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An Exonic Splicing Silencer Downstream of the 3′ Splice Site A2 Is Required for Efficient Human Immunodeficiency Virus Type 1 Replication

Joshua M. Madsen[1] and C. Martin Stoltzfus[1,2]*

Interdisciplinary Program in Molecular Biology[1] and Department of Microbiology,[2] Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242

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Alternative splicing of the human immunodeficiency virus type 1 (HIV-1) genomic mRNA produces more than 40 unique viral mRNA species, of which more than half remain incompletely spliced within an HIV-1-infected cell. Regulation of splicing at HIV-1 3′ splice sites (3′ss) requires suboptimal polypyrimidine tracts, and positive or negative regulation of splicing occurs through binding of cellular factors to *cis*-acting splicing regulatory elements. We have previously shown that splicing at HIV-1 3′ss A2, which produces *vpr* mRNA and promotes inclusion of HIV-1 exon 3, is repressed by the hnRNP A/B-dependent exonic splicing silencer ESSV. Here we show that ESSV activity downstream of 3′ss A2 is localized to a 16-nucleotide element within HIV-1 exon 3. HIV-1 replication was reduced by 95% when ESSV was inactivated by mutagenesis. Reduced replication was concomitant with increased inclusion of exon 3 within spliced viral mRNA and decreased accumulation of unspliced viral mRNA, resulting in decreased cell-associated p55 Gag. Prolonged culture of ESSV mutant viruses resulted in two independent second-site reversions disrupting the splice sites that define exon 3, 3′ss A2 and 5′ splice site D3. Either of these changes restored both HIV-1 replication and regulated viral splicing. Therefore, inhibition of HIV-1 3′ss A2 splicing is necessary for HIV-1 replication.

In contrast to the alternative splicing of most cellular mRNA, splicing of retroviral mRNA results in the cytoplasmic accumulation of incompletely spliced and unspliced viral mRNA. Incompletely spliced viral mRNA is necessary for the expression of the Env, Vpu, Vpr, and Vif proteins, and the accumulation of unspliced viral mRNA is necessary for expression of the *gag* and *pol* gene products and also serves as the genomic viral mRNA encapsidated within progeny virions. Completely spliced viral mRNA is required for expression of the regulatory viral proteins Tat, Rev, and Nef. More than 40 unique incompletely and completely spliced viral mRNA species are generated through the alternative splicing of the HIV-1 primary transcript within an HIV-1-infected cell (23, 26).

HIV-1 3′ splice sites (3′ss) are used with differing efficiencies in part because viral polypyrimidine tracts (PPT) are interspersed with purines (27, 30), leading to decreased affinity for the essential cellular splicing factor U2AF65 (for a review of cellular splicing see reference 12). In addition, the efficiency of HIV-1 splicing is also regulated by both positive and negative *cis* elements within the viral genome that act to promote or repress splicing. To date, four exonic splicing silencers (ESS) and one intronic splicing silencer (ISS) have been identified within the viral genome (Fig. 1). Utilization of HIV-1 3′ss A2 by the spliceosome is negatively regulated by ESSV, 3′ss A3 by ESSp and ESS2, and 3′ss A7 by the ISS and ESS3 (2, 3, 5, 16, 29, 31).

We have previously characterized ESSV as a 24-nucleotide (nt) ESS element within HIV-1 exon 3, downstream of HIV-1 3′ss A2. Utilization of HIV-1 3′ss A2 leads to the accumulation of the incompletely spliced viral mRNA encoding the viral accessory protein Vpr and promotes the inclusion of noncoding exon 3 within both the incompletely and completely spliced viral mRNA species. We have also shown that 3′ss A2 utilization is repressed by inhibition of U2AF65 recognition of the 3′ss A2 PPT through the binding of cellular hnRNP A/B proteins to ESSV (5, 10).

Although the inhibition of HIV-1 3′ss by viral ESS elements has been investigated with great detail both in vitro and within the context of subgenomic HIV-1 constructs, the importance of ESS elements with regard to HIV-1 replication has not been investigated. To this end, we have disrupted ESSV by mutagenesis and studied the consequences of these mutations on HIV-1 replication. Our data indicate that maintenance of ESSV is necessary not only for appropriate 3′ss utilization but also for the accumulation of wild-type levels of unspliced viral mRNA, Gag protein production, and production of virus particles.

MATERIALS AND METHODS

Plasmids. The infectious HIV-1 molecular clone pNL4-3 (accession no. M19921) was obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (1), and plasmids pCMV-110 β-galactosidase (β-gal) and pΔPSP have been described previously (16). The plasmid pEMPSP was created by insertion of the 1,192-nucleotide EcoRI/MluI fragment of pΔPSP into pCMV5 (obtained from M. F. Stinski, University of Iowa). Mutant ESSV derivatives of pΔPSP were generated within pEMPSP with the corresponding oligonucleotide primer pairs shown in Table 1, using a QuikChange site-directed mutagenesis kit (Stratagene). The 1,192-nucleotide EcoRI/MluI fragment of pEMPSP was inserted into pΔPSP. pNL4-3 mutants were generated using oligonucleotides shown in Table 1 by site-directed mutagenesis, followed by insertion of the 441-nucleotide EcoRI/PflMI fragment of pEMPSP into pNL4-3. Plasmids pNEVM and pNEU were also generated by site-directed

* Corresponding author. Mailing address: Department of Microbiology, Carver College of Medicine, University of Iowa, Iowa City, IA 52242. Phone: (319) 335-7793. Fax: (319) 335-9006. E-mail: marty-stoltzfus@uiowa.edu.

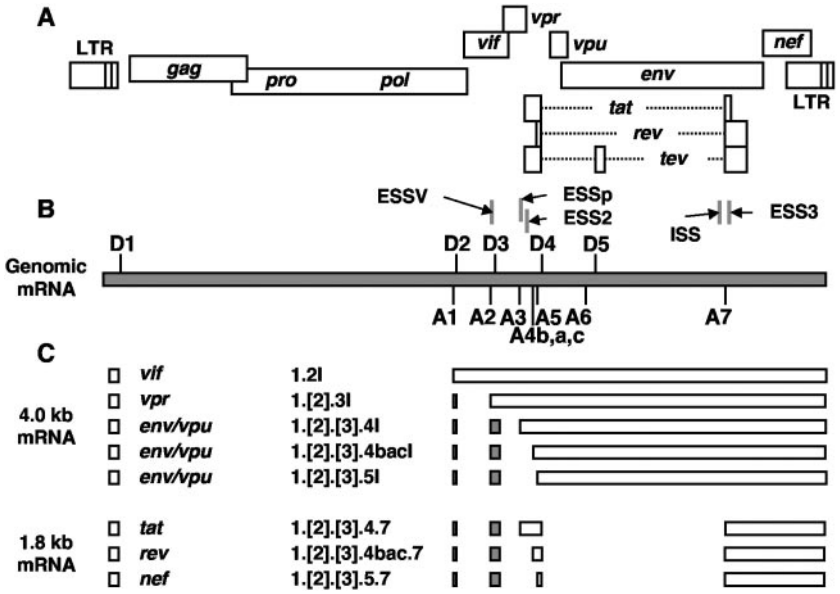

FIG. 1. (A) Spliced RNA species produced within HIV-1-infected cells. HIV-1 genes are shown relative to the viral long terminal repeats (LTRs). (B) The viral genomic or unspliced mRNA showing the location of the 5′ss and 3′ss within the infectious plasmid pNL4-3. The locations of negative splicing elements ESSV, ESSp, ESS2, ISS, and ESS3 are indicated by gray boxes. (C) The HIV-1 incompletely and completely spliced viral mRNAs (4.0-kb and 1.8-kb size classes, respectively) are shown as open boxes. Spliced mRNAs are denoted by the translated open reading frames and by exon content. The incompletely spliced mRNAs, denoted with an I, are differentiated from completely spliced mRNAs by inclusion of the intron between 5′ss D4 and 3′ss A7. Either one or both of noncoding exons 2 and 3 (shown as gray-shaded exons) are differentially included within all 1.8- and 4.0-kb mRNA species with the exception of *vif* (1.2I).

mutagenesis of pEMPSP, through two sequential PCR steps using the primer sets shown in Table 1.

Cell culture. HeLa and 293T cells were obtained from the American Type Culture Collection and maintained as previously described (5). Jurkat, H9, and HeLa-CD4-LTR-βgal (MAGI) cells were obtained from the NIH AIDS Research and Reference Reagent Program (19, 22, 24, 25, 32). Jurkat and H9 cells and were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. MAGI cells were cultured as previously described (19).

Transfections. HeLa cells were transiently transfected with pΔPSP derivatives by calcium phosphate precipitation as previously described (5). HeLa cells were transfected with pNL4-3 mutants using either Geneporter (Gene Therapy Systems, Inc.) or Lipofectine PLUS (Invitrogen) according to the manufacturer's specifications. Geneporter transfections were performed using 10^6 HeLa cells seeded 24 h before transfection in a 100-mm dish, 10 μg DNA, and 25 μl Geneporter. Lipofectine PLUS transfections were performed using 6×10^5 HeLa cells seeded 24 h before transfection in a six-well plate, 1.75 μg DNA, 20 μl PLUS reagent, and 1.25 μl Lipofectine. Calcium phosphate precipitations were performed using 10^6 293T cells seeded 24 h before transfection in a 60-mm dish, using 14 μg DNA and 2× HEPES-buffered saline as described previously (4). Transient transfections were harvested 48 to 72 h posttransfection.

RT assays and virus production. Cell-free supernatants were assayed for reverse transcriptase (RT) activity by [α-^{32}P]dTTP incorporation, as described previously (34), and [α-^{32}P]dTTP incorporation was quantitated using an Instant

TABLE 1. Oligonucleotides used for site-directed mutagenesis[a]

Clone	Source	Sequence	nt no.
15/16	NL4-3	GAAATACCATATTCTGACGTATAGTTAGTCCTAGGTGTGAATATC	5402–5446
16/17		GAAATACCATATTATTACGTATAGTTAGTCCTAGGTGTGAATATC	
18/19		GAAATACCATATTAGGCAGTATAGTTAGTCCTAGGTGTGAATATC	
20/21		GAAATACCATATTAGGACTGATAGTTAGTCCTAGGTGTGAATATC	
22/23		GAAATACCATATTAGGACGTCGAGTTAGTCCTAGGTGTGAATATC	
24/25		GAAATACCATATTAGGACGTATGATTAGTCCTAGGTGTGAATATC	
26/27		GAAATACCATATTAGGACGTATAGAAAGTCCTAGGTGTGAATATC	
28/29		GAAATACCATATTAGGACGTATAGTTCTTCCTAGGTGTGAATATC	
30/31		GAAATACCATATTAGGACGTATAGTTAGGACTAGGTGTGAATATC	
32/33		GAAATACCATATTAGGACGTATAGTTAGTCAAAGGTGTGAATATC	
34/35		GAAATACCATATTAGGACGTATAGTTAGTCCTCTGTGTGAATATC	
36/37		GAAATACCATATTAGGACGTATAGTTAGTCCTAGTGGTGAATATC	
NEVM1		CCATATTAGGACGTATAGTGATTCCCAAGTGTGAATATCAAGCAGG	5408–5453
NEVM2		GGACGTATAGTGCTGACCAAGTGTGAATATCAAGCAGG	5416–5453
NEU1		CCATATTAGGACGTATAGTATCGCCTAGGTGTGAATATCAAGCAGG	5408–5453
NEU2		CCATATTAGGACGTATAGTATCGCCACGTTGTGAATATCAAGCAGG	5408–5453
VMRV6		CTGCACTATTTTGATTGTTTTTCAATCTGCTATAAGAAATACC	5365–5409
VMRV2		GCAGGACATACCAAGGCAGGATCTCTACAGTACTTGGC	5449–5486

[a] Oligonucleotides represent the sense primer and are shown in the 5′-to-3′ orientation. The corresponding complementary antisense primers are not shown, and primers are shown with respect to pNL4-3.

Imager (Packard). NL4-3 virus was produced by collecting supernatants from HeLa cells transfected with pNL4-3 as described above. MVP5180 virus (accession no. L20571) was obtained from the NIH AIDS Research and Reference Reagent Program (15). Viral titers were determined by infecting MAGI indicator cell lines as described previously (19).

Infections. H9 cells (10^7 cells) were infected at a multiplicity of infection of 0.001 with either NL4-3 or MVP5180 in 1 ml RPMI 1640 containing 10 μg/μl Polybrene for 2.5 h at 37°C. Infected H9 cells were resuspended in 25 ml RPMI 1640 containing 2 μg/ml Polybrene.

RNA isolation and RT-PCR. Total RNA was isolated using Tri-Reagent as described by the manufacturer (Molecular Research Center, Inc.). Total cellular RNA was reverse transcribed as described previously (5) with the following modifications: 1.33 mM each deoxynucleoside triphosphate (dNTP), 0.66 U RNasin (Amersham), and 3 μg bovine serum albumin. Poly(A)$^+$ mRNA was isolated from 10^6 infected H9 cells 3 days postinfection, using a Dynabead mRNA DIRECT kit (Dynal), according to the manufacturer's protocol. Poly(A)$^+$ mRNA was reverse transcribed using the Dynabeads as a template in a 120-μl reaction mixture containing 1× first-strand buffer, 0.25 mM each dNTP, 1 U RNasin, and 600 U Moloney murine leukemia virus RT for 1 h at 37°C. Dynabeads were serially diluted and amplified with oligonucleotide primers BA1 and BA4, complementary to β-actin, as described previously (14) in a reaction identical to that described for amplification of HIV-1 mRNA with the exception that 2.5 mM $MgCl_2$ was used for the β-actin PCR. Reactions were normalized to β-actin, and 1 to 5 μl Dyanbeads was used to analyze spliced viral mRNA species. The 1.8-kb HIV-1 mRNA species were amplified using the BSS and SJ4.7A primer pairs, and the 4.0-kb HIV-1 mRNA was amplified using the BSS and KPNA primer pairs (23). Group O mRNA was amplified using primers representing group O sequences. Spliced HIV-1 mRNA was amplified as previously described (5) with the following modifications: 1.5 mM $MgCl_2$, 0.2 mM each dNTP, and 1 U AmpliTaq Gold polymerase. PCRs were verified by polyacrylamide gel electrophoresis (PAGE) as previously described (5), in the presence of 10 μCi of [α-^{32}P]dTTP; 0.02 mM cold dTTP; and 0.2 mM each dATP, dCTP, and dGTP.

Northern blotting. Ten micrograms of total cellular RNA from pNL4-3-transfected HeLa cells was fractionated by electrophoresis, transferred to a Nytran membrane (Schleicher & Schuell), and incubated with a radiolabeled probe generated from the 422 nucleotide XhoI/BamHI restriction fragment of pNL4-3, which was random primed using Ready-To-Go DNA labeling beads (Amersham) and [α-^{32}P]dCTP. The blot was then stripped as previously described (28) and probed using a radiolabeled β-actin probe generated from the β-actin PCR product described above.

Immunoblotting. Twenty micrograms of protein from transfected HeLa cells was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted. HIV-1 patient sera (obtained from J. Stapleton, University of Iowa Veteran Affairs Medical Center) and monoclonal antibody E7 against β-tubulin (Developmental Studies Hybridoma Bank, University of Iowa) were diluted 1:500. Monoclonal antibody 1DG, against HIV-1 gp120 (NIH AIDS Research and Reference Reagent Program), was diluted 1:1,000, and monoclonal antibody 40-1a (Developmental Studies Hybridoma Bank, University of Iowa) against β-gal was diluted 1:50. Antibodies were incubated at room temperature overnight. Peroxidase-conjugated secondary antibodies were incubated at 1:50,000 for both goat anti-human immunoglobulin G (IgG; Cappel) and peroxidase-conjugated sheep anti-mouse IgG (Amersham). Blots were developed using an ECL Plus Western blotting detection system (Amersham), and autoradiography was performed.

Identification of revertants and genomic DNA isolation. Reversion experiments were performed in six-well plates by transfecting 2×10^6 Jurkat cells with 3 μg of pNEVM and 25 μl Geneporter (Gene Therapy Systems, Inc.) according to the manufacturer's specifications as previously described (9). Three days posttransfection the cultures were split 1:10 into a 12-well dish. Every 3 to 4 days cultures were passaged at a 1:10 dilution in RPMI supplemented as above, and supernatants were analyzed for reverse transcriptase activity. Of the two cultures that became RT positive, reverse transcriptase activity was restored between 36 and 56 days posttransfection within the NEVM revertant 2 (Rev 2)-infected culture and between 25 and 56 days posttransfection within the NEVM revertant 6 (Rev 6) culture.

Genomic DNA harvested 50 days posttransfection was isolated from Rev2- and Rev6-infected cells and treated with proteinase K (4). HIV-1 exon 3 was then amplified from 1.5 μg genomic DNA by PCR using primers Rev S (^{5084}G GATGAGGATTAACACATGG^{5103}) and Rev A (^{5891}GCAGTTTTAGGCTG ACTTCC^{5872}) (nucleotides with respect to pNL4-3) in a 50-μl reaction mixture containing the following: 1× AmpliTaq Gold buffer (Applied Biosystems), 25 pmol Rev S, 25 pmol Rev A, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, and 1.25 U *Pfu* Turbo (Stratagene). PCR was performed for 10 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min followed by 20 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 3 min and then 72°C for 10 min. The resulting PCR product was amplified using the pGEMT Vector System II (Promega), according to the manufacturer's recommendations, and plasmids from two independent colonies representing Rev 2- and Rev 6-transformed JM109 cells were sequenced. The only mutations identified within the 800 nucleotides of the HIV-1 genome sequenced were those within either 3′ss A2 or 5′ss D3, as described in Results. Nucleotide changes within 3′ss A2 and 5′ss D3 were introduced into pNEVM by the site-directed mutagenesis strategy described above, using primer pairs VMRV6 and VMRV2 (Table 1), respectively, to generate plasmids pVMRV6 and pVMRV2, corresponding to the sequences of the revertants identified.

RESULTS

ESSV activity is localized to a 16-nt element downstream of HIV-1 3′ss A2. The exonic splicing silencer ESSV has previously been mapped to a 24-nt element, containing three PyUAG motifs, downstream of 3′ss A2. Mutation of these three elements resulted in increased *vpr* mRNA splicing and increased inclusion of exon 3 within the spliced viral mRNAs, in the context of a *gag-pol*-deleted HIV-1 clone (5). To more precisely localize the sequences necessary for splicing silencer activity in vivo, ESSV was disrupted by dinucleotide substitutions within the context of the *gag-pol*-deleted HIV-1 clone, pΔPSP (Fig. 2A). Alterations in 3′ss utilization were detected by RT-PCR analysis of spliced viral mRNAs after transient transfection of HeLa cells (Fig. 2B). Analysis of HIV-1 mRNA species within the 1.8-kb size class revealed that, although the disruption of the most 5′ PyUAG motif within the 24-nt ESSV (Fig. 2B, 15/16) only slightly increased the inclusion of exon 3 within either *nef* or *rev* mRNA, compared to wild-type ESSV (Fig. 2C), mutagenesis of the two most 3′ PyUAG motifs (Fig. 2B and C, 26/27, 28/29, 32/33, and 34/35) resulted in a five- to eightfold increase in HIV-1 exon 3 inclusion compared to wild-type ESSV. Mutagenesis of the nucleotides corresponding to mutants 16/17 and 18/19 decreased the inclusion of HIV-1 exon 3 (Fig. 2B and C), suggesting the possibility that these mutations may disrupt a putative positive splicing element. Furthermore, mutagenesis of the AUAG sequence immediately preceding the second PyUAG motif (Fig. 2B; 22/23 and 24/25) also relieved repression of splicing at HIV-1 3′ss A2. Taken together, these data suggest that most of the inhibitory activity of ESSV is localized to a 16-nt element, containing three (Py/A)UAG motifs, downstream of HIV-1 3′ss A2.

Inactivation of HIV-1 ESSV inhibits viral replication. In order to determine if ESSV is necessary for HIV-1 replication, we generated two mutants of the infectious molecular clone pNL4-3 in which the two downstream (Py/A)UAG motifs within ESSV were disrupted (Fig. 3A, mutants NEVM and NEU). The first mutant, NEVM, represents the sequence of the highly divergent ESSV of the group O strain MVP5180. Mutant NEU was designed to maintain the Vif amino acid sequence of NL4-3 while disrupting ESSV, in order to rule out the possibility that differences in Vif expression could affect mutant viral replication. HeLa cells were transfected with either pNEVM or pNEU, and virus particle production was determined by measuring reverse transcriptase activity within the culture supernatant. Both mutants exhibited greater-than-20-fold reductions in particle production compared to pNL4-3 (Fig. 3B).

To investigate the cause of the dramatic reduction in virus

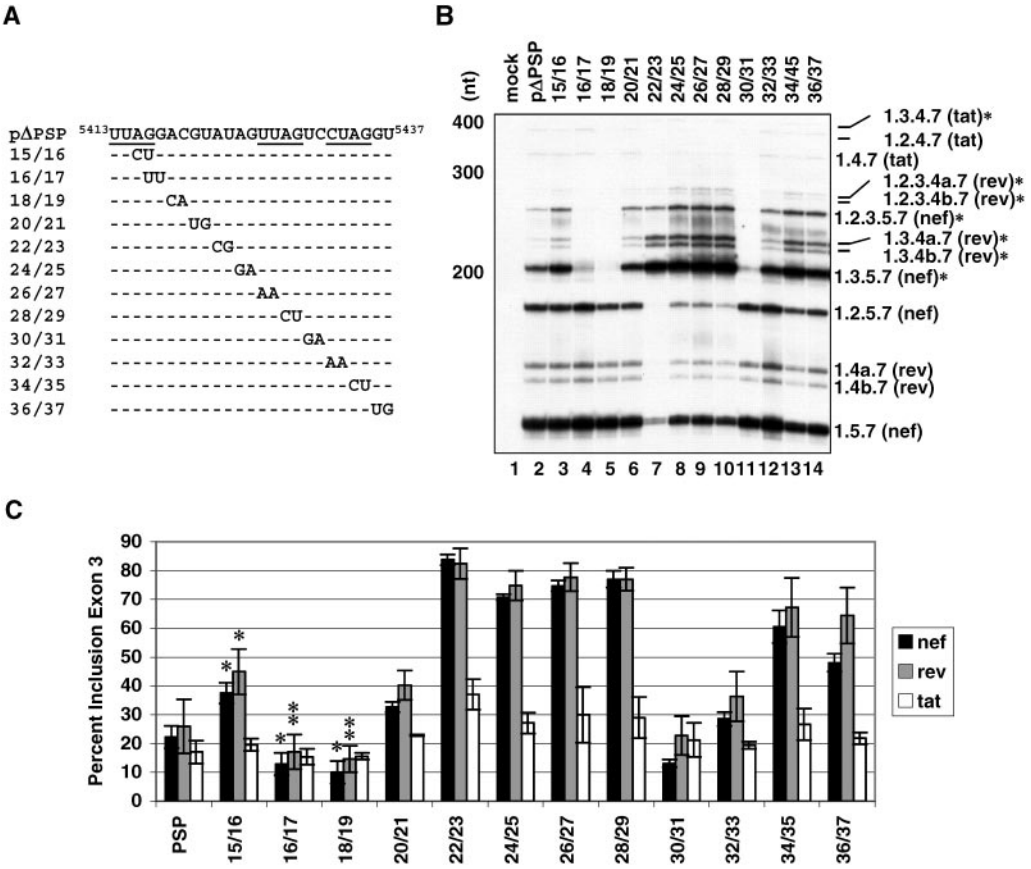

FIG. 2. Localization of splicing silencer activity within the NL4-3 ESSV. (A) The HIV-1 sequence within the context of the *gag-pol*-deleted plasmid pΔPSP was mutagenized by dinucleotide substitutions spanning ESSV. The previously identified PyUAG motifs within ESSV are underlined. (B) Total RNA samples from HeLa cells 48 h after transfection with the indicated plasmids were analyzed by RT-PCR using primers specific for completely spliced viral mRNA. HIV-1 RNA species are indicated on the right side of the gel by exon content and the mRNA to which they encode, and mRNAs spliced at 3′ss A2 are indicated by asterisks. (C) The percent exon 3 inclusion within each viral mRNA is represented by the net cpm of viral mRNA species containing exon 3 divided by the total cpm of the respective *nef*, *rev*, and *tat* mRNA species. The levels of amplified *tat* mRNA products were relatively low, and the observed differences were not statistically significant. Thus, *tat* mRNA species were excluded from this analysis. Percent inclusion of exon 3 was determined from at least three independent experiments. Asterisks indicate a significant difference when compared the respective mRNA species from pΔPSP-transfected cells from three independent experiments (*, $P < 0.01$; **, $P > 0.1$ [as determined by Student's *t* test]).

particle production in the absence of ESSV, we first analyzed viral mRNA splicing in cells transfected with the mutant ESSV plasmids. Analysis of spliced viral mRNA within transfected cells indicated that there was an increase in the inclusion of HIV-1 exon 3 within both the completely and incompletely spliced viral mRNA classes in cells transfected with either mutant. Specifically, the *nef* (1.3.5.7), *rev* (1.3.4a/b/7), and *tat* (1.3.4.7) exon 3-containing mRNAs increased relative to the respective mRNA species lacking exon 3 (*nef*, 1.5.7 and 1.2.5.7; *rev*, 1.4a/b.7; *tat*, 1.4.7 and 1.2.4.7) (Fig. 3C, compare lane 2 to 3 and 4). Similarly, the steady-state levels of incompletely spliced *env* mRNA species (1.3.5I, 1.3.4b/aI, and 1.3.4I), containing exon 3, and the *vpr* mRNA (1.3I) spliced at 3′ss A2 increased in both mutants relative to wild-type-transfected cells (Fig. 3D, compare lane 2 to 3 and 4).

In order to determine if the overall level of viral splicing changed in response to increased exon 3 inclusion, Northern blot analysis of viral mRNA was performed on total RNA from transfected cells. In contrast to pNL4-3-transfected cells, where ~50% of viral RNA remains unspliced, the unspliced viral RNA within pNEVM- and pNEU-transfected cells was decreased to a low level (Fig. 3E). The dramatic decrease in unspliced viral RNA in pNEVM- and pNEU-transfected cells correlated with a corresponding decrease in cell-associated HIV-1 Gag proteins (p55, CA, and MA) compared to pNL4-3-transfected cells (Fig. 3F). In contrast, transient transfection of all three viral constructs exhibited similar levels of HIV-1 Env expression, when the levels of a cotransfected β-gal control plasmid were taken into consideration. The wild-type expression levels of HIV-1 Env suggest that the decrease in unspliced viral mRNA and Gag protein is not due to suboptimal expression of HIV-1 Rev. Furthermore, transient expression either of HIV-1 Tat or Rev had no effect on levels of virus production in pNEVM- and pNEU-transfected cells, demonstrating that decreased regulatory protein expression is not responsible for the observed defect in virus replication (data not shown). Together these results suggest that the inability to produce reverse-transcriptase-competent virions is caused by

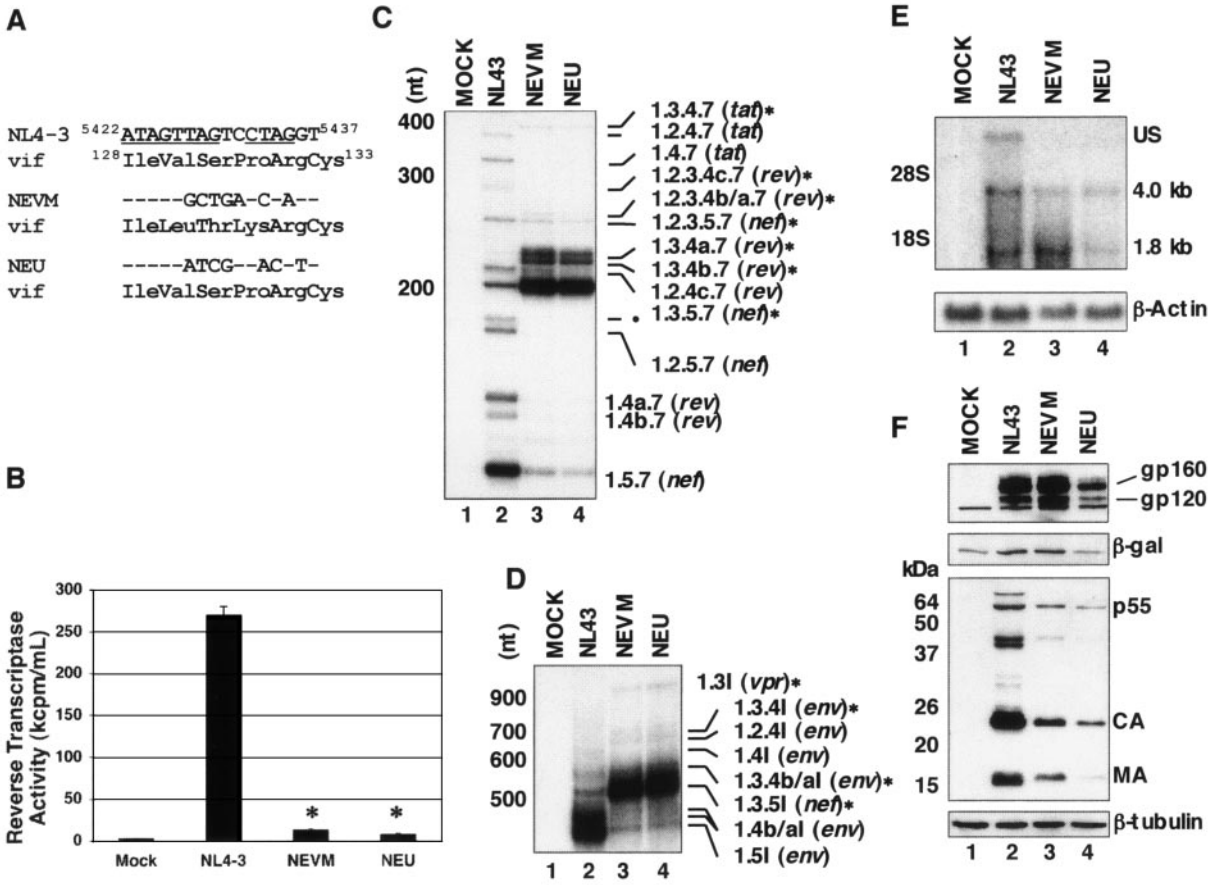

FIG. 3. ESSV is required for regulated splicing and efficient replication of NL4-3. (A) ESSV within pNL4-3 was mutagenized as shown for either the pNEVM or pNEU mutant. The respective coding sequence for the viral accessory protein vif is shown, and the nucleotide numbering reflects that of NL4-3. (B) Reverse transcriptase activity within cell-free supernatants from HeLa cells transfected with ESSV mutants. Asterisks indicate a significant difference when compared to mock-transfected cells from three independent experiments ($P < 0.01$ by Student's t test). Total cellular RNA was subjected to RT-PCR using primers specific for either the completely (C) or incompletely (D) spliced viral mRNA. Labels on the right refer to the exon content of the viral mRNA and mRNA identity, and asterisks indicate mRNA spliced at 3′ss A2. The band labeled with a closed circle was not reproducible in different experiments and does not correspond to an expected PCR product amplified from a known viral mRNA species under these conditions. (E) Total cellular RNA was subjected to Northern analysis with a radiolabeled probe complementary to all HIV-1 mRNA. The positions of the size classes of HIV-1 mRNA (unspliced [US], 4.0 kb, and 1.8 kb) are indicated. The blot was stripped and probed with a radiolabeled probe complementary to β-actin. (F) Protein from transfected HeLa cells was subjected to Western analysis using antibodies to gp160, β-galactosidase, β-tubulin, or serum from an HIV-1-infected patient.

the overuse of a viral 3′ss by the cellular splicing machinery, resulting in an insufficient pool of unspliced viral mRNA and, consequently, decreased HIV-1 Gag expression.

Selection of second-site revertants restoring pNEVM replication. If the replication defect associated with the pNEVM mutant was caused by an increase in exon 3 inclusion, long-term culture of pNEVM-transfected cells would be expected to select for second-site revertants that would restore efficient viral replication. The ability of pNEVM-transfected cells to produce a marginally significant amount of reverse transcriptase-competent virions (Fig. 3B) suggested that virus replication was occurring at a low level in the absence of ESSV. Low levels of virus replication allowed us to select for second-site reversions after transient transfection of Jurkat cells. Two independent cultures of Jurkat cells contained nearly wild-type levels of reverse transcriptase activity within the viral culture supernatant after 25 to 50 days after transfection of pNEVM (data not shown). Analysis of the integrated viral genomes within these cultures revealed no changes within the mutagenized ESSV sequence. However, the integrated viral genomes from Rev 2-infected cells contained a single point mutation within the viral donor splice site D3 such that the invariant GU was mutagenized to GC ($^{+1}$G/GUAGG^{-5} to $^{+1}$G/GCAGG^{-5}). Similarly, the viral acceptor splice 3′ss A2 was disrupted within the integrated viral genomes from Rev 6-infected cells, deleting the invariant AG ($^{-4}$UCAG/AA^{+2} to $^{-4}$UC●●/AA^{+2}) (Fig. 4A).

To investigate whether the disruption of 3′ss A2 or 5′ss D3 was sufficient to restore inefficient splicing in the absence of ESSV, we disrupted these splicing signals in the context of the ESSV mutant, pNEVM, by site-directed mutagenesis and analyzed spliced HIV-1 mRNA species within transiently transfected HeLa cell cultures by RT-PCR. Disruption of 3′ss A2 (VMRV6) or 5′ss D3 (VMRV2) restored virus production within transiently transfected cells to levels similar to that of pNL4-3-transfected cells (Fig. 4B). Analysis of spliced viral

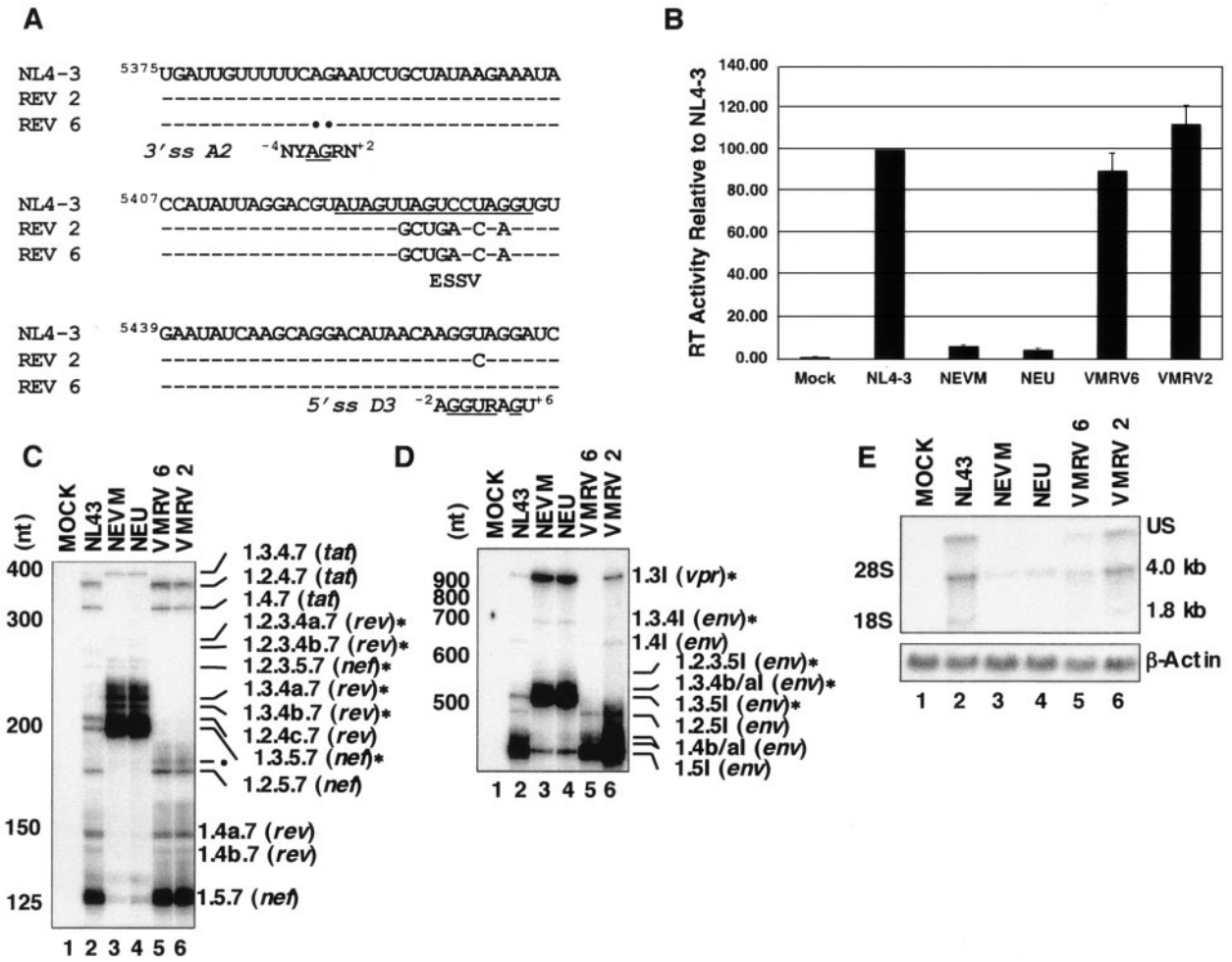

FIG. 4. Revertants reestablishing balanced splicing are selected after long-term culture of ESSV mutants. (A) Nucleotide changes identified within HIV-1 exon 3 represented below the respective sequence of pNL4-3 were identified upon sequencing integrated proviral genomes from revertant Jurkat cultures. The seven-nucleotide disruption of ESSV is shown below the wild-type ESSV sequence, which is underlined, and the single point mutation within 5′ss D3 in Rev2 or the dinucleotide deletion within 3′ss A2 in Rev6 is shown above the metazoan consensus 5′ss or 3′ss sequence, respectively. (B) Reverse transcriptase activity within cell-free supernatants from 293T cells transfected with ESSV and splice site mutants. Data are representative of three independent experiments. (C and D) Analysis of viral mRNA amplified by RT-PCR using primers specific for either the completely (C) or incompletely (D) spliced viral mRNA and using total cellular mRNA from cells which had been transfected with plasmids pVMRV2 and pVMRV6, which represent the respective reversions identified in panel A. Labels on the right refer to the exon content of the viral mRNA and mRNA identity, and asterisks indicate mRNA spliced at 3′ss A2. The band labeled with a closed circle was not reproducible in different experiments and does not correspond to an expected PCR product amplified from a known viral mRNA species under these conditions. (E) Northern analysis of transfected cellular RNA with a radiolabeled probe complementary to all HIV-1 mRNA (unspliced [US], 4.0 kb, and 1.8 kb), as well as a radiolabeled probe complementary to β-actin after stripping the blot.

mRNA by RT-PCR revealed the failure to include HIV-1 exon 3 within either completely or incompletely spliced viral mRNAs (Fig. 4C and D, lanes 5 and 6) in VMRV6- and VMRV2-transfected cells when compared to NL4-3 (Fig. 4C and 4D, lane 2). Furthermore, cells transfected with VMRV6 did not accumulate *vpr* mRNA due to the absence of 3′ss A2 (Fig. 4D, lane 5). However, in the absence of both ESSV and 5′ss D3, we observed an accumulation of the incompletely spliced *vpr* mRNA (Fig. 4D, lane 6), which suggests that splicing of *vpr* mRNA can still occur in the absence of splicing at 5′ss D3. Disruption of either 3′ss A2 or 5′ss D3 in the presence of the NEVM mutation resulted in the accumulation of unspliced viral mRNA, as observed in NL4-3-transfected cells (Fig. 4E). Taken together, these results demonstrate that dis-

TABLE 2. Alignment of ESSV elements[a]

Group	Subtype	ESSV
M	B	^{4964}ATAG TTAGTC CTAGGT^{4979}
	A	^{4745}G--- ----C- ----T-^{4760}
	C	^{4745}---- ---T-- ------^{4760}
	D	^{4957}---- ------ ------^{4972}
	F	^{4681}-G-A ----C- ----T-^{4696}
	G	^{4772}---- ----C- ----T-^{4787}
N		^{5016}CC-- -GTTA- ------^{5031}
O		^{5274}-G-- -ACTGA -C-A--^{5289}

[a] The consensus HIV-1 subtype B ESSV is shown, and the three (Py/A)TAG motifs are underlined. Viral sequences (21) were divided between groups and into subtypes and aligned, and diverging nucleotides are shown. Numbering and nucleotides represent the consensus sequence from each respective group M subtype or group O HIV-1 and group N strain CM.YBF30.

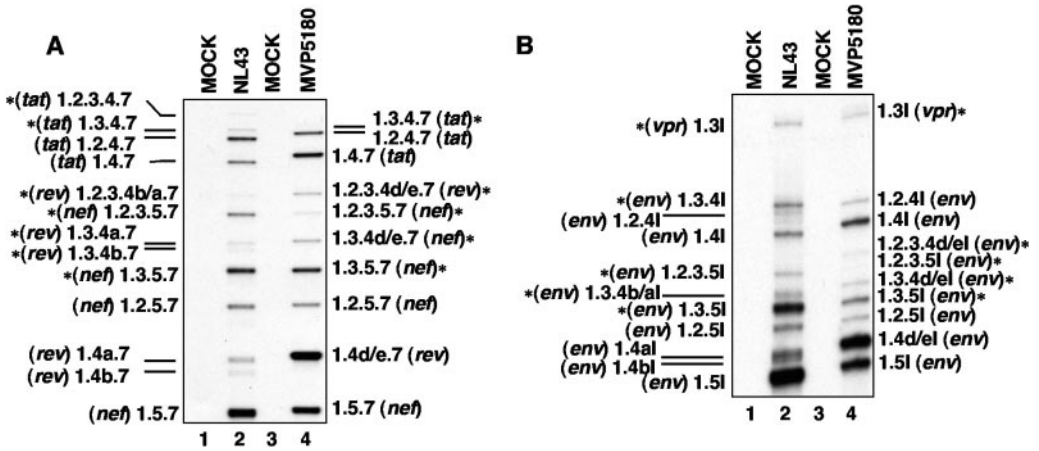

FIG. 5. Comparison of spliced mRNAs from cells infected with NL4-3 and group O virus MVP5180. Poly(A)$^+$ mRNA was purified from infected H9 cells and analyzed by RT-PCR. cDNA from infected cells, normalized to β-actin mRNA levels, was subjected to PCR using primers specific for either the completely spliced viral mRNA (A) or the incompletely spliced viral mRNA (B). mRNA identity is indicated as well as exon content, and mRNAs spliced at 3′ss A2 are indicated by asterisks. Lane 1 in panels A and B represents mock-transfected cells PCR amplified with primers complementary to NL4-3 completely or incompletely spliced RNA, and lane 3 in panels A and B represents oligonucleotide primers complementary to MVP5180 mRNAs. Group O strain MVP5180 mRNAs contain 13 extra nucleotides downstream of 3′ss A3 (relative to NL4-3), and therefore MVP5180 mRNA species containing exon 4 migrate more slowly than NL4-3 mRNA containing exon 4.

ruption of splicing at either 3′ss A2 or 5′ss D3 is sufficient to restore regulated splicing and nearly wild-type virus production in the absence of ESSV.

ESSV is not conserved between group M and group O HIV-1. Alignment of the respective 16-nt ESSV elements from various strains of group M HIV-1 revealed that ESSV was conserved between group M HIV-1 strains (Table 2). Within the group M strains of HIV-1, the three (Py/A)UAG motifs in the ESSV elements from clades B, D, and G are conserved, and the ESSV sequences of clades A, C, and F diverge by only one or two nucleotides within the three (Py/A)UAG motifs. In contrast, the respective ESSV sequences from both group O and group N HIV-1 diverge markedly from those of group M HIV-1. The first two of three (Py/A)UAG motifs are absent within group N ESSV, and all three (Py/A)UAG motifs are absent within group O HIV-1. Thus, a (Py/A)UAG-dependent ESSV at this location within the HIV-1 genome appears to be a characteristic of group M HIV-1 strains but not of group N or O HIV-1 strains.

Splicing at 3′ss A2 is inhibited in group O HIV-1-infected cells. The NEVM mutation, which represents the group O HIV-1 substitutions within two of the group M HIV-1 ESSV (Py/A)UAG motifs, was shown to relieve ESSV inhibition of a heterologous, upstream acceptor splice site in an in vitro splicing assay performed in HeLa nuclear extract (data not shown). Relief of ESSV inhibition confirmed the absence of silencer activity within the pNEVM ESSV. Furthermore, the observed divergence in ESSV between group M and group O HIV-1 suggested that HIV-1 3′ss A2 may not be regulated within group O HIV-1-infected cells and predicts that group O HIV-1-infected cells would have increased exon 3 inclusion and *vpr* mRNA levels when compared to group M HIV-1-infected cells. To test this hypothesis, we analyzed RNA isolated from H9 cells infected with either NL4-3 or the group O HIV-1 strain MVP5180. RT-PCR analysis of spliced viral mRNA revealed that there was no increase in the inclusion of exon 3 in group O *nef*, *rev*, and *tat* mRNAs (Fig. 5A, lanes 2 and 4) or *env* mRNAs (Fig. 5B, lanes 2 and 4) when compared to group M mRNAs. Furthermore, the steady-state levels of the incompletely spliced *vpr* mRNA (Fig. 5B, 1.3I) did not appear to be different between group M- and group O-infected cells. These results suggest that, although ESSV is not conserved between group O and group M HIV-1, utilization of the MVP5180 3′ss A2 is inhibited to a similar extent as NL4-3 3′ss A2, preventing exon 3 inclusion in group O HIV-1 mRNA.

DISCUSSION

In this report, we have shown for the first time that one of the hnRNP A/B-dependent exonic splicing silencers (ESSV) within the HIV-1 genome is required for efficient virus replication. ESSV has previously been shown to specifically repress splicing at HIV-1 3′ss A2 (5). Disruption of ESSV results in an increased level of *vpr* mRNA and an almost complete inclusion of the noncoding exon 3, which is flanked by 3′ss A2 and 5′ss D3. Our data indicated that a sequence necessary for ESSV activity downstream of HIV-1 3′ss A2 is localized to a 16-nt element within the *vif* coding sequence. We have previously shown that a 24-nt sequence containing ESSV was sufficient to inhibit utilization of a heterologous 3′ss in HeLa cell nuclear extracts (5). It has previously been shown that other HIV-1 ESS elements and the ISS element contain PyUAG repeats that are necessary for binding of members of the hnRNP A/B protein family (5, 8, 31, 37). We showed in this report that an AUAG motif, in addition to the two PyUAG motifs, is important for ESSV activity. Similarly, an AUAG motif and a PyUAG motif are also present within the 20-nt hnRNP A1 "winner" high-affinity binding site that was established by the SELEX method (6). Furthermore, the most 5′ nucleotides of the 16-nt ESSV element share homology with two of the putative ESS clusters recently identified by a computational ap-

proach (36). Thus, our data define the consensus sequence for HIV-1 ESS motifs described to date as (Py/A)UAG.

Although ESSV is highly conserved in the group M HIV-1 strains, it is not conserved in the group O strains where all three (Py/A)UAG motifs are disrupted. In contrast to ESSV, the locations and sequences of 3′ss A2 and 5′ss D3 are highly conserved in both group O and M HIV-1 strains (21). However, in spite of the absence of ESSV, we showed that the group O strain MVP5180 included exon 3 and produced *vpr* mRNA at levels comparable to the group M strain NL4-3. Inhibition of a HIV-1 3′ss A2 in the absence of a (Py/A)UAG-dependent ESSV suggests that the group O viruses may contain other splicing silencer elements, either upstream or downstream of 3′ss A2, that act to repress splicing.

We observed that mutation of ESSV results in a decreased level of unspliced viral RNA, a reduction in Gag protein levels, and a decreased production of virus particles within transiently transfected cells. The accumulation of unspliced RNA and efficient virus production were restored to ESSV mutants by inactivation of either 3′ss A2 or 5′ss D3, which prevents the increased 3′ss utilization exhibited by the ESSV mutant. HIV-1 isolate IIIB has a unique splicing pattern since it includes an exon within the *env* gene (exon 6), through utilization of cryptic 3′ss A6 (13). In a separate report, a single base mutation within a putative ESS in exon 6 resulted in an increase in inclusion of exon 6 (33). Furthermore, overall splicing of viral RNA was increased, which was correlated with decreased Gag expression (33). Recent results have indicated that, rather than inactivating an ESS as originally proposed, the mutation within exon 6 created a positively acting exonic splicing enhancer (ESE) that acts to increase splicing at the splicing acceptor flanking exon 6 (7). It was not clear from the studies of Wentz et al. (33) whether excessive splicing or the observed decrease in Rev protein levels is primarily responsible for a decrease in unspliced RNA levels and the replication-defective phenotype. In our study, the ESSV mutant produced Rev at sufficient levels to express wild-type levels of Env protein, whose expression requires Rev-dependent transport, indicating that decreased unspliced viral mRNA was not a result of decreased Rev expression.

In the case of avian sarcoma virus (ASV), mutations that improved the branch point of the *env* 3′ss caused a defective virus phenotype which was restored by second-site reversions of the branch point sequence itself or mutations within the ESE element downstream of the *env* 3′ss (11, 17, 18). Mutations that improve the PPT of the ASV *src* 3′ss also caused excessive splicing and a defective virus phenotype. Efficient virus replication was restored by deletion mutations that decreased the length of the PPT (35).

In addition to the inability of the ESSV mutants to accumulate unspliced RNA, these mutations might be predicted to impair virus replication by increasing levels of spliced mRNA compared to NL4-3-transfected cells. Instead, we found that the levels of 4.0-kb and 1.8-kb spliced viral mRNAs in ESSV mutant-transfected cells were similar to wild type (Fig. 3E). The most likely explanation for the decrease in spliced viral mRNA is that the increased inclusion of exon 3 into HIV-1 mRNAs resulted in reduced mRNA stability. The potential influence of HIV-1 exon 3 on viral mRNA stability is consistent with a previous report which showed that the expression of HIV-1 cDNA constructs containing HIV-1 mRNA 5′ leaders with exon 3 are reduced compared to similar constructs lacking exon 3 (20). An alternate explanation for the observed decrease in spliced viral mRNA is that the mutations we have generated within ESSV may disrupt a stability element. We believe that the disruption of a putative stability element is unlikely since the ESSV mutation in both revertant viruses was not altered, yet the revertants accumulated both spliced and unspliced viral mRNA at levels similar to wild-type NL4-3-transfected cells.

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