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Elements Distinct from Human Immunodeficiency Virus Type 1 Splice Sites Are Responsible for the Rev Dependence of *env* mRNA

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In the absence of the viral regulatory protein Rev, the human immunodeficiency virus type 1 *gag/pol* and *env* mRNAs are inefficiently expressed, since nucleocytoplasmic transport, stability, and polysomal loading are impaired. It has been suggested that splicing is necessary for Rev function and that the low expression of the unspliced and intermediate spliced mRNAs in the absence of Rev is associated with specific splice sites. Previous studies identified distinct RNA elements within the *gag/pol* region responsible for low expression that are not associated with splice sites. Here we study the determinants for Rev dependence of the authentic *env* mRNA. We demonstrate that upon removal of all the utilized splice sites, the *env* mRNA is still Rev dependent and Rev responsive for expression in human cells. We have identified several regions within the *env* mRNA that inhibit expression of a *gag-env* hybrid mRNA. Elimination of one of these elements, located within the Rev-responsive element, did not result in virus expression, supporting our model that several independently acting elements are responsible for the downregulatory effect. By analogy to the RNA elements within the *gag/pol* region, we propose that elements unrelated to utilized splice sites are responsible for the posttranscriptional regulation of *env* mRNA.

Human immunodeficiency virus type 1 (HIV-1) uses an elaborate posttranscriptional regulatory system that involves both nuclear and cytoplasmic events to control expression of its viral mRNAs (for reviews, see references 7, 12, 14, 16, 25, and 34 and references therein). HIV-1 produces two subsets of mRNAs: the small multiply spliced species encoding Tat, Rev, and Nef and the intermediate spliced and unspliced mRNAs expressing the structural and accessory proteins Gag, Pol, Vif, Vpr, Vpu, and Env. The latter group is expressed very poorly in the absence of Rev. Rev interacts directly with a distinct RNA element, termed the Rev-responsive element (RRE), within the *env* coding region, which is present in the unspliced and partially spliced mRNAs. The presence of Rev regulates these mRNAs at the posttranscriptional level, resulting in their efficient expression. This finding led to the proposal that the mRNAs encoding viral structural proteins are expressed poorly, because they contain elements (named INS, IR, or CRS) that prevent their expression (6, 10, 15, 22, 26, 28, 31). The presence of such elements is proposed to be responsible for the instability, nuclear retention, and inefficient translation of these RNAs. Some INS elements have been identified in the *gag* and *pol* regions of HIV-1 (6, 22, 27, 31). Several of the INS elements located within the *gag* region have been characterized in detail (27, 31). These elements span regions of about 200 to 300 nucleotides (nt) with no obvious sequence homology. Clustered point mutations changing the nucleotide but not the amino acid composition resulted in Rev-independent *gag* expression (28). It was suggested that these elements are RNA recognition sites for cellular factors, which, upon binding, are responsible for the inefficient expression of these mRNAs (28). The viral regulatory factor Rev counteracts the action of the cellular factors, resulting in a high level of expression of all the RRE-containing mRNAs.

Another model for Rev function proposes that poor expression of the HIV mRNAs is caused by inefficient splicing (5). In this model, inefficiently used splice sites (such as retroviral splice sites) are responsible for nuclear retention, while Rev dissociates the precursor mRNAs from the spliceosomes, thereby allowing their transport to the cytoplasm. According to this model, a pair of functional splice sites is required for the retention of the mRNAs. This idea was further supported by experiments showing that removal of the splice donor upstream of the *env* AUG abolished expression of *env* even in the presence of Rev (21). The data suggested that this mRNA was not recognized either by the splicing machinery or by Rev. These studies were performed with hybrid constructs based on the highly replicative simian virus 40 vector system studied in monkey Cos-1 cells. Our previous studies on subgenomic HIV-1 *gag* expression plasmids demonstrated that such mRNAs depend on Rev for expression even in the absence of any functional splice sites (2, 8, 11). In addition, a hybrid *env* expression vector not able to form spliced RNA was shown to depend on Rev for its expression (15). These findings contradict the conclusion that splicing is necessary for Rev function (5, 21). Since the subset of the intermediate HIV-1 mRNAs undergoes partial splicing, it is possible that determinants responsible for their Rev dependence, compared with the *gag/pol* mRNA or a hybrid *env* mRNA, are different from those from the authentic cDNA.

To further assess the role in splicing of Rev function, we have studied the Rev dependence of the authentic *env* encoding mRNA 1.5E in human cells. We show that HIV-1 *env*

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expression is Rev dependent also in the absence of any functional splice sites. Therefore, our results contradict previous data suggesting that the presence of a splice site upstream of the *env* coding region is necessary for efficient *env* expression (21). Furthermore, we have identified several regions within the *env* open reading frame (ORF) that are responsible for inhibition of expression. These regions do not appear to contain splice sites. Our data suggest the presence of RNA binding sites for cellular factors inhibiting expression, similar to those in the *gag/pol* region that are not associated with splice sites.

MATERIALS AND METHODS

Recombinant plasmids. pNL15E contains the HIV-1 5′ long terminal repeat (LTR) promoter, the complete *env* cDNA 1.5E, and the HIV 3′ LTR including the polyadenylation signal (30). Generated from the molecular clone pNL4-3 (1), pNL15E lacks the splice acceptor site for exon 6D, which is used in the production of the *tev* mRNA (3). To mutagenize the splice sites, the *env* ORF was subcloned as a *Sal*I-*Bam*HI fragment from pBS15E (30) into *Sal*I-*Bam*HI sites of pM13mp19, resulting in pM13env. The splice donor at nt 5592 was changed by site-directed mutagenesis (changing GCAGTA to GaAtTc, and thus introducing an *Eco*RI site) from uracil-containing single-stranded DNA as described before (19, 32). The numbering follows the HXB2R sequence (24). The oligonucleotide used also changed the *vpu* AUG to ACG; therefore, all the subsequent constructs were *vpu* mutants. The *env* ORF was cloned back into the expression vector as a *Sal*I-*Bam*HI fragment, which resulted in p1.5ESD$^-$. The splice acceptors were deleted by site-directed mutagenesis from pM13envSD$^-$ by looping out 42 nt encompassing nt 7884 to 7926 and inserting AGT, which generated a *Sca*I site. gp160 produced from this construct has an amino acid change (Asp to Thr) followed by a 13-amino-acid deletion. pNL15EDSS was generated by cloning the *Sal*I-*Bam*HI fragment back into the expression vector as described above.

To facilitate further mutagenesis, pNL15DSS was cloned as a *Pst*I fragment into the *Pst*I site of the Bluescript KS$^-$ vector, resulting in pBS15EDSS. Single-stranded uracil-containing DNA was prepared as described above and used for mutagenesis. The gp120 expression plasmid p120DSS was generated by inserting termination codons in all reading frames followed by the restriction sites for *Nru*I and *Mlu*I in the *env* ORF at the processing site (32). p120DR has a deletion (*Mlu*I in the polylinker to *Hpa*I at nt 8200) that removes the 5′ portion of the gp41 ORF, including the RRE, leaving the HIV-1 3′ LTR. To create p120pA, the region 3′ of the stop codon of gp120 in p120DSS was removed (from *Mlu*I to *Xba*I in the polylinkers) and replaced by the simian virus 40 polyadenylation signal (cloned as a blunt-ended *Eco*RI fragment containing the *Bcl*I-*Bam*HI fragment). p120R330, p120R270, and p120RD345 contain RRE330, RRE270, and RRED345 (2, 33), respectively, inserted into the *Nru*I site of p120pA.

p37M1-10 contains the HIV-1 5′ LTR promoter and p37^{gag} gene followed by multiple unique restriction sites (*Bam*HI, *Xba*I, *Xho*I, *Apa*I, and *Asp* 718) and the HIV-1 3′ LTR providing the polyadenylation signal (nt 8561 to 9265 according to the numbering of HXB2R [24]). p37M1-10 contains multiple point mutations within the p37^{gag} gene, which destroy the identified inhibitory or instability elements (27, 28, 31) and is expressed at high levels in the absence of Rev. Different fragments (fragments 1 to 6) of the *env* ORF were amplified by PCR with oligonucleotides providing a unique cloning site in their tails and inserted into the polylinker located downstream of the p37^{gag} gene in p37M1-10. Fragment 1 spans 409 bp (nt 5606 to 6014) plus *Bam*HI sites; fragment 2 spans 432 bp (nt 6004 to 6435) plus *Spe*I sites; fragment 3 spans 444 bp (nt 6435 to 6878) plus *Xba*I sites; fragment 4 spans 388 bp (nt 6879 to 7266) plus *Xho*I sites; fragment 5 spans the *Sty*I fragment of 330 bp (nt 7266 to 7595) encompassing the RRE; fragment 6 spans the *Bam*HI-*Kpn*I fragment of 541 bp (nt 8021 to 8561) and was cloned directionally by deleting 35 bp from the polylinker. Fragments 1+2, 1+2+3, and 4+5e span 830, 1,273, and 1,046 bp, respectively, and were amplified with the same oligonucleotides as described above. Fragments 1 to 4 and the combined fragments were amplified from the HIV-1 molecular clone pLW2.4 (a gift from G. Shaw), whereas fragments 5 and 6 were from the molecular clone pNL4-3.

Mutations in the RRE were introduced by site-directed mutagenesis into pBSHIV.SX. This plasmid contains the HIV-1 *Sal*I-to-*Xho*I fragment (nt 5332 to 8443) from pNL4-3 (1) inserted into Bluescript KS$^-$. Three oligonucleotides that were designed to change the nucleotide composition of the RRE without affecting the *env* ORF were introduced, resulting in pBSHIVR^{234}. The mutant RRE (RRE^{234}) encompassing the *Sty* 330 fragment was PCR amplified, subcloned into the *Eco*RV site of Bluescript KS$^+$, and then cloned directionally into the *Cla*I-*Xho*I sites of p37M1-4, resulting in p37M1-4/R^{234}. p37M1-4 contains the p37^{gag} gene with a mutated INS-1 element inserted between the HIV-1 LTRs (28) and lacks part of the polylinker downstream of the 3′ LTR (*Sma*I-*Xba*I). This construct produces Gag in the absence of Rev. Insertion of RRE (*Sty* 330 fragment) 3′ to the *gag* gene generated p37M1-4/RRE. The following oligonucleotides were used (capital letters indicate introduced changes): agcgtcaatgacCctTacCgt GcaAgcTCgCcaGCtGCtgtctggtatag (oligonucleotide 2) (nt 7366 to 7415), tgcagcagcagaacaaCCtgctCCgCgcAatCgaAgc CcaGcagcatctgttgcaa (oligonucleotide 3) (nt 7416 to 7471), and ccaggcaagaatcctTgcCgtTgaGagGtaTctGaaAgaCcaGcaAct Tcttgggatttggggttgc (oligonucleotide 4) (nt 7498 to 7564). pNL4-3R^{234} contains the mutant RRE^{234} inserted as a *Nhe*I-*Bam*HI fragment from pBSHIVR^{234} into NL4-3 (1), replacing the homologous region. pNL4-3R^{234}/RRE has the wild-type RRE (*Sty* 330 fragment) inserted into the *Xho*I site (nt 8443), thereby destroying the *nef* gene.

Cells and transfections. HLtat is a HeLa-derived cell line that constitutively expresses HIV-1 Tat (29). The cells were transfected by the calcium phosphate coprecipitation technique as described elsewhere (11, 13), using plasmid DNA purified on Qiagen columns according to the manufacturer's instructions. The cells were incubated with the precipitates for 4 h. To study the effect of Rev, the transfection mixtures contained 1 μg of the Rev-expressing plasmid pLsrev (23) or pL3crev (11). The luciferase expression plasmid pRSVluc (9) was included in the transfection mixtures, and subsequent measurements of luciferase activity in cell lysates were performed to control for transfection efficiency. Similar amounts of luciferase were produced within each experiment. One day later, total RNA and protein were isolated from duplicate plates as described elsewhere (15, 18).

Analyses of mRNA and protein produced from transfected cells. Twenty micrograms of RNA was subjected to Northern (RNA) blot analysis as described elsewhere (15). HIV-1 mRNAs were detected with probes spanning the 3′ end of the mRNA (nt 8443 to 9118 and 6158 to 7924 of the molecular clone HXB2). The blots were rehybridized with a β-actin probe as described elsewhere (8). gp160 was visualized on Western blots (immunoblots) as described elsewhere (15), by using a mixture of HIV-1 patient plasma (Scripps Laboratory, Inc.) and rabbit anti-gp120 antiserum. To measure gp120, the cells

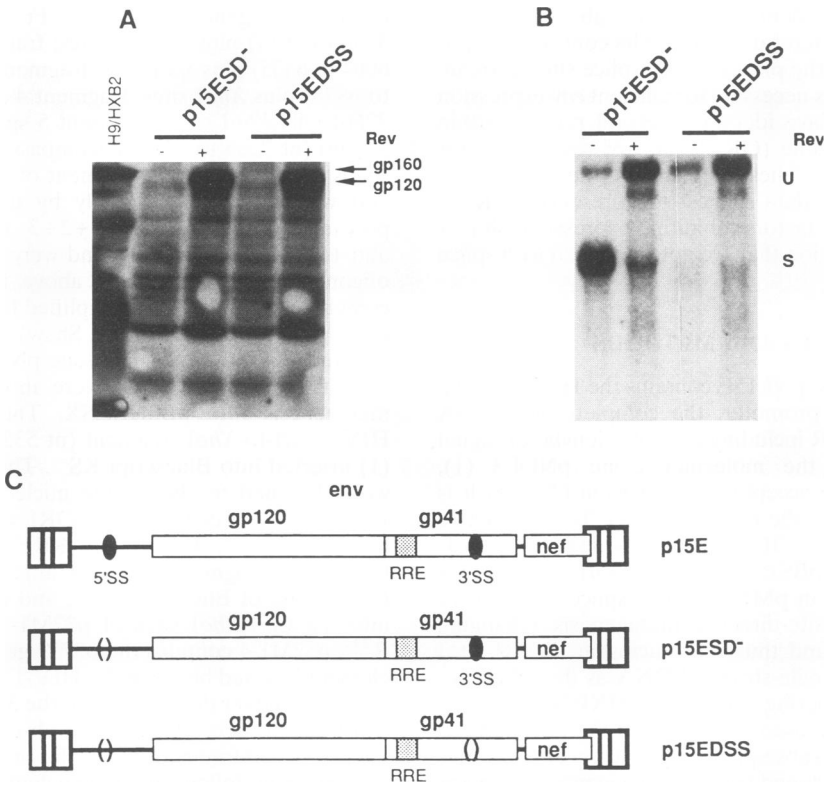


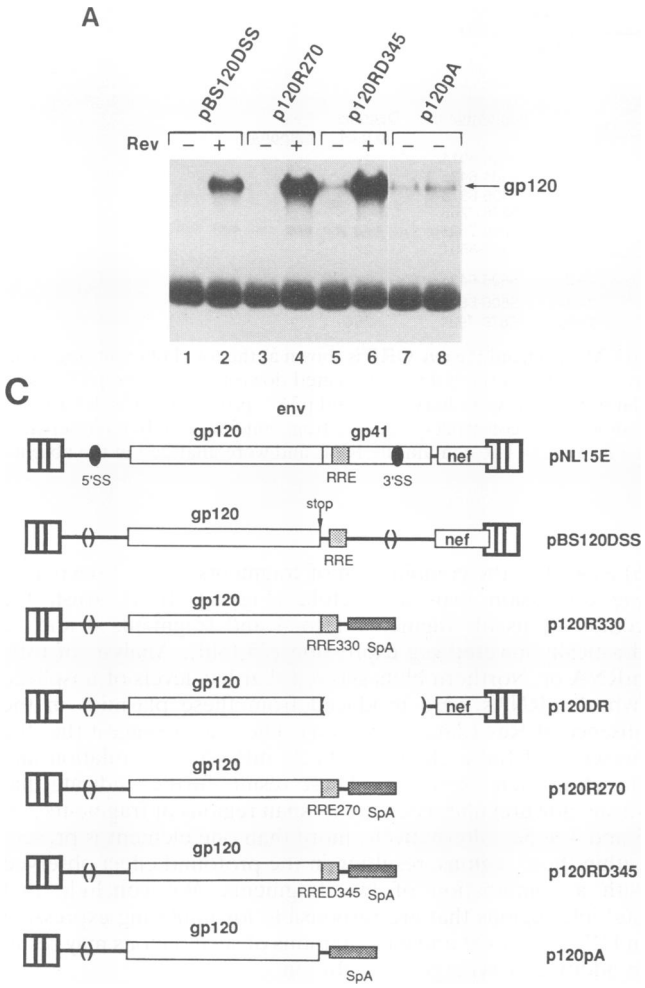
FIG. 1. Env expression is Rev dependent in the absence of utilized splice sites. HLtat cells were transfected with the splice site-mutated *env* expression plasmids p15ESD$^-$ and p15EDSS in the absence or presence of a *rev* expression plasmid (pL3srev). One day later, the cells were harvested for analyses of RNA and protein. (A) Cell-associated Env was visualized by Western blot analysis using a mixture of HIV-1 patient sera and rabbit anti-gp120 antiserum. (B) Total RNA was extracted and analyzed on Northern blots. The blots were hybridized with a nick-translated probe spanning the *Xho*I-*Sac*I fragment (nt 8443 to 9118) of HXB2. U, unspliced mRNA; S, spliced mRNA. (C) Structures of *env* expression plasmids. The plasmids that lack splice sites were generated from p15E as described in Materials and Methods. 5'SS, 5' splice site; 3'SS, 3' splice site.

were biosynthetically labeled for 5 h with 200 μCi of [^{35}S]cysteine per ml. Env was immunoprecipitated from the culture medium with HIV-1 patient plasma. The proteins were separated on 10% denaturing polyacrylamide gels and visualized after autoradiography. Production of p37^{gag} was measured by an HIV-1 p24^{gag} antigen capture assay (Coulter). The expression of Gag from these plasmids was compared with Gag production of p37M1-10D or p37M1-4.

RESULTS

The expression of *env* is Rev dependent in the absence of any utilized splice sites. Previous experiments suggested a dependence of *env* expression on the splice sites upstream of the coding region (21). To study *env* expression in detail, we used a cDNA expression plasmid pNL15E (29), producing authentic 1.5E *env* mRNA (Fig. 1C). We had previously shown that in the presence of Rev the 1.5E mRNA produces Vpu and Env from the unspliced mRNA while in the absence of Rev this mRNA is further spliced, resulting in the 1.5.7 mRNA which produces only Nef (29). To study the effect of splicing on *env* expression, we changed the splice donor at nt 5592 by site-directed mutagenesis (changing GCAGTA to GaAtTc), which resulted in p15ESD$^-$ (Fig. 1C). Duplicate plates of HLtat cells were transfected with p15ESD$^-$ in the absence and presence of a Rev-producing plasmid. One day later, total cellular protein was extracted, and Env was detected on Western blots using HIV-1 patient serum (Fig. 1A). Env protein was produced only in the presence of Rev. Total RNA was analyzed by Northern blot analysis (Fig. 1B) by hybridization to a probe detecting the 3' ends of the mRNAs. Despite the mutation of the splice donor, the 15ESD$^-$ mRNA was efficiently spliced in the absence of Rev, producing a small mRNA (Fig. 1B). We reverse transcribed, PCR amplified, and cloned the cDNA corresponding to this mRNA. Sequence analysis revealed that the spliced mRNA was generated by the use of an alternative splice donor located 13 nt 3' of the mutated splice donor at nt 5605 (TACATgtaatg) and the common splice acceptor site at nt 7925. We confirmed that this mRNA produced Nef (data not shown). Therefore, in contrast to published results (21), this splice donor-mutated construct (p15ESD$^-$) produced a spliceable mRNA and expressed high levels of Env in the presence of Rev.

To rule out any effect of splicing on Rev dependence of *env* expression, we mutated the splice acceptor site at nt 7925. Since our previous analysis of cDNA clones had revealed that in addition to this splice acceptor site (SA7) there are two additional splice acceptor sites (SA7A and SA7B) located at nt 7897 and 7901 (29), we removed a region of 42 bp encompassing nt 7884 to 7926 by site-directed mutagenesis, which resulted in p15EDSS (Fig. 1C). Northern blot analysis confirmed that the 15EDSS mRNA was not spliced (Fig. 1B). Despite the removal of all functional splice sites from p15EDSS, this mRNA responds to the presence of Rev, resulting in high



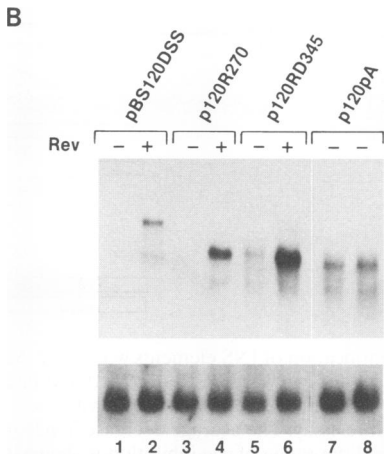


FIG. 2. Env production from the gp120 expression plasmids is Rev dependent. Duplicate plates of HLtat cells were transfected with the indicated plasmids in the absence or presence of a *rev* expression plasmid (pLsrev). One day later, the cells were harvested for analyses of protein and RNA. (A) Protein production was measured by immunoprecipitation after labeling for 5 h with 200 μCi of [^{35}S]cysteine. Secreted processed Env (gp120) was immunoprecipitated from the culture medium by a mixture of HIV-1 patient sera and rabbit anti-gp120 antiserum. (B) Total RNA was extracted and analyzed on Northern blots. The blots were hybridized with a nick-translated HIV-1-specific probe spanning nt 6158 to 7924 (upper panel) or with a β-actin-specific probe (lower panel). Lanes 7 and 8, duplicate transfections with p120pA. (C) Structures of *env* expression plasmids. SS, splice site.

levels of Env production (Fig. 1A). We have previously studied expression of *env* with a recombinant plasmid producing gp160 (15). In this construct (pL3ten), the tissue plasminogen activator leader peptide was linked to the *env* ORF starting at nt 5893 and ending at nt 8561. Thus, the construct lacks the region of the splice donor sites and the *env* leader peptide and contains the simian virus 40 polyadenylation signal. This plasmid generated only unspliced mRNA and depended on Rev for efficient Env expression.

These results, taken together with data obtained by studying *gag* expression (27, 28, 31), suggest that the presence of inefficiently utilized or unutilized splice sites is not the primary determinant for Rev dependency. We know that at least two unused splice sites are present in the 15EDSS mRNA (the alternative splice donor at nt 5605 and the splice donor of exon 6D at nt 6269). Therefore, we cannot rule out that initial spliceosome formation, which does not lead to the execution of splicing, could occur. It is possible that this interaction is sufficient to retain the mRNA in the nucleus and, since no splicing occurs, would lead to degradation of the mRNA. Alternatively, it is possible that splice site-independent inhibitory RNA elements (INS) similar to those within the *gag/pol* region are responsible for Rev dependency (28, 31).

Identification of downregulatory elements within gp120 mRNA. To distinguish between these possibilities, we designed a series of constructs that allowed the identification of negatively acting elements located within the coding region of gp120 or gp41. First, we introduced a stop codon at the cleavage site between the extracellular gp120 and the transmembrane gp41 at nt 7301 in pBS15EDSS, resulting in pBS120DSS (Fig. 2C). HLtat cells were transfected with this plasmid in the absence and presence of Rev, and then Env protein and mRNA production was analyzed. Northern blot analysis showed that the BS120DSS mRNA accumulates efficiently only in the presence of Rev (Fig. 2B, upper panel). To confirm the presence of intact mRNA in the absence of Rev, the Northern blot was rehybridized with a β-specific probe (Fig. 2B, lower panel). Immunoprecipitation of biosynthetically labeled protein from the culture medium of cells transfected with pBS120DSS confirmed the production of high levels of gp120 only in the presence of Rev (Fig. 2A). The release of gp120 was very efficient, since only barely detectable amounts remained associated with the cells (data not shown). This finding rules out the possibility that the translation of the gp41 portion of the *env* cDNA is responsible for the defect in *env* expression.

Next, we removed the part of the region 3′ of the stop codon of gp120 from pBS120DSS by deleting the 5′ portion of gp41 including the RRE but leaving the HIV-1 3′ LTR as a polyadenylation signal. The resulting plasmid (p120DR) (Fig. 2C) produced very low levels of Env (data not shown). To rule out any effect of the polyadenylation signal on gp120 mRNA accumulation, we replaced the complete region 3′ to the stop codon of gp120 (consisting of gp41, including the RRE and 3′ LTR) with the simian virus 40 polyadenylation signal, resulting in p120pA (Fig. 2C). Similarly to the data obtained for p120DR, this construct produced low levels of gp120 mRNA and protein in the absence of Rev (Fig. 2A and B, upper panel, lanes 7 and 8) upon transfection into HLtat cells. This construct cannot respond to Rev, since it lacks the RRE. Taken

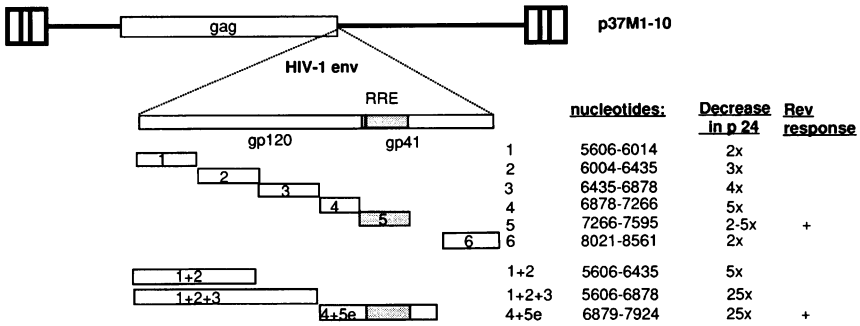


FIG. 3. Identification of INS elements within *env*. Schematic structure of p37M1-10D and the *env* ORF is shown at the top. Different fragments (fragments 1 to 6) of *env* were PCR amplified and inserted into unique restriction sites in the polylinker located downstream of the $p37^{gag}$ gene in p37M1-10D. HLtat cells were transfected with these constructs. One day later, the cells were harvested, and $p24^{gag}$ production was determined by antigen capture assay. The average decrease in p24 in three experiments is shown. The construct containing fragment 5 was analyzed in separate experiments, and the range of *gag* inhibition is shown. Constructs with fragments 5 and 4+5e contain the RRE and were analyzed in the absence and presence of Rev.

together, these results demonstrate the presence of a major INS-like sequence within the gp120 portion of *env*. p120pA produces higher levels of mRNA than pBS120DSS (compare lanes 7 and 8 with lane 1). We speculate that additional INS-like elements located within the gp41 region (see also Fig. 3) that are present only in pBS120DSS are responsible for this effect.

To study the effect of Rev on the mRNA produced from p120pA, we inserted different RREs (RRE330, RRE270, and RRED345 [33]) downstream of the gp120 stop codon, resulting in p120R330, p120R270, and p120RD345, respectively (Fig. 2C). RRE330 and RRE270 are intact RREs and differ in the length of the long stem of the predicted RRE structure. RRED345 has an internal deletion but retains the Rev interaction site. We previously showed that all three RREs mediate the Rev response to similar extents (3, 33). The expression of gp120 in the absence and presence of Rev was analyzed by Northern blots and immunoprecipitations. Only in the presence of Rev were high levels of gp120 protein and mRNA produced from p120R330 (data not shown), p120R270 (Fig. 2A and B), and p120RD345 (Fig. 2A and B). We had previously reported that the presence of Rev increases not only the cytoplasmic levels of RRE-containing mRNAs but, very importantly, also their polysomal loading and hence their expression (8). This explains the finding that similar levels of mRNA (Fig. 2B, upper panel; compare lane 2 with lanes 5, 7, and 8) produce different amounts of Env (Fig. 2A; compare lane 2 with lanes 5, 7, and 8).

These data further support our observation that major INS-like elements are located in the gp120 region. In the presence of the RRE in *cis* and Rev in *trans* the downregulatory effect of these elements can be overcome efficiently.

Localization of the regions responsible for Rev dependence of the HIV-1 *env* mRNA. To locate elements within the *env* ORF that have a downregulatory effect in vivo, we designed the following approach. The *env* ORF was subdivided into different consecutive fragments. These fragments and combinations thereof were PCR amplified with synthetic oligonucleotides and inserted downstream of the mutated $p37^{gag}$ gene in the indicator plasmid p37M1-10. p37M1-10 expresses the HIV-1 $p37^{gag}$ gene in a Rev-independent manner, since all its inhibitory elements were eliminated (27, 28, 31). Upon transfection into HLtat cells, this plasmid produced high levels of $p37^{gag}$ that were quantitated by HIV-1 $p24^{gag}$ antigen capture assay. The presence of the individual fragments (fragments 1 to 6) as well as the combination of fragments 1 and 2 decreased *gag* expression two- to fivefold (Fig. 3). In contrast, the combinations of fragments 1 to 3 and fragments 4 and 5e drastically lowered *gag* expression (25-fold). Analysis of total mRNA on Northern blots showed that low levels of unspliced hybrid mRNAs were produced from these plasmids in the absence of Rev (data not shown). These data suggest that the presence of these elements affects mRNA accumulation and therefore their expression. These results further indicate that strong downregulatory elements span regions of fragments 2 + 3 and 4 + 5e. Alternatively, more than one element is present within these regions, resulting in the profound effect observed with a combination of two fragments. We conclude that multiple regions that are responsible for inhibiting expression in HIV-1 *env* exist and combinations of such regions may result in additive or synergistic inhibition.

It is interesting that the insertion of fragment 5 alone (the intact RRE or RRE330) affected *gag* expression to a lower level compared with that for fragment 4+5e. Cotransfection of the expression vectors containing fragment 5 or 4+5e together with a Rev-producing plasmid resulted in an increase of *gag* mRNA and protein production to levels similar to the level produced by the parent plasmid p37M1-10 (data not shown).

To address whether the RRE element has a major contribution in the downregulatory effect observed, we designed oligonucleotides that change the nucleotide sequence but do not affect the amino acid composition of Env. The nucleotide composition of the RRE element was changed in three regions by site-directed mutagenesis (for details, see Materials and Methods), resulting in mutant RRE^{234}. To test whether the downregulatory effect of the RRE was eliminated, we inserted the mutant RRE^{234} as a 330-bp *Sty*I fragment (corresponding to fragment 5) into the indicator plasmid p37M1-4, generating $p37M1\text{-}4R^{234}$. HLtat cells were transfected with $p37M1\text{-}4R^{234}$, and Gag production was measured the following day. Figure 4A shows that insertion of the wild-type RRE330 lowered *gag* expression ninefold compared with that for the parent plasmid. In contrast, insertion of the mutant RRE^{234} did not lower *gag* expression. Therefore, the nucleotide changes in regions corresponding to oligonucleotides 2, 3, and 4 within the RRE eliminated its downregulatory effect. In addition, we inserted the mutant RRE^{234} into the fully Rev-dependent plasmid pNLgag (33), which contains the complete HIV-1 *gag* gene expressed from the LTR promoter. Since the presence of Rev did not increase expression from this construct (data not

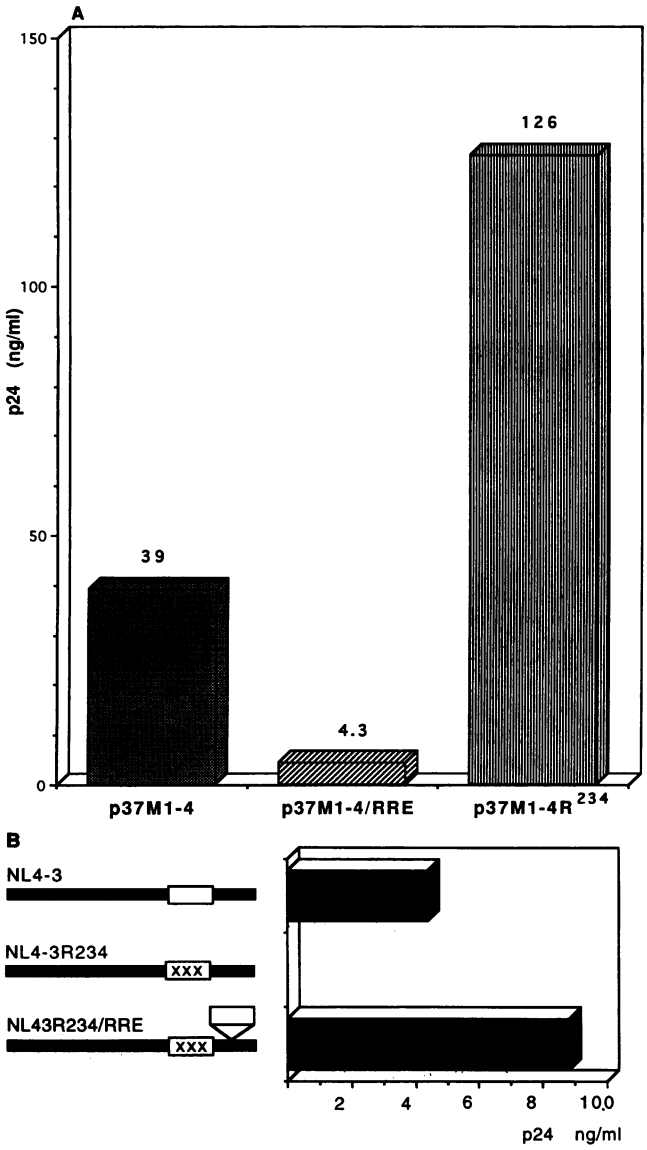


FIG. 4. Multiple point mutations within RRE eliminate its downregulatory effect. Mutant RRE, RRE^{234}, was inserted into the *gag* expression plasmid p37M1-4 and the molecular clone pNL4-3, resulting in p37M1-$4R^{234}$ and pNL4-$3R^{234}$, respectively. pNL4-$3R^{234}$/RRE contains both the mutant and the wild-type RREs. Open box, wild-type RRE; × × ×, mutant RRE^{234}. HLtat cells were transfected with these plasmids, and the wild-type and mutant RRE-containing plasmids were compared in terms of Gag production. Gag production from the different *gag* expression plasmids (A) and that from the molecular clones (B) are shown.

shown), we conclude that the introduced mutations destroyed the secondary structure of RRE, thereby abolishing the Rev interaction.

We then inserted mutant RRE^{234} into an otherwise intact provirus clone, generating pNL4-$3R^{234}$. Upon transfection into HLtat cells (Fig. 4B) or the lymphoid cell line HPB-ALL (data not shown) no Gag production, and hence no virus expression, was detected. These experiments demonstrate that the elimination of the downregulatory effect of RRE did not result in expression. Furthermore, Rev cannot activate expression of pNL4-$3R^{234}$, since its interaction sites on the mutant RRE were destroyed (see above). Insertion of the intact RRE into the *Xho*I site located within the *nef* gene of pNL4-$3R^{234}$ generated pNL4-$3R^{234}$/RRE. Upon transfection into HLtat cells, high levels of Gag expression, comparable to those produced by wild-type pNL4-3, were produced from this construct (Fig. 4B). In this case, Rev-mediated HIV expression occurs via the introduced RRE element that counteracts the negative determinants on the viral mRNAs. These data further support our findings that multiple elements causing the low expression of the unspliced and intermediate spliced mRNAs are present within HIV-1. The elimination of one element is not sufficient to render HIV-1 mRNAs Rev independent in human cells.

DISCUSSION

The experiments presented here show that the expression of the intermediate spliced *env* mRNA 1.5E in human cells depends on Rev even in the absence of any functional splice sites. This observation is in agreement with data obtained previously from experiments in which the gp160 ORF was expressed from a hybrid recombinant plasmid that produced low levels of an unspliced mRNA in the absence of Rev (15). Studying *env* expression, Brighty and Rosenberg (4) similarly concluded that splicing is not the determinant for Rev dependence in *Drosophila* cells. Taken together, these data strongly suggest that elements distinct from splice sites are responsible for the inefficient expression of these mRNAs.

Our data contradict another model for Rev function, proposed by Chang and Sharp (5). Studying the expression of an HIV *env*–β-globin hybrid mRNA, the authors suggest that the presence of splice sites in the unspliced and intermediate spliced viral mRNAs is responsible for nuclear retention resulting in unstable mRNAs. Rev dissociates the precursor mRNAs from the spliceosomes, allowing their transport to the cytoplasm. This idea was further supported by experiments performed by Lu et al. (21), showing that removal of the splice donor upstream of the *env* AUG resulted in unstable mRNA that did not respond to Rev. We mutated the same splice donor as described by Lu et al. (21) (changing the splice donor region from GCAGTA to GaAtTc compared with GCAtTA). However, this mutated mRNA was efficiently spliced by using an alternative splice donor located 13 nt downstream of the mutated splice site and depended on Rev for expression. It is possible that the different expression vectors and/or cell lines studied are responsible for the observed difference. The constructs studied by Lu et al. (21) were based on a highly replicative vector system that expressed an *env*–β-globin hybrid mRNA and were studied in monkey Cos-1 cells. Our results are based on the expression of the authentic *vpu/env* cDNA 1.5E in human HeLa cells. This cDNA encodes an mRNA identical to the one produced by the HIV-1 provirus in the infected cells.

Our findings on intermediate spliced mRNA 1.5E are similar to our data previously obtained by studying the determinants regulating the expression of the *gag/pol* genes (11, 15, 27, 28, 31), suggesting that elements independent of splice sites are responsible for low expression. Subsequently, several regions within *gag/pol* responsible for the low expression were characterized in detail (27, 28, 31). Several of these elements, called INS, were eliminated by changing their nucleotide composition, resulting in Rev-independent *gag* expression. All identified INS elements spanned long regions of 200 to 300 nt; multiple nucleotide changes were necessary to destroy their downregulatory effect. Our studies on the *gag-env* hybrid mRNAs revealed that combinations of fragments 2 and 3 and

fragments 4 and 5e (Fig. 3) synergistically lowered *gag* expression, while each fragment alone only marginally affected *gag* expression. These results suggest that strong downregulatory elements span regions of fragments 2 or 3 and 4 or 5e. Alternatively, more than one element is present within these regions, resulting in the pronounced effect observed with combinations of two fragments. Previous studies had proposed that either of these regions is responsible for the low expression (10, 26). In contrast, our results suggest that a combination of these regions is responsible for the low expression of the *env* mRNA. Experiments are in progress to introduce point mutations that alter the nucleotide composition of these regions, applying the same method used to inactivate downregulatory elements within the *gag/pol* region (27, 28). Since the downregulatory effect of these INS-like elements is present in the absence of viral factors, we propose, by analogy to the INS elements in *gag/pol*, that these elements are RNA binding sites of cellular factor(s). Experiments are ongoing to determine the underlying cellular mechanism responsible for this downregulation and to compare the mechanisms of function of the different elements.

To regulate viral expression, the lentiviruses and the human T-cell leukemia virus family of retroviruses depend on Rev or Rex factors. The requirement for Rev dependence has been studied best with HIV-1. An interesting question is why some of these viral mRNAs contain multiple downregulatory elements. Since the *env* region is present in all the Rev-dependent mRNAs (unspliced and intermediate spliced), this region could be sufficient to confer posttranscriptional regulation. In this context, Brighty and Rosenberg demonstrated that, in *Drosophila* cells, only the RRE is responsible for low expression in the absence of Rev and necessary for Rev-mediated expression (4). The idea that the RRE has a dual function is intriguing. Studies with human cells demonstrated that the insertion of the RRE in various constructs such as HIV-1 mutant *gag* genes p17M1-4 (36), p37M1-10 (Fig. 3), and p37M1-4 (Fig. 4A); HIV-1 *tat*; and Rous sarcoma virus *gag* (our unpublished data) decreased expression 2- to 10-fold by affecting the RNA levels. In all these examples, the presence of Rev counteracted this RRE-mediated decrease, resulting in a proportional increase of expression. Nucleotide changes within the RRE that were designed to eliminate its downregulatory effect were inserted into a molecular clone of HIV-1. As anticipated from the studies of subgenomic hybrid mRNAs, such an RRE mutant virus could not be expressed. The data presented here demonstrate that the RRE is not the only downregulatory element. We identified other regions whose presence affects *env* mRNA accumulation in an independent or additive manner in human cells.

Rev-independent expression of *gag* and *env* was reported with either the dihydrofolate reductase selection system (20) or the vaccinia virus expression system (17, 35). The dihydrofolate reductase selection in chinese hamster ovary cells results in high amplification of the introduced gene. Vaccinia virus replicates and expresses its proteins in the cytoplasm and, therefore, bypasses the nucleus. These differences may explain the Rev-independent production of the viral structural proteins in these systems.

Interestingly, a plasmid containing only the gp120 ORF, such as p120pA, does not produce high levels of Env in human cells (Fig. 2), while a gp120-encoding plasmid is expressed at high levels in *Drosophila* cells (4). The RRE seems to be the only negative determinant within *env* mRNA in the *Drosophila* system. In contrast, introduction of a mutant RRE into the complete virus has no positive effect in human cells. Taken together, these data suggest that determinants for low expression and/or their downregulatory strength may differ in different cell types. This conclusion is in agreement with our model proposing that cellular factors interact with these RNA elements and affect the posttranscriptional regulation of the mRNAs. We speculate that these factors may act differently in human and *Drosophila* cells. Their identification may lead to understanding posttranscriptional HIV-1 regulation in more detail and may also shed light on the posttranscriptional regulation of cellular mRNAs.

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REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
2. **Benko, D. M., R. Robinson, L. Solomin, M. Mellini, B. K. Felber, and G. N. Pavlakis.** 1990. Binding of trans-dominant mutant rev protein of human immunodeficiency virus type 1 to the cis-acting rev responsive element does not affect the fate of viral mRNA. New. Biol. **2:**1111–1122.
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.
4. **Brighty, D., and M. Rosenberg.** A cis-acting repressive sequence that overlaps the Rev responsive element of HIV-1 regulates nuclear retention of env mRNAs independently of splice signal. Submitted for publication.
5. **Chang, D. D., and P. A. Sharp.** 1989. Regulation by HIV Rev depends upon recognition of splice sites. Cell **59:**789–795.
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. **Cullen, B. R.** 1991. Human immunodeficiency virus as a prototypic complex retrovirus. J. Virol. **65:**1053–1056.
8. **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.
9. **de Wet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani.** 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. **7:**725–737.
10. **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.
11. **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:**1495–1499.
12. **Felber, B. K., and G. N. Pavlakis.** 1993. Molecular biology of HIV-1: positive and negative regulatory elements important for virus expression. AIDS **7**(Suppl.):S51–S62.
13. **Graham, F. J., and A. J. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456–460.
14. **Greene, W. C.** 1990. Regulation of HIV-1 gene expression. Annu. Rev. Immunol. **8:**453–475.
15. **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.
16. **Haseltine, W. A., and F. Wong-Staal.** 1991. Genetic structure and regulation of HIV, p. 1–537. Raven Press, New York.
17. **Karacostas, V., K. Nagashima, M. A. Gonda, and B. Moss.** 1989. Human immunodeficiency virus-like particles produced by a vaccinia virus expression vector. Proc. Natl. Acad. Sci. USA **86**:8964–8967.
18. **Krawczyk, Z., and C. Wu.** 1987. Isolation of RNA for dot hybridization by heparin-DNase I treatment of whole cell lysate. Anal. Biochem. **165**:20–27.
19. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**:488–492.
20. **Lasky, L. A., J. E. Groopman, C. W. Fennie, P. M. Benz, D. J. Capon, D. J. Dowbenko, G. R. Nakamura, W. M. Nunes, M. E. Renz, and P. W. Berman.** 1986. Neutralization of the AIDS retrovirus by antibodies to a recombinant envelope glycoprotein. Science **233**:209–212.
21. **Lu, X., 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.
22. **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.
23. **Mermer, B., B. K. Felber, M. Campbell, and G. N. Pavlakis.** 1990. Identification of *trans*-dominant HIV-1 rev protein mutants by direct transfer of bacterially produced proteins into human cells. Nucleic Acids Res. **18**:2037–2044.
24. **Myers, G., B. Korber, J. A. Berzofsky, R. Smith, and G. N. Pavlakis.** 1991. Human retroviruses and AIDS. A compilation and analysis of nucleic acid and amino acid sequences. Los Alamos National Laboratory, Los Alamos, N.Mex.
25. **Pavlakis, G. N., S. Schwartz, D. M. D'Agostino, and B. K. Felber.** 1991. Structure, splicing, and regulation of expression of HIV-1: a model for the general organization of lentiviruses and other complex retroviruses, p. 41–63. *In* R. Kennedy, F. Wong-Staal, and W. C. Koff (ed.), Annual review of AIDS Research. Marcel Dekker, Inc., New York.
26. **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.
27. **Schneider, R., et al.** Rev-independent expression of Gag of HIV-1. Unpublished data.
28. **Schwartz, S., M. Campbell, G. Nasioulas, J. Harrison, B. K. Felber, and G. N. Pavlakis.** 1992. Mutational inactivation of an inhibitory sequence in human immunodeficiency virus type 1 results in Rev-independent *gag* expression. J. Virol. **66**:7176–7182.
29. **Schwartz, S., B. K. Felber, D. M. Benko, E. M. Fenyö, 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.
30. **Schwartz, S., B. K. Felber, E. M. Fenyö, 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.
31. **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.
32. **Schwartz, S., B. K. Felber, and G. N. Pavlakis.** 1992. Mechanism of translation of monocistronic and multicistronic human immunodeficiency virus type 1 mRNAs. Mol. Cell. Biol. **12**:207–219.
33. **Solomin, L., B. K. Felber, and G. N. Pavlakis.** 1990. Different sites of interaction for Rev, Tev, and Rex proteins within the Rev-responsive element of human immunodeficiency virus type 1. J. Virol. **64**:6010–6017.
34. **Vaishnav, Y., and F. Wong-Staal.** 1991. The biochemistry of AIDS. Annu. Rev. Biochem. **60**:577–630.
35. **Willey, R. L., D. H. Smith, L. A. Lasky, T. S. Theodore, P. L. Earl, B. Moss, D. J. Capon, and M. A. Martin.** 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J. Virol. **62**:139–147.
36. **Zolothukin, A. S., J. B. Harford, and B. K. Felber.** Rev of human immunodeficiency virus and Rex of the human T-cell leukemia virus type I can counteract a cellular RNA instability element of the transferrin receptor mRNA. Submitted for publication.