX1Δ2 plus pSVSX3Δ3 (lanes 3) is shown. RNAs were separated on a formaldehyde-containing agarose gel which was blotted and fluorographed.

accumulated in the nucleus in the absence of *rev*. In addition to the two high-molecular-weight *env* RNAs with (3.9 kb) and without (3.2 kb) the β-globin intron, a third band was observed in total RNA. This band may represent a splicing intermediate or by-product containing the intron from the envelope region. Hybridization of these blots with the chicken actin cDNA probe used in Fig. 5C gave equivalent signals in lanes 1 and 2 and in lanes 3 and 4 (data not shown), demonstrating that equal amounts of RNA were compared.

To directly examine nuclear RNA, another experiment was performed. As before, cells were transfected with either pSVSX1Δ2 alone or together with a vector which supplied *rev*. In this case, the *rev*-containing vector (pSVSX3Δ3) was similar to pSVSX1Δ3. It was made from pSVSX3 (see above) which lacks part of the first coding exon of *tat*. RNA was isolated from both nuclei and cytoplasm and analyzed on the Northern blot shown in Fig. 7. An oligonucleotide mapping within the first coding exon of *tat* was used as a hybridization probe. This probe would not be expected to hybridize to RNA from the *rev*-supplying vector pSVSX3Δ3 since this vector was deleted in the region complementary to the probe.

Figure 7A shows poly(A)$^+$ RNA isolated from cytoplasm, whereas Fig. 7B shows RNA isolated from nuclei. As before, greatly reduced levels of *env* RNA were seen in the cytoplasmic RNA preparations of cells transfected with pSVSX1Δ2 alone (lane 2A) compared with the levels seen when pSVSX1Δ2 was transfected together with the vector which expressed *rev* (lane 3A). However, in contrast to this, nuclear RNA from cells transfected with pSVSX1Δ2 alone (lane 2B) contained considerable amounts of *env* RNA, whereas only barely detectable levels of *env* RNA were found in the nuclear preparations from cells cotransfected with pSVSX1Δ2 and the *rev*-containing vector (lane 3B). As expected, no RNA could be detected in either the cytoplasmic or nuclear preparations from the cells transfected with pSVSX3Δ3 alone (lanes 1A and 1B). It was also observed that there was considerably more of the smaller *tat*/*rev* mRNA in the cytoplasmic preparations from cells transfected with pSVSX1Δ2 alone (lane 2A) compared with the levels observed in the cotransfection (lane 3A). Very little of this RNA was detected in the nuclear preparations whether or not *rev* was present. As before, a chicken actin cDNA probe was used to demonstrate equivalent RNA loading (data not shown).

DISCUSSION

The data presented here demonstrate that HIV envelope protein expression from a vector with a heterologous promoter and polyadenylation signal requires *rev*. This mimics the situation in HIV-infected cells, in which it has been shown that *rev* is necessary for both *env* and *gag* expression (9, 30). In infected cells, the study of *env* expression is complicated by the fact that expression of *env*, as well as of all the other viral genes, is also regulated by *tat*. In our system, *tat* is not required since the viral long terminal repeat is not used. We have thus been able to study the function of *rev* separated from any effect of *tat*. Furthermore, the analysis has been greatly facilitated by the large amounts of viral protein and RNA obtained in this system compared with the levels seen in virus-infected cells.

Expression of *env* from pSVSX1 seems to occur in a highly unorthodox manner. Not only does it require the *rev* gene, but our results also suggest that the protein is being made from an mRNA in which *env* is the fourth open reading frame downstream from *tat*, *rev*, and *vpu*. Thus the proteins encoded by *vpu* and the first coding exons of *tat* and *rev* could also be made from this message. As a consequence of this, these proteins would also be regulated by *rev*. In this context, it should be noted that Malim et al. (19) recently reported the synthesis of a truncated tat protein from a construct similar to ours. The expression of this protein was *rev* dependent. It remains to be determined whether these proteins are made from an analogous mRNA during HIV infection.

In the absence of *rev*, severely reduced levels of steady-state *env* mRNA were observed in the cytoplasm, whereas total *env* RNA levels did not appear to be dramatically affected. Analysis of nuclear RNA showed large amounts of *env* RNA in the absence of *rev* but only minute amounts in the presence of *rev*. In contrast to this, accumulation in the nucleus of the spliced species was not observed. Thus the most likely explanation for our results is a specific accumulation of *env* RNA in the nucleus when *rev* is not present. This implies that there must be some property of the *env* RNA which is not shared by the spliced RNA species encoding *tat* and *rev* and which makes transport of *env* RNA to the cytoplasm *rev* dependent. This property could be either the presence of a discrete linear sequence of nucleotides in the *env* RNA or a specific secondary structure. Our data show that the sequences present in pSVSX1Δ4 are sufficient to make *env* expression *rev* dependent, since this construct failed to express cytoplasmic *env* mRNA. Rosen et al. recently showed that positioning sequences from the *env* region downstream of the *CAT* gene had a negative effect on *CAT* expression (27). Their experiments suggested that there might be several regions within the *env* gene that exerted this negative effect. Two of these regions are not present in pSVSX1Δ4. Further studies are clearly necessary to define the exact regions involved in *rev* dependence.

Our experiments show that the function of *rev* requires yet another region in the *env* gene. This element maps entirely

within a *Bgl*II-*Bam*HI fragment (nucleotides 7620 to 8474). This region must be *cis*-acting, since pSVSX1, which can express *env*, failed to complement pSVSX1Δ4 (Fig. 5A, lane 4). These results are in accordance with those of Rosen et al. (27), who mapped sequences important for the *rev* response close to the 5′ end of this fragment. We have further shown that the *rev*-responsive element can be moved but that it has to be present in the correct orientation for *rev* to function. Similar data utilizing *CAT* and *gag* hybrid constructs have also recently been described by G. Pavlakis (personal communication). These data are consistent with *rev* binding directly to the *rev*-responsive element in the RNA or to cellular proteins that interact with this element. *rev* could also work by regulating the expression of the genes encoding such proteins. In this context, it is interesting to note that the rev protein appears to be associated with the nucleoli (4) and that a region of nonrandom secondary structure has been predicted in the area of the HIV RNA where we have mapped the *rev*-responsive element (17).

Several reports have interpreted results to indicate that *rev* regulates *env* expression through differential splicing. Feinberg et al. (9), utilizing *rev*-defective clones of virus, reported that the levels of *env* and *gag* RNA in total RNA preparations from cells transfected with these clones were severely reduced, while there was a concomitant increase in the levels of doubly spliced RNAs. Likewise, Malim et al. (19), utilizing constructs containing the *env* region, showed that the levels of cytoplasmic spliced RNA were increased in the absence of *rev* compared with when *rev* was present. Also in our experiments, the amount of small spliced mRNA in cytoplasmic preparations was increased in the absence of *rev* (cf. Fig. 5A, lanes 1 and 3; also Fig. 7A, lanes 2 and 3). These results are not in conflict with our hypothesis that the primary function of *rev* is to act as a regulator of nuclear export. Accumulation of nuclear RNA could make a larger number of molecules available for splicing, which in turn could lead to increased amounts of the spliced species. If these spliced molecules are more stable than the unspliced *env* RNA species, higher steady-state levels would result. A primary effect of *rev* on transport is also consistent with the fact that expression of *env*-containing RNA has been shown to be *rev* dependent even from vectors unable to splice (16, 27).

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