Inhibitory Activity of the Equine Infectious Anemia Virus Major 5' Splice Site in the Absence of Rev

WEI TAN,¹ MARTIN SCHALLING,² CUIPING ZHAO,¹ MATTIAS LUUKKONEN,¹ MIA NILSSON,²
EVA MARIA FENYÖ,¹ GEORGE N. PAVLAKIS,³ AND STEFAN SCHWARTZ^{1*}

*Microbiology and Tumorbiology Center, Karolinska Institute, 171 77 Stockholm,¹ and Neurogenetics Unit,
Department of Molecular Medicine, Karolinska Hospital, 171 76 Stockholm,² Sweden, and
National Cancer Institute—Frederick Cancer Research and Development Center,
ABL-Basic Research Program, Frederick, Maryland 21702-1201³*

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The major 5' splice site of equine infectious anemia virus (EIAV) conforms to the consensus 5' splice site in eight consecutive positions and is located immediately upstream of the *gag* AUG. Our results show that the presence of this 5' splice site on the EIAV *gag* mRNA decreases Gag production 30- to 60-fold. This is caused by inefficient nuclear mRNA export and inefficient mRNA utilization. Inhibition could be overcome by providing human immunodeficiency virus type 1 Rev/Rev-responsive element, human T-cell leukemia virus type 1 Rex/Rex-responsive element, or simian retrovirus type 1 constitutive transport element. In addition, inhibition could be abolished by introducing single point mutations in the 5' splice site or by moving the 5' splice site away from its natural position immediately upstream of the *gag* AUG. This demonstrates that both maintenance of a perfect consensus 5' splice site and its proper location on the mRNA are important for inhibitory activity of the EIAV major 5' splice site.

Studies of the human immunodeficiency virus type 1 (HIV-1) Rev protein have revealed that Rev is required for efficient expression of *Gag*, *Pol*, and *Env* proteins (38). Rev acts posttranscriptionally by interacting with an RNA sequence named the Rev-responsive element (RRE) which is located on the *gag*, *pol*, and *env* mRNAs (25, 35, 51, 60). In the absence of Rev, the unspliced and singly spliced HIV-1 mRNAs encoding viral structural proteins are sequestered in the nucleus (25, 27, 36, 51), are unstable (27, 50), and are not translated into proteins (5, 20, 45). It has been suggested that unspliced and singly spliced HIV-1 mRNAs are trapped in the nucleus as a result of spliceosome formation on unutilized splice sites on these mRNAs (15).

On the basis of the ability of synthetic peptides of Rev to inhibit splicing of cellular mRNAs *in vitro*, it has been suggested that Rev acts by inhibiting splicing, thereby facilitating transport of unspliced and singly spliced mRNAs from the nucleus to the cytoplasm (41). However, HIV-1 mRNAs contain sequences that act *in cis* to inhibit splicing, and these sequences are active in the absence of Rev (3, 4, 70, 71). As a result, high levels of unspliced mRNAs are also detected in the absence of Rev.

Results from several laboratories have shown that HIV-1 mRNAs contain *cis*-acting, intragenic inhibitory sequences (10, 18, 49, 54, 60, 63, 65) that inhibit production of viral structural proteins in the absence of Rev. Such sequences have been mapped within the *gag*, *pol*, and *env* regions of HIV-1 mRNAs. Inhibitory sequences located within the HIV-1 p17^{gag} coding sequence were inactivated by the introduction of multiple point mutations (63, 65). These silent mutations, which did not affect the amino acid sequence of the HIV-1 *Gag* protein, resulted in constitutive high *Gag* production independently of Rev (63), thereby showing that the inhibitory sequences were

directly involved in inhibition of *Gag* production in the absence of Rev. These sequences acted at least in part by reducing mRNA stability (63, 65). Taken together, previously published results are consistent with the idea that Rev interacts with RRE-containing mRNAs and redirects these mRNAs from pathways resulting in nuclear retention and degradation mediated by inhibitory elements. It has been shown that Rev is able to directly promote nuclear export of RRE-containing mRNAs microinjected into *Xenopus* oocyte nuclei (29). Furthermore, conjugates between bovine serum albumin and peptides constituting the activation domain of Rev can inhibit Rev-mediated nuclear mRNA export in *Xenopus* oocytes (28). Recently, cellular proteins with homology to nucleoporins were shown to interact with the activation domain of Rev, supporting a role for Rev in nuclear mRNA export (8, 31).

To date, members of the lentivirus family including HIV-1, HIV-2, simian immunodeficiency virus, visna virus, caprine arthritis encephalitis virus, feline immunodeficiency virus, and equine infectious anemia virus (EIAV) have been shown to encode Rev proteins (19). These Rev proteins act on RNA-responsive elements located within the *env* gene. The oncoretroviruses human T-cell leukemia virus types 1 and 2 (HTLV-I and -II) and bovine leukemia virus (19) encode Rev-like proteins termed Rex. These proteins bind to RNA elements named Rex-responsive elements (RREs) located within the viral long terminal repeat (LTR). Interestingly, the HTLV-I Rex protein can replace HIV-1 Rev, but not vice versa (59). Mason-Pfizer monkey virus is a D-type retrovirus which causes immunodeficiency in newborn rhesus monkeys. It has recently been shown that the 3' untranslated region (UTR) of Mason-Pfizer monkey virus contains a constitutive transport element (CTE) that can substitute for HIV-1 Rev/RRE (9). An element with similar function was also found in the 3' UTR of simian retrovirus type 1 (SRV-1) (85). This element can replace Rev/RRE in the context of the whole virus, resulting in Rev-independent virus expression. These results suggest that eucaryotic cells produce a protein with Rev-like properties and that cellular mRNAs may be regulated by similar mechanisms.

* Corresponding author. Mailing address: Microbiology and Tumorbiology Center, Karolinska Institute, P.O. Box 280, 171 77 Stockholm, Sweden. Phone: 468 728 6312. Fax: 468 331 399. Electronic mail address: stefan.schwartz@mtc.ki.se.

To gain further insight into the posttranscriptional mechanisms that regulate expression of complex retroviruses, we investigated the requirements for efficient expression of the EIAV Gag protein. EIAV is an ungulate lentivirus and the etiologic agent of equine infectious anemia. In general, EIAV shares many biological features with HIV-1, such as lifelong persistent infection in the natural host, restricted viral expression during an asymptomatic period, and a relatively high rate of antigenic variation and periodic emergence of neutralization-resistant variants despite the presence of a host immune response (16, 17, 57). As a complex retrovirus, the EIAV genome contains several short open reading frames (ORFs) in addition to the *gag*, *pol*, and *env* genes common to all retroviruses (see Fig. 1A). These short ORFs are translated from multiply spliced mRNAs (6, 12, 56, 61, 74). ORF S1 encodes a transcriptional transactivator named Tat, which acts on a 25-nucleotide (nt) RNA sequence named the Tat-responsive element (14, 22, 23), located downstream of the transcriptional start site on the EIAV LTR (23, 67, 68). The EIAV Tat protein appears to be functionally and structurally homologous to HIV-1 Tat (13, 21, 22). ORF S2 encodes a protein whose function is unknown, and a Rev protein is encoded by ORF S3 (52). The EIAV RRE has not been mapped in detail but appears to be a bipartite element located within the *env* region (52).

In the present study, we identified sequences that inhibit expression of EIAV Gag in the absence of Rev. One inhibitory RNA sequence is located immediately upstream of the EIAV *gag* gene and coincides with the major 5' splice site on the EIAV genome. Interestingly, the EIAV 5' splice site has a perfect homology to the consensus 5' splice site, implying strong binding to the U1 small nuclear RNA (snRNA) component of the cellular splicing machinery. Introduction of single point mutations at positions -2, -1, +1, +2, +3, +4, or +5 in the 5' splice site sequence abolished the inhibitory activity and gave rise to high levels of Gag protein. Inhibition could be overcome by providing the HIV-1 Rev/RRE, the HTLV-1 Rex/RxRE, or the SRV-1 CTE in *cis*. We provide evidence that the unutilized splice site acts by retaining the EIAV *gag* mRNA in the nucleus and by affecting the efficiency of utilization of mRNAs containing the 5' splice site.

MATERIALS AND METHODS

Plasmid constructions. To construct pE15, the EIAV matrix p15^{gag} coding sequence (nt 446 to 817) was first PCR amplified from the EIAV plasmid p8 (73) (kindly provided by N. R. Rice) by using oligonucleotide 7227 (5'-CAGCGCGC CAAGATGGGAGACCTTTG-3'), which introduced a *Bss*HII site (underlined) upstream of the translational start codon of *gag*, and oligonucleotide 7228 (5'-CTATTAATATTCTTCAGAGGGCTC-3'), which introduced a translational stop codon (underlined). The first nucleotide of the EIAV LTR is designated +1 (73). The PCR fragment was subcloned into pNL17 (65) digested with *Sal*I and *Eco*RI and filled in with Klenow DNA polymerase. This resulted in pE15. To generate pG15R, EIAV sequences between nt 189 and 817 were PCR amplified with oligonucleotides 8921 (5'-CAGCGCGCGCACTCAGATTCTG CGG-3') and 7228 and were subcloned into pBluescript (Stratagene). The p15^{gag} coding sequence was transferred as an *Xho*I-*Eco*RI fragment into pNL17R (65) digested with *Sal*I and *Eco*RI. pG15 was generated by digestion of pG15R with *Asp* 718 and then by religation.

To generate pE55R, the EIAV p55^{gag} gene (nt 446 to 1906) was first PCR amplified with oligonucleotides 7227 and 7420 (5'-TTACTCCACAACTGTC CAGG-3') and subcloned into *Eco*RV-digested pBluescript (Stratagene), resulting in pT7E55. The EIAV *gag* sequence was then transferred as a *Sal*I-*Eco*RI fragment into pNL17R (65) digested with *Sal*I and *Eco*RI. pE55 was generated by partial digestion of pE55R with *Asp* 718 and by religation. To generate pG55R, EIAV sequences between nt 189 and 1906 were PCR amplified with oligonucleotides 8921 and 7420 and were subcloned into pBluescript (Stratagene), resulting in pT7G55. The EIAV *gag* sequence was then transferred as a *Sal*I-*Eco*RI fragment into pNL17R (65) digested with *Sal*I and *Eco*RI. pG55 was generated by partial digestion of pG55R with *Asp* 718 and then by religation. The HIV-1 5' LTR sequence in plasmid pE55 or pG55 starts at nt 1 and ends at nt

711 (numbering of the HIV-1 sequence refers to the sequence of pNL43) (1). To construct pCE55, a *Sal*I-*Eco*RI fragment containing the EIAV p55^{gag} gene (nt 446 to 1906) from pE55 was first cloned downstream of the human cytomegalovirus immediate-early promoter. pCE55 was thereafter generated by insertion of a *Bam*HI-*Bcl*II fragment containing the simian virus 40 early poly(A) signal downstream of the EIAV p55^{gag} gene. To generate pCG55, the EIAV sequences in pCE55 were removed by partial digestion with *Sal*I-*Eco*RI and were replaced with a *Sal*I-*Eco*RI fragment obtained from pG55 (nt 189 to 1906).

To construct pG55RxRE, the HTLV-1 RxRE was transferred as an *Eco*RI-*Xho*I fragment from pBS-RxRE (80) to pG55 digested with *Eco*RI and *Xho*I. To construct pG55CTE, the SRV-1 CTE was PCR amplified with oligonucleotides 12745 (5'-GCATCAACGCGTGTGACGGATCCAGACCACCTCCCTGCG GAG-3') and 13827 (5'-GCATCAGCGGCGCCTCGAGTCTAGACAAATC CCTCGGAAGCTGCG-3') (85) and was cloned directly into pG55 digested with *Eco*RI and filled in with Klenow DNA polymerase.

Plasmid pΔ186 was generated by digestion of pG55 with *Bss*HII and *Sma*I, filling in of staggered 5' ends by using Klenow DNA polymerase, and religation. pΔ233 was constructed by partial digestion of pG55 with *Sal*I and *Msc*I, filling in, and religation. pESD(-)G was constructed by first PCR amplifying sequences upstream of the EIAV *gag* AUG (nt 189 to 442) by using oligonucleotides 8921 and EIAVSD(-) (5'-GTCTCCCATTTTCGGCGCGCTCTGTGTTCTG-3'). The oligonucleotide EIAVSD(-) introduced a *Bss*HII restriction site (underlined), which resulted in four substitutions and insertion of a C nucleotide upstream of the 5' splice site. pESD(-)G was generated by cloning of the *Bss*HII-digested PCR fragment into the *Bss*HII site located immediately upstream of the EIAV *gag* gene in plasmid pE55.

To construct pG55ΔE, sequences upstream of the EIAV 5' splice site were mutated by site-directed mutagenesis on uracil-containing single-stranded DNA of pT7G55 with oligonucleotide ΔE (5'-GTCTCCCATTTACCTGGGCCCCGG AACACCTCC-3') as described elsewhere (48), in which an *Apa*I site (underlined) was introduced and a 15-nt sequence immediately upstream of the 5' splice site was deleted. The mutated EIAV sequence was transferred as a *Bss*HII-*Asp* 718 fragment into *Bss*HII-*Asp* 718-digested pNL17R (65), generating plasmid pG55ΔE.

To construct plasmids with multiple or single point mutations in the EIAV 5' splice site CAGGTAAG (nt 438 to 445), site-directed mutagenesis on uracil-containing single-stranded DNA of pT7G55 was performed as described elsewhere (48) with the following oligonucleotides (mutations introduced by the oligonucleotides are indicated by lowercase letters): -1C-2C-3T (5'-GTCTCC CATCTTACggaTCTCTCTG-3'), +3G+4C (5'-GTCTCCCATCgcACTGTGCTCTCTG-3'), -3T (5'-GTCTCCCATCTTACCTaTCTCTCTG-3'), -2C (5'-G TCTCCCATCTTACCGTCTCTCTG-3'), -1C (5'-GTCTCCCATCTTACgT GTCCTCTG-3'), +1A (5'-GTCTCCCATCTTAcTGTCTCTCTG-3'), +2A (5'-GTCTCCCATCTTCTCTCTCTCTG-3'), +3G (5'-GTCTCCCATCTeA CCTGTCTCTCTG-3'), +4C (5'-GTCTCCCATCTaCTGTCTCTCTCTG-3'), +5A (5'-GTCTCCCATTTACCTGTCTCTCTG-3'). Mutants were screened by sequencing, and mutated fragments were transferred as *Bss*HII-*Apa*I fragments into pG55 digested with *Bss*HII and *Apa*I. This cloning step resulted in the plasmids pG55-3T-2C-1C, pG55+3G+4C, pG55-3T, pG55-2C, pG55-1C, pG55+1A, pG55+2A, pG55+3G, pG55+4C, and pG55+5A, respectively.

To construct pESDSD(-)G, a sequence containing the EIAV 5' splice site (nt 189 to 445) was PCR amplified with oligonucleotides 8921 and EAS (5'-GGGCGCGCCTTACCTGTCTCTCTGTGTTCT-3') and was inserted in the *Stu*I site at nucleotide position 237 in plasmid pESD(-)G. To construct pESD13G, a sequence containing the EIAV 5' splice site was first excised with *Bss*HII and then cloned into *Bss*HII-digested pE55. pESD32G and pESD86G, respectively, were constructed by transferring a *Bss*HII-digested and Klenow filled-in fragment containing the 5' splice site into *Msc*I- or *Bss*HII-digested and Klenow-filled-in pESD(-)G.

The HIV-2 *gag* coding sequence (nt 535 to 2109) was PCR amplified from the infectious HIV-2 molecular clone HIV-2ISY (30) by using oligonucleotides 7105 (5'-CATGCGCGCAGATTGTGGGAGATGGGC-3') and 7422 (5'-GAGAAA CCTTTGTCTGGTATC-3'). A *Bss*HII restriction site (underlined) was introduced 12 nt upstream of the HIV-2 *gag* ATG (underlined) through oligonucleotide 7105. The PCR fragment was first inserted into *Eco*RV-digested pBluescript (Stratagene) and was subsequently transferred as a *Bss*HII-*Asp* 718 fragment to pNL17R (65) partially digested with *Bss*HII and *Asp* 718, generating pH2GR. pH2G was generated by digestion of pH2GR with *Asp* 718 and then by religation. To construct pESDH2G and pESDH2GR, a *Bss*HII fragment containing the EIAV 5' splice site (see above) was subcloned into the *Bss*HII site immediately upstream of HIV-2 *gag* ATG in pH2G and pH2GR, resulting in pESDH2G and pESDH2GR, respectively. To construct pESDM17, a *Bss*HII fragment containing the EIAV 5' splice site was subcloned into *Bss*HII-digested plasmid p17M1234 (63). Plasmid pM17ESD was constructed by cloning the EIAV 5' splice site-containing sequence downstream of the p17^{gag} gene in plasmid p17M1234 (63).

The HIV-1 Rev expression plasmid pNL14A7 (64), the HIV-1 p37^{gag} expression plasmid pC37M (63), and the HTLV-1 Rex expression plasmid pL3Rex (80) have been described previously.

Cells and transfections. HLtat cells (64), a HeLa-derived cell line constitutively producing HIV-1 Tat, were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Transfections were performed by the calcium

phosphate coprecipitation technique (34) as previously described (77). When the vaccinia virus-T7 RNA polymerase expression system was employed, cells were infected with 0.5×10^6 PFU of recombinant vaccinia virus vTF7-3 (33) expressing T7 RNA polymerase 1 to 2 h prior to transfection. pNLCAW (76), pC37M (63), or pCSIX (76) was included as an internal control in each transfection experiment, and alkaline phosphatase activity was determined as previously described (76).

Western blotting (immunoblotting). Immunoblotting was performed as described previously (76, 77). Briefly, proteins were electrophoretically separated on 12 or 15% polyacrylamide-sodium dodecyl sulfate (SDS) gels and then transferred onto nitrocellulose membranes. Blots were blocked for 1 h at 37°C with 10% nonfat milk in phosphate-buffered saline (PBS) containing 0.3% Tween 20 (PBS-T). After being washed three times in PBS-T buffer, the blots were incubated for 1 h at 37°C with either the HIV-1- or HIV-2-positive patient serum (1:3,000 dilution) (kindly provided by J. Albert and R. Thorstenson) or rabbit hyperimmune sera directed against EIAV p15^{gag} or p26^{gag} (37) (1:5,000 dilution) (kindly provided by S. Oroszlan). Horseradish peroxidase (HRP)-conjugated donkey anti-human immunoglobulin G (IgG) or HRP-conjugated goat anti-rabbit IgG (1:10,000 dilution) was used as a secondary antibody. Specific proteins were visualized with enhanced chemiluminescence detection reagents (Amersham).

RNA extractions and Northern (RNA) blotting. Total RNA was prepared from transfected HLtat cells (64) 24 h posttransfection by the heparin-DNase procedure as previously described (77). A 20-μg amount of each RNA sample was separated on a 1% denaturing agarose gel and blotted onto nitrocellulose filters. After baking at 80°C for 2 h, the membranes were hybridized to a [³²P]UTP-labeled riboprobe, generated from pT7G55, at 55°C overnight. The filters were washed three times with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS and three times with 0.1× SSC–0.1% SDS at 60°C. The dried filters were exposed to X-ray film at –70°C with intensifying screens.

Cytoplasmic and nuclear poly(A)⁺ mRNAs were isolated by Dynabeads Oligo(dT)₂₅ (Dynal A.S.) as described (77). Briefly, transfected cells were lysed for 5 min on ice in lysis buffer (10 mM Tris-HCl [pH 7.5], 0.14 M NaCl, 5 mM KCl, 0.65% Nonidet P-40). Cytoplasmic and nuclear fractions were separated by centrifugation at 8,000 × g for 2 min. The pellets containing nuclei were washed once with lysis buffer and resuspended in lysis buffer lacking Nonidet P-40, and then an equal volume of lysis buffer containing 1% SDS was added. After incubation for 10 min on ice, the samples were frozen on dry ice and then thawed at room temperature and subjected to low-speed centrifugation to remove cellular debris. Supernatants obtained from the cytoplasmic and nuclear fractions were incubated with equal volumes of 2× binding buffer (20 mM Tris-HCl [pH 7.5], 1.0 M LiCl, 2 mM EDTA, 0.5% SDS) containing 400 μg of Dynabeads Oligo(dT)₂₅ (Dynal A.S.). After being washed three times, poly(A)⁺ mRNAs were eluted from the beads with elution buffer (2 mM EDTA [pH 7.5]) at 65°C for 2 to 3 min and stored at –70°C until use.

In situ hybridization. In situ RNA hybridization was performed as previously described (62). Briefly, the 46-mer antisense oligonucleotide probe E46 (5'-C GTCACCTTCTCTAACTTCTTGAGCGCCTTGCTCCATGTCAGGG-3') complementary to the EIAV p15^{gag} sequence (nt 454 to 499) was end labeled by using ³²S-dATP and terminal deoxynucleotidyltransferase. At 24 h posttransfection, cells in each plate were washed twice with PBS and then hybridized with 10⁷ cpm of probe in 900 μl of hybridization buffer (50% formamide, 4× SSC, 500 μg of salmon sperm DNA per ml, 0.2 M dithiothreitol, 1× Denhardt's solution, 1% sarcosyl, 10% dextran sulfate) at 42°C overnight. The cells were rinsed once with 1× SSC at room temperature and four times at 55°C. After being washed once in water for 1 min, the cells were dehydrated for 1 min in 60% ethanol and then for 1 min in 90% ethanol. The air-dried cells were covered with NTB2 nuclear emulsion (Kodak, Rochester, N.Y.) and exposed for 2 weeks before development.

RT-PCR. Reverse transcription (RT)-PCR was performed as previously described (77). Briefly, cytoplasmic and nuclear poly(A)⁺ mRNAs were reverse transcribed at 42°C for 1 h in a total volume of 30 μl. Reaction without reverse transcriptase was performed in parallel and served as a control for the absence of plasmid DNA contamination. A 5-μl amount of the cDNA product was PCR amplified in a 100-μl reaction volume with oligonucleotides EP (5'-AGGTG ACGGTACAAGGGTCTCAGAAA-3') and EW (5'-CCCACCATGTTCTTTC AAAGGC-3'), which detected cDNA of unspliced EIAV *gag* mRNA, oligonucleotides ACTINS (5'-TGAGCTGCGTGTGGCTCC-3') and ACTINA (5'-GG CATGGGGGAGGGCATAACC-3'), which specifically amplify cDNA of spliced actin mRNA, or ACTINS-1 (5'-CCAGTGGCTTCCCACTG-3') and ACTINA, which detect cDNA of unspliced actin mRNA.

Oligonucleotides EW and ACTINA were end labeled with [³²P]ATP prior to use. PCR was performed in a total volume of 100 μl for 20 to 25 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min with a final extension at 72°C for 10 min. A 10-μl sample from each RT-PCR was analyzed by electrophoresis on 5% polyacrylamide gels.

RESULTS

The EIAV 5' UTR inhibits EIAV Gag production. We have previously demonstrated that the HIV-1 matrix p17^{gag} coding

region contains inhibitory sequences that act posttranscriptionally to repress HIV-1 Gag production in the absence of Rev (63, 65). EIAV is a complex retrovirus but has a relatively simple genomic organization compared with that of HIV-1 (Fig. 1A). In this study, we investigated whether the EIAV p15^{gag} matrix gene contains inhibitory sequences similar to those found in the HIV-1 p17^{gag} coding sequence. We first generated two plasmids designed to produce the EIAV p15^{gag} matrix protein. Plasmid pE15 contains the EIAV p15^{gag} coding sequence (nt 446 to 817) (nucleotide numbering of the EIAV sequences follows that used by Stephens et al. [73] and starts at the first nucleotide of the EIAV LTR), while plasmid pG15 contains the complete EIAV 5' UTR (nt 189 to 445) in addition to the p15^{gag} gene (Fig. 1B). Transcription from these plasmids is under control of the HIV-1 LTR promoter. To investigate whether the p15^{gag} protein was produced from plasmids pE15 and pG15, these plasmids were separately transfected into HLtat cells (64), a HeLa derivative constitutively producing HIV-1 Tat protein. A CAT-producing plasmid pNLCAW (76) was included in transfections and served as an internal control for transfection efficiency and the levels of CAT protein were measured by capture enzyme-linked immunosorbent assaying as previously described (76). Cells were harvested 24 h posttransfection, and the production of EIAV p15^{gag} protein was assessed by Western immunoblotting. Figure 1C (left panel) shows that high levels of p15^{gag} protein were produced from cells transfected with pE15, while p15^{gag} produced from pG15 was undetectable. These results indicated that inhibitory sequences are not contained within the EIAV p15^{gag} matrix coding sequence and suggested that an inhibitory sequence either overlaps the EIAV 5' UTR and the EIAV *gag* gene or is located in the EIAV 5' UTR.

Since inhibitory sequences were not found in the EIAV matrix p15^{gag} coding region, we then investigated whether such sequences were present in other parts of the EIAV p55^{gag} gene. Therefore, we generated plasmid pE55, which contains the complete EIAV p55^{gag} gene, and pG55, which contains the EIAV 5' UTR in addition to p55^{gag} coding sequence (Fig. 1B). Western blot analysis revealed that high levels of EIAV p55^{gag} were produced from cells transfected with pE55 (Fig. 1C, right panel), while pG55 did not produce detectable levels of p55^{gag}. These experiments suggested that sequences in the EIAV 5' UTR are required for inhibition of Gag production. Western blot analysis of serial 2-fold dilutions of cell lysates from cells transfected with pE55 showed that p55^{gag} levels produced from pE55 were 30- to 60-fold higher than those produced from pG55 (Fig. 1D). We also investigated whether the inhibitory effect was dependent on the promoter or poly(A) signal used in plasmid pG55. Therefore, the HIV-1 LTR sequences in plasmids pE55 and pG55 were replaced by human cytomegalovirus immediate-early promoter and simian virus 40 early poly(A) signal, resulting in plasmids pCE55 and pCG55, respectively (Fig. 1B). As shown in Fig. 1C (right panel), similarly low levels of p55^{gag} were produced from pCG55 and pG55, while high levels of Gag were produced from pCE55 and pE55. These results demonstrate that inhibition caused by the presence of the EIAV 5' UTR could be reproduced with plasmids with different promoters and poly(A) signals.

The inhibitory effect could be overcome by HIV-1 Rev and RRE, by HTLV-I Rex and RxRE, or by SRV-1 CTE. To determine whether the EIAV inhibitory sequence was important for Rev regulation, we investigated whether inhibition could be overcome by providing HIV-1 Rev in *trans* and RRE in *cis*. The HIV-1 RRE sequence was introduced within the transcription unit downstream of the EIAV *gag* coding sequence in plasmid pG15, pE55, or pG55, generating plasmids pG15R, pE55R,

FIG. 2. Inhibitory effect could be overcome by HIV-1 Rev and RRE, by HTLV-I Rex and RxRE, or by the SRV-1 CTE. (A) Structures of EIAV p15^{gag} and p55^{gag} expression plasmids. Black boxes, HIV-1 LTR sequences; open boxes, EIAV sequences. Numbers indicate nucleotide positions in the EIAV genome (the first nucleotide of the EIAV LTR is designated +1) (73). (B) Western blot analysis of EIAV p15^{gag} (left panel) and p55^{gag} (right panel) production from HLTat cells transfected with the indicated Gag expression plasmids in the absence (-) or presence (+) of HIV-1 Rev. Immunoblotting was performed as described in the legend to Fig. 1C. The positions of EIAV Gag proteins are indicated on the left. (C) Western blot analysis of EIAV p55^{gag} production from cells transfected with pG55R in the absence or presence of Rev protein. Cell lysates from cells transfected with G55R in the presence of Rev were serially twofold diluted and analyzed by Western immunoblotting as described in the legend to Fig. 1C. The position of EIAV p55^{gag} is indicated on the left. (D) Western blot analysis of EIAV p55^{gag} production from HLTat cells transfected with indicated Gag expression plasmids in the absence or presence of HTLV-I Rex or HIV-1 Rev. Immunoblotting was performed as described in the legend to Fig. 1C. The positions of EIAV p55^{gag} are indicated on the left. (E) Northern blot analysis of total RNA isolated from cells transfected with pG55R or pG15R in the absence (-) or presence (+) of Rev. A 20-μg amount of each RNA sample was run on 1% agarose-formaldehyde gels, transferred onto nitrocellulose membranes, and hybridized with a [³²P]UTP-labeled riboprobe specifically detecting a portion of the EIAV gag coding sequence. The positions of 28S and 18S are indicated on the right. T, total RNA.

retention of the EIAV gag mRNAs. For this purpose, RNA in situ hybridizations were performed. An antisense oligonucleotide probe complementary to the EIAV p55^{gag} mRNA was hybridized to cells transfected with pG55R in the absence or presence of Rev. The results of in situ hybridizations revealed that in the absence of Rev, most of the transfected cells displayed a pattern of nuclear retention of the EIAV gag mRNA (Fig. 3A), while in the presence of Rev, EIAV gag mRNAs were primarily localized in the cytoplasm (Fig. 3C). However, a fraction of the cells transfected in the absence of Rev dis-

played an apparent cytoplasmic staining (Fig. 3B), and in the presence of Rev, a small fraction of cells with the EIAV gag mRNAs retained in the nucleus could be observed (Fig. 3D). To compare the difference in RNA distribution, the number of cells displaying a pattern of either nuclear retention or cytoplasmic accumulation was determined. We found that in the absence of Rev, 87% of the positive cells showed hybridization primarily over the nucleus, while in the remaining 13% of the cells the EIAV gag mRNAs were present in high levels also in the cytoplasm. However, in the presence of Rev, 98% of the

FIG. 3. Intracellular localization of EIAV *gag* mRNAs detected by in situ hybridization. An antisense oligonucleotide was hybridized to cells transfected with pG55R in the absence or presence of Rev protein. EIAV *gag* mRNAs produced from pG55R in the absence of Rev were located primarily in the nucleus (A), although cytoplasmic localization of hybridization signals could be observed in a portion of cells (B). EIAV *gag* mRNAs produced from pG55R in the presence of Rev were predominantly localized in the cytoplasm (C); however, a small percentage of cells with hybridization signals primarily over the nucleus could be detected (D).

cells showed primarily cytoplasmic accumulation of EIAV *gag* mRNAs.

To confirm the results obtained by in situ hybridizations, cytoplasmic poly(A)⁺ mRNA was extracted from cells transfected with pG55R in the absence or presence of Rev and was analyzed by RT-PCR. The poly(A)⁺ mRNAs were serially diluted 4-fold and were subjected to RT and then to PCR amplification with oligonucleotides specifically amplifying a portion of the EIAV p15^{gag} coding sequence. Cytoplasmic EIAV *gag* poly(A)⁺ mRNA levels produced from pG55R in the presence of Rev were approximately 4-fold higher than those produced in the absence of Rev (Fig. 4, upper panel), while production of EIAV p55^{gag} from pG55R increased by ~60-fold in the presence of Rev (Fig. 2C). Therefore, by in situ hybridization and by RT-PCR, we could show that there was approximately 4- to 7-fold more cytoplasmic poly(A)⁺ EIAV *gag* mRNA in the presence of Rev than in the absence of Rev. To control for the cell fractionation technique, we also analyzed spliced and unspliced actin mRNAs. The results showed that unspliced actin mRNAs were found primarily in the nuclear fractions (Fig. 4, bottom panel), demonstrating that nuclear leakage did not occur, while spliced actin mRNA was found in both nuclear and cytoplasmic fractions (Fig. 4, bottom panel). We concluded that Rev acted not only by increasing the levels

of EIAV *gag* mRNAs in the cytoplasm, but also by improving the translatability of the *gag* mRNAs. This supports the idea that Rev is a multifunctional protein or that the action of Rev has multiple effects.

The negative effect on p55^{gag} production could be bypassed by transcription in the cytoplasm. We then wished to test whether transcription in the cytoplasm could bypass the inhibition exerted by the EIAV sequences. For this purpose, we employed the vaccinia virus-T7 RNA polymerase-based expression system (33). T7 RNA polymerase expressed from the recombinant vaccinia virus vTF7-3 is located in the cytoplasm, since it lacks a nuclear localization signal (24). As a result, transcription directed by T7 RNA polymerase occurs in the cytoplasm. Two expression plasmids were constructed: pT7G55, which contains the EIAV p55^{gag} gene and upstream sequence (nt 189 to 1906), and pT7E55, which contains the EIAV p55^{gag} gene (nt 446 to 1906) but lacks the EIAV inhibitory sequence (Fig. 5A). In both plasmids, the EIAV p55^{gag} gene is under the control of the bacteriophage T7 promoter specifically recognized by T7 RNA polymerase. To evaluate EIAV p55^{gag} production, HLTat cells were first infected with recombinant vaccinia virus vTF7-3 (33) and then transfected with pT7G55 and pT7E55. Western blot analysis revealed a two- to four-fold difference in p55^{gag} production between these

FIG. 4. RT-PCR analysis of cytoplasmic and nuclear poly(A)⁺ mRNAs. Top panel, PCR amplification of the cDNA synthesized from serially fourfold-diluted cytoplasmic poly(A)⁺ mRNA isolated from cells transfected with pG55R in the absence (-) or presence (+) of HIV-1 Rev protein; bottom panel, PCR amplification of cDNA from spliced and unspliced cytoplasmic or nuclear poly(A)⁺ actin mRNAs. The numbers of cycles used for PCR amplification of cDNAs were adjusted to detect cDNA in the linear range of the assay. N, nuclear RNA; C, cytoplasmic RNA; actin-S, spliced actin mRNA; actin-U, unspliced actin RNA. Oligonucleotides were end labeled with [γ -³²P]ATP prior to PCR. The PCR products were analyzed on 5% polyacrylamide gels and then by autoradiography. Positions of the amplified DNA fragments are indicated on the left.

two plasmids (Fig. 5B and data not shown), while parallel transfections with pG55 and pE55 showed a great difference in Gag production (Fig. 5B). These results demonstrated that the negative effect exerted by the EIAV inhibitory sequence could be partially relieved by transcription in the cytoplasm, indicating that nuclear factors are required for efficient inhibition of EIAV Gag production. This is in agreement with the negative effect on nuclear mRNA export. However, it should be noted that the 5' ends of the mRNAs produced from pT7G55 and pT7E55 are different, and this may potentially affect the expression levels of the EIAV p55^{gag} protein.

Inhibitory activity coincides with the major EIAV 5' splice site. We then wished to map and characterize the EIAV inhibitory element. As shown in Fig. 6A, plasmid pG55 contains 254 nt upstream of the *gag* AUG, which are absent in plasmid pE55. Progressive 5' end deletions were introduced in this region. A total of 186 or 233 nt was deleted from the 5' end of the EIAV sequence in plasmid pG55, generating plasmid p Δ 186 or p Δ 233, respectively (Fig. 6A). Western blot analysis revealed that similarly low levels of EIAV Gag were produced from plasmids p Δ 186, p Δ 233, and pG55, while high levels of Gag were produced from pE55 (Fig. 6B), indicating that the inhibitory sequence is located downstream of nt 421 (Fig. 6A). Since the EIAV major 5' splice site is present in this region, it was mutated by introduction of multiple point mutations,

A

B

FIG. 5. Expression of EIAV p55^{gag} with the vaccinia virus-T7 RNA polymerase expression system and in vitro translation system. (A) Structures of EIAV p55^{gag} expression plasmids driven by the bacteriophage T7 promoter. Stippled boxes, bacteriophage T7 promoter; open boxes, EIAV sequences. Bent arrows show the directions of transcription driven by the T7 promoter. Numbers indicate nucleotide positions in the EIAV genome (the first nucleotide of the EIAV LTR is designated +1) (73). (B) Western blot analysis of EIAV p55^{gag} production from pT7G55- or pT7E55-transfected HLtat cells that are infected with recombinant vaccinia virus, vTF7-3, producing T7 RNA polymerase (left panel), or from HLtat cells transfected with pG55 or pE55 (right panel), respectively.

changing the EIAV 5' splice site containing sequence from ACAGGTAAG to cgcGccAAG. This altered the highly conserved GT dinucleotide in the 5' splice site to CC. The mutant plasmid, named pESD(-)G (Fig. 6A), produced high levels of EIAV Gag, comparable to those produced by pE55 (Fig. 6B), demonstrating that the EIAV 5' splice site was required for inhibition of Gag production in the absence of Rev.

Inhibitory activity of the EIAV 5' splice site could be abolished by single point mutations. We first wished to exclude the possibility that sequences upstream of the EIAV 5' splice site contributed to inhibition of EIAV Gag expression. Since we have shown that the EIAV 5' UTR sequence upstream of nt 421 is not required for inhibition (Fig. 6A and B), a 15-nt sequence (nt 423 to 437) located immediately upstream of the EIAV 5' splice site was deleted from plasmid pG55, generating plasmid pG55 Δ E (Fig. 7A). This plasmid did not produce elevated levels of p55^{gag} (Fig. 7B), demonstrating that upstream of the EIAV *gag* AUG, the 5' splice site is the only sequence required for inhibition of Gag expression.

Compared with the invariable G and T at positions +1 and +2, positions -3, -2, -1, +3, +4, and +5 on the 5' splice site may vary without inactivating the 5' splice site. In fact, many functional 5' splice sites on cellular mRNAs deviate from the consensus sequence at positions other than +1 and +2 (53). Inspection of this 5' splice site revealed that it has a perfect complementarity to the U1 snRNA in eight consecutive nucleotide positions (Fig. 7A). To investigate if maintenance of a perfect consensus 5' splice site is required for inhibition, we first changed the three nucleotides (CAG) at positions -3 to -1 immediately upstream of GT in the 5' splice site to TCC by site-directed mutagenesis, generating plasmid pG55-3T-2C-1C (Fig. 7A). In addition, the 2 nt (AA) at positions +3 and +4 immediately downstream of the invariable GT dinucleotide were mutated to GC, generating plasmid pG55+3G+4C (Fig. 7A). Both pG55-3T-2C-1C and pG55+3G+4C produced high levels of Gag, comparable to those produced from pE55 (Fig.

A

B

FIG. 6. Sequences containing the EIAV 5' splice site inhibit EIAV p55^{gag} production. (A) Structures of EIAV p55^{gag} expression plasmids with deletions upstream of the EIAV *gag* AUG or with mutations within the major 5' splice site. Black boxes, the HIV-1 LTR sequences; open boxes, the EIAV sequences; stippled circles, the major 5' splice site of EIAV; SD, 5' splice site. Numbers indicate nucleotide positions in the EIAV genome (the first nucleotide of the EIAV LTR is designated +1) (73). The parentheses indicate deletions; XX, the mutations introduced in the EIAV 5' splice site. The sequences encoding the 5' splice site in pG55 or the mutated splice site in pESD(-)G are displayed. Mutated nucleotides in the 5' splice site are indicated by lowercase letters, while the nonmutated nucleotides are indicated by capital letters. (B) Western blot analysis of EIAV p55^{gag} production from cells transfected with pG55, pΔ186, pΔ233, pE55, and pESD(-)G. Immunoblotting was performed as described in the legend to Fig. 1C.

7B). These results showed that mutations in the variable positions of the 5' splice site could abolish inhibitory activity even if the highly conserved GT dinucleotide was left intact, thus demonstrating that nucleotides at these positions also contribute to inhibition of EIAV Gag expression.

To evaluate the importance of each nucleotide position in the 5' splice site for inhibition, each of the eight nucleotides in the 5' splice site was mutated one by one as shown in Fig. 7A. The resulting plasmids were named pG55-3T, pG55-2C, pG55-1C, pG55+1A, pG55+2A, pG55+3G, pG55+4C, and pG55+5A, respectively (Fig. 7A). Western blot analysis revealed that high levels of p55^{gag} were produced from all of the mutants, respectively, except for pG55-3T (Fig. 7C). Although the EIAV Gag levels produced from pG55-2C were slightly lower than those produced from pE55, they were still substantially higher than those produced from pG55, indicating that nucleotide A at position -2 in the 5' splice site contributes to inhibition to a lower extent than other positions of nucleotides in the 5' splice site. Interestingly, among these mutant plasmids, only pG55-3T did not produce elevated levels of p55^{gag}. The difference in Gag production between pG55-3T and pG55+1A was 30- to 60-fold (Fig. 7D), while the difference in cytoplasmic EIAV poly(A)⁺ *gag* mRNA levels was approxi-

mately 4-fold (data not shown). All mutants produced similar levels of p55^{gag} when they were expressed with the vaccinia virus T7 RNA polymerase-based expression system (data not shown). Taken together, these experiments established that the full activity of the EIAV inhibitory sequence is critically dependent on the seven nucleotides (AGGTAAG) of the 5' splice site.

A minimal distance between the EIAV 5' splice site and the *gag* AUG is required for inhibitory activity. The natural position of the EIAV major 5' splice site is immediately upstream of the EIAV *gag* translational start codon (Fig. 7A). This organization is unique for EIAV among the retroviruses. We then investigated whether the inhibitory effect could be abolished by increasing the distance between the EIAV 5' splice site and the EIAV *gag* AUG. We generated plasmids pESD13G, pESD32G, pESD86G, and pESDSD(-)G (Fig. 8A), in which the 5' splice site was placed 13, 32, 86, and 216 nt upstream of the EIAV *gag* AUG, respectively. Figure 8B shows that plasmid pESD13G produced similarly low levels of p55^{gag} as pG55, while high levels of p55^{gag} were produced from plasmids pESD32G, pESD86G, and pESDSD(-)G, respectively. It could be argued that utilization of the splice sites would result in *gag* mRNAs lacking the 5' splice site that could potentially produce high levels of p55^{gag}. Northern blots of total RNA extracted from cells transfected with pE55, pESD13G, or pESD86G showed that similar levels of unspliced *gag* mRNAs were produced among them (Fig. 8C). To further verify that the EIAV 5' splice site is not utilized in pESD13G, we analyzed the structures of cytoplasmic poly(A)⁺ mRNAs produced from pESD13G, pESD32G, or pESD86G by RT-PCR. DNA bands corresponding in size to unspliced *gag* mRNA were detected (Fig. 8D). The appearance of these bands was dependent on reverse transcriptase. In parallel PCR reactions, the corresponding plasmid DNAs were used as DNA templates. The amplified DNAs obtained from these reactions had the same sizes as the corresponding DNAs generated by RT-PCR (Fig. 8D), demonstrating that mRNAs produced from pESD13G, pESD32G, and pESD86G were not spliced. In conclusion, these results demonstrated that the negative effect of the EIAV 5' splice site was lost when the distance between the EIAV 5' splice site and the *gag* coding sequence increased to 32 nt or more, indicating that close proximity of the EIAV 5' splice site to the *gag* coding sequence was required for strong inhibition.

The EIAV 5' splice site inhibits heterologous *gag* gene expression. To test if the EIAV 5' splice site could inhibit expression of a heterologous retroviral *gag* gene, we cloned the EIAV splice site-containing sequence (nt 189 to 445) 21 nt upstream of the HIV-2 *gag* gene in plasmid pH2G (Fig. 9A), generating plasmid pESDH2G (Fig. 9A). Western blot analysis revealed that plasmid pH2G produced high levels of HIV-2 p55^{gag} (Fig. 9B), while low levels of HIV-2 Gag were produced from pESDH2G, demonstrating that the EIAV 5' splice site could inhibit HIV-2 Gag production. To test if this inhibition could be overcome by the HIV-1 Rev and RRE, RRE was inserted downstream of the HIV-2 *gag* gene in plasmid pESDH2G, resulting in plasmid pESDH2GR (Fig. 9A). Since the Gag levels produced from pESDH2GR in the presence of Rev were similar to those produced from pH2G (Fig. 9B), the results demonstrated that the 5' splice site of EIAV could confer Rev dependence to a heterologous *gag* gene. Production of HIV-2 p55^{gag} was not substantially increased from plasmid pH2GR in the presence of Rev (Fig. 9B), demonstrating that the HIV-2 *gag* gene does not contain endogenous inhibitory sequences. The HIV-1 p37^{gag} levels produced from the

FIG. 7. The EIAV inhibitory sequence coincides with the major 5' splice site on the EIAV genome. (A) Structures of EIAV p55^{gag} expression plasmids. Black boxes, HIV-1 LTRs; open boxes, EIAV sequences; stippled circle, EIAV 5' splice site; SD, 5' splice site. The nucleotide sequence of the EIAV 5' splice site is shown on the top. Numbers indicate nucleotide positions in the EIAV genome (the first nucleotide of the EIAV LTR is designated +1) (73). Parentheses indicate sequence which was deleted. Multiple and single point mutations within the 5' splice site are displayed. ATG is the translational start codon of the EIAV gag gene. The names of the plasmids are indicated on the right. (B) Western blot analysis of EIAV p55^{gag} production from mutated plasmids. (C) Western blot analysis of EIAV p55^{gag} production from plasmids with single point mutations in the 5' splice site. (D) Quantitative comparison of EIAV p55^{gag} production from cells transfected with pG55-3T or pG55+1A. Cell lysates obtained from cells transfected with pG55+1A were serially twofold diluted and analyzed by Western immunoblotting as described in the legend to Fig. 1C.

internal control plasmid pC37M (63) did not vary significantly among these samples (Fig. 9B).

In contrast to HIV-2 gag, HIV-1 gag contains strong intragenic inhibitory sequences (18, 49, 65). One inhibitory sequence mapped to the HIV-1 p17^{gag} coding region (65). Introduction of 28 point mutations inactivated this inhibitory element (63). To test if the EIAV 5' splice site could inhibit production of HIV-1 p17^{gag} from plasmid p17M1234, in which the inhibitory HIV-1 sequences were mutated (Fig. 9A), the EIAV 5' splice site was inserted 18 nt upstream of the mutated HIV-1 p17^{gag} gene in plasmid p17M1234 (63), generating plasmid pESDM17 (Fig. 9A). As shown in Fig. 9C, the presence of the EIAV splice site upstream of the HIV-1 p17^{gag} AUG resulted in a dramatic decrease in p17^{gag} production, while pM17ESD (Fig. 9A), in which the EIAV splice site is present downstream of the gag sequence, produced high levels of p17^{gag}, comparable to those produced by p17M1234 (Fig. 9C). These results confirmed that the inhibitory activity of the EIAV 5' splice site is position dependent.

DISCUSSION

In the present study, we have shown that EIAV mRNAs contain negative regulatory elements that inhibit production of EIAV Gag in the absence of Rev. Deletion and mutation

analysis mapped an inhibitory RNA sequence to the EIAV major 5' splice site located immediately upstream of the gag AUG.

The EIAV 5' splice site is complementary to the U1 snRNA in eight consecutive nucleotide positions (58). Introduction of single point mutations in the 5' splice site could abolish inhibitory activity. Mutations at position -3 of the 5' splice site had no effect on inhibition, demonstrating that this nucleotide position does not contribute to inhibition. This is also the least conserved position in a 5' splice site (53). It has been shown that single point mutations within a 5' splice site are well tolerated (2) and that the vast majority of naturally occurring 5' splice sites deviate from the consensus 5' splice site in one or several positions (53), but they remain functionally intact. However, a 5' splice site can be improved by increasing its complementarity to U1 snRNA (83), indicating that the strength of U1 binding is an important determinant of the efficiency of splicing (84). Therefore, one explanation for the loss of inhibitory activity, when point mutations were introduced in the EIAV 5' splice site, is that Watson-Crick base-pairing interactions between the EIAV mRNA and U1 snRNA are not maximized. However, cotransfections of pG55+5A with pU1+4 (84), which contains a U1 snRNA sequence that

FIG. 8. The inhibitory effect of the EIAV 5' splice site was abolished when the distance between the EIAV 5' splice site and the gag AUG increased to 32 nt or more. (A) Structures of EIAV p55^{gag} expression plasmids, with the EIAV 5' splice site located at various positions upstream of the EIAV gag AUG. Black boxes, the HIV-1 LTRs; open boxes, EIAV sequences; stippled circle, EIAV 5' splice site; SD, 5' splice site. Numbers indicate nucleotide positions in the EIAV genome (the first nucleotide of the EIAV LTR is designated +1) (73). XX, mutations introduced in the EIAV 5' splice site (see Fig. 6A). The numbers of nucleotides located between the EIAV 5' splice site and the gag AUG are indicated on the right. (B) Western blot analysis of Gag production from the EIAV Gag expression plasmids as indicated. (C) Northern blot analysis of total RNA extracted from cells transfected with pG55, pESD13G, or pESD86G. The EIAV gag RNA was detected as described in the legend to Fig. 3A. (D) RT-PCR analysis of cytoplasmic poly(A)⁺ mRNAs isolated from cells transfected with pESD13G, pESD32G, or pESD86G. cDNAs were PCR amplified with oligonucleotides specific for the EIAV gag gene. The same set of oligonucleotides were employed in a parallel PCRs with DNAs of plasmids pESD13G, pESD32G, pESD86G, and pG55 as templates. The amplified products were analyzed on 5% polyacrylamide gels and visualized by ethidium bromide staining. M, DNA size marker.

is complementary to the EIAV 5' splice site mutated to A at position +5, did not result in inhibition of EIAV Gag production (data not shown). Although we cannot rule out that the U1 snRNA is involved in inhibition of gene expression, our data suggest that additional factors are required for inhibition. For example, it was recently shown that the small nuclear RNA U5 interacts with positions -2 and -3 of the 5' splice site (55, 69) and that U6 interacts with positions +4, +5, and +6 in the 5' splice site (40, 46). U5 and U6 presumably bind to the 5' splice site after release of U1 (43). Therefore, various point mutations in the 5' splice site may affect different steps in spliceosome assembly. In addition to the snRNPs, other cellular components interact with 5' splice sites. One of them, ASF/SF2, binds specifically to the 5' splice site in vitro, and it was proposed that it aids in recognition of pre-mRNA 5' splice sites (26, 86). Binding of ASF/SF2 was negatively affected by single point mutations in the 5' splice site sequence (86), lending further support to the idea that mutation of one nucleotide position in the 5' splice site may affect more than one step in spliceosome formation.

How a 5' splice site is recognized is not well understood (39). For example, it has been reported that when splice sites are inserted into different locations in the pre-mRNA, they are often not used. On the basis of these and other observations,

many investigators concluded that sequences in addition to the consensus 5' splice site are required for 5' splice site selection. Numerous mRNAs contain purine-rich sequences, termed splicing enhancers (11, 78, 82), that act in *cis* to stimulate splicing. Proteins of the SR family of splicing factors have been shown to bind to exonic enhancers (44, 72), and in one case the U1 snRNA cross-linked to such a sequence (81). It is believed that early steps in spliceosome formation are affected (72), and it has been shown that SR proteins that bind to splicing enhancers act by stimulating binding of U1 small nuclear ribonucleoprotein to the 5' splice site (26, 42). Exonic sequences that are required for efficient splicing have been identified in the retroviruses Rous sarcoma virus (32) and HIV-1 (70). One explanation for the position-dependent inhibitory effect of the EIAV 5' splice site is that the p15^{gag} encoding region contains a sequence that is required for efficient recognition of the EIAV 5' splice site by the cellular splicing machinery.

It has been shown that insertion of HIV-1 splice sites in a β -globin mRNA resulted in nuclear entrapment of this mRNA (15). Simultaneous mutations in both the HIV-1 5' and 3' splice sites eliminated Rev responsiveness and resulted in efficient transport of the mRNA from the nucleus to the cytoplasm (15). However, these mRNAs contained only the splice sites from HIV-1 but not the intragenic inhibitory sequences in

FIG. 9. The EIAV 5' splice site inhibits HIV-2 p55^{gag} or HIV-1 p17^{gag} expression. (A) Structures of HIV-2 p55^{gag} or HIV-1 p17^{gag} expression plasmids. Black boxes, the HIV-1 LTRs; shaded boxes, HIV-2 p55^{gag} coding sequences; stippled circles, EIAV 5' splice site; open boxes without extra specification, EIAV sequences; 17M1234, mutated HIV-1 p17^{gag} gene; SD, 5' splice site. The numbers of the first and last nucleotides of the EIAV sequences inserted in the constructs are indicated. Nucleotide numbering starts at the first nucleotide of the EIAV LTR (73). (B) Western blot analysis of cell lysates from cells transfected with pH2G, pH2GR, pESDH2G, or pESDH2GR in the absence (–) or presence (+) of HIV-1 Rev. Proteins were separated on 12% polyacrylamide-SDS gel and transferred onto nitrocellulose membrane. The membrane was incubated for 1 h with HIV-2-positive patient serum (1:1,000) and then with HRP-conjugated anti-human IgG antiserum (1:10,000). The HIV-2 p55^{gag} protein was visualized with enhanced chemiluminescence detection reagents. The HIV-2 serum cross-reacted with HIV-1 p37^{gag} produced from pC37M, which served as an internal control for transfection efficiency. (C) Western blot analysis of cell lysates from cells transfected with p17M1234, pESDM17, and pM17ESD. After transfer of the proteins onto nitrocellulose membranes, the HIV-1 p17^{gag} protein was detected with HIV-1-positive patient serum (1:3,000) and then with HRP-conjugated anti-human IgG antiserum. The HIV-1 p17^{gag} was visualized with enhanced chemiluminescence detection reagents.

the *env* coding region (10, 54). Mutation of the HIV-1 splice sites in *env* cDNAs does not result in constitutive Env production, suggesting that Env expression is regulated at several levels (54). The importance of splice sites for gene expression of retroviruses has been documented also by other investigators. Using HIV-1 Env expression plasmids, Lu et al. (47) showed that mutation or deletion of the 5' splice site upstream of the *env* ORF resulted in a dramatic decrease in steady-state levels of unspliced HIV-1 *env* mRNA in transfected cells and abolished Env production in the presence of Rev. In their expression system, the presence of the 5' splice site was required for Env expression (47). This is in contrast to the results obtained here, where we show that mutating the EIAV 5' splice site gave rise to high levels of Gag independent of Rev, demonstrating that the presence of an intact EIAV 5' splice site is required for Rev-dependent regulation but is not a prerequisite for efficient production of EIAV Gag protein. Interestingly, our data show that HIV-2 and EIAV do not contain inhibitory sequences in the *gag* coding region that act independently of the EIAV 5' splice site. This is in contrast to HIV-1, in which strong inhibitory sequences are located in the p17^{gag} coding sequence (63, 65). These sequences inhibit Gag production in the absence of unutilized splice sites. Similarly,

inhibitory sequences identified in the HIV-2 Pol region were not present on the corresponding location in simian immunodeficiency virus (18). Therefore, the exact location of inhibitory sequences is not conserved among the different lentiviruses. Furthermore, it is likely that the different inhibitory sequences act at different levels in the mRNA processing pathway.

Inhibitory sequences of HTLV-I and -II have also been mapped to the region upstream of the *gag* AUG (7, 66). The inhibitory effect could be overcome by HTLV I or -II Rex proteins. The inhibitory sequences were localized adjacent to the major 5' splice site, but mutation of the HTLV-II 5' splice site did not eliminate inhibition (7). This suggests that not all 5' splice sites of retroviruses have inhibitory activity. This is in agreement with the results presented here, which show that the inhibitory activity of an unutilized splice site is strongly influenced by its primary sequence and its exact location on the mRNA. This may also explain why unutilized splice sites from simple retroviruses, such as Rous sarcoma virus, do not inhibit gene expression (63).

Our results generated by RT-PCR and in situ hybridization show that in the presence of Rev, cytoplasmic EIAV *gag* poly(A)⁺ mRNA levels increased by approximately 4- to 7-fold, while the production of EIAV Gag protein increased by

30- to 60-fold. This indicates that the presence of the EIAV 5' splice site affects not only subcellular distribution of EIAV *gag* mRNAs, but also their utilization, indicating that the EIAV inhibitory element may act at different levels after RNA transcription. It was recently reported that EIAV *gag* mRNAs are retained in the nucleus in the absence of Rev (52). Inefficient mRNA utilization has been observed for Rev-dependent HIV-1 mRNAs in the absence of Rev (5, 20, 45). The EIAV mRNAs may be exported through a nonproductive pathway or may be associated with nuclear factors when they enter the cytoplasm. Indeed, spliceosomal proteins can function as repressors of translation in vitro (75). Alternatively, the RNA sequence upstream of the EIAV *gag* AUG may form a secondary structure which may hinder migration of ribosomes on the EIAV mRNA. However, this is less likely, since high levels of Gag were produced when the mRNAs were expressed in the cytoplasm, which indicates that inhibition requires nuclear factors. The initiator codon for the EIAV *tat* ORF is located at nucleotide position 354 upstream of the *gag* AUG and could therefore inhibit translation of the EIAV *gag* ORF (12). The EIAV *gag* levels did not increase when the *tat* start codon was deleted from plasmid pG55 (compare pG55 with pΔ186). This is most likely due to the fact that *tat* translation initiation occurs at a non-AUG codon, CUG, which allows a large fraction of the ribosomes to continue scanning for a downstream AUG (12).

Inhibitory sequences on retrovirus mRNAs may be important for establishment of latent infections, thereby allowing the virus to escape the immune surveillance of the host. We have recently proposed that a complex retrovirus lacking inhibitory sequences would not be dependent on Rev (63). Expression of such a viral genome from a promoter regulated solely by cellular factors, e.g., the promoter of a simple retrovirus, may result in constitutive expression and failure of the virus to escape the immune response. This would constitute a novel approach for vaccines (79). It has been proposed that the fact that simple retroviruses have not been isolated from humans and ungulates reflects the ability of humans and ungulates to mount a protective immune response to infection with simpler retroviruses but not to that with complex retroviruses (79). Therefore, a simple retrovirus producing HIV-1 Gag, Pol, and Env may be used as vaccine, either as a live attenuated virus or as an inactivated virus.

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REFERENCES

- 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.
- Aebi, M., H. Hornig, R. A. Padgett, J. Reiser, and C. Weissmann. 1986. Sequence requirements for splicing of higher eukaryotic nuclear pre-mRNA. *Cell* **47**:555-565.
- Amendt, B. A., D. Hesslein, L.-J. Chang, and C. M. Stoltzfus. 1994. Presence of negative and positive *cis*-acting RNA splicing elements within and flanking the first *tat* coding exon of human immunodeficiency virus type 1. *Mol. Cell. Biol.* **14**:3960-3970.
- Amendt, B. A., Z.-H. Si, and C. M. Stoltzfus. 1995. Presence of exon splicing silencers within the human immunodeficiency virus type 1 *tat* exon 2 and *tat*-*rev* exon 3: evidence for inhibition mediated by cellular factors. *Mol. Cell. Biol.* **15**:4606-4615.
- Arrigo, S. J., and I. S. Y. Chen. 1991. Rev is necessary for translation but not cytoplasmic accumulation of HIV-1 *vif*, *vpr*, and *env/vpu* 2 RNAs. *Genes Dev.* **5**:808-819.
- Beisel, C. E., J. F. Edwards, L. L. Dunn, and N. R. Rice. 1993. Analysis of multiple mRNAs from pathogenic equine infectious anemia virus (EIAV) in an acutely infected horse reveals a novel protein, Ttm, derived from the carboxy terminus of the EIAV transmembrane protein. *J. Virol.* **67**:832-842.
- Black, A. C., I. S. Y. Chen, S. Arrigo, C. T. Ruland, T. Alligiamiento, E. Chin, and J. D. Rosenblatt. 1991. Regulation of HTLV-II gene expression by Rex involves positive and negative *cis*-acting elements in the 5' long terminal repeat. *Virology* **181**:433-444.
- Bogerd, H. P., R. A. Fridell, S. Madore, and B. R. Cullen. 1995. Identification of a novel cellular cofactor for the Rev/Rex class of retroviral regulatory proteins. *Cell* **82**:485-494.
- Bray, M., S. Prasad, J. W. Dubay, E. Hunter, K. T. Jeang, D. Rekosh, and M. L. Hammariskjöld. 1994. A small element from the Mason-Pfizer monkey virus genome makes human immunodeficiency virus type 1 expression and replication Rev-independent. *Proc. Natl. Acad. Sci. USA* **91**:1256-1260.
- Brighty, D. W., and M. Rosenberg. 1994. A *cis*-acting repressive sequence that overlaps the Rev-responsive element of human immunodeficiency virus type 1 regulates nuclear retention of *env* mRNAs independently of known splice signals. *Proc. Natl. Acad. Sci. USA* **91**:8314-8318.
- Caputi, M., G. Casari, S. Guenzi, R. Tagliabue, A. Sidoli, C. A. Melo, and F. E. Baralle. 1994. A novel bipartite enhancer modulates the differential processing of the human fibronectin EDA exon. *Nucleic Acids Res.* **22**:1018-1022.
- Carroll, R., and D. Derse. 1993. Translation of equine infectious anemia virus bicistronic *tat*-*rev* mRNA requires leaky ribosome scanning of the *tat* CTG initiation codon. *J. Virol.* **67**:1433-1440.
- Carroll, R., L. Martarano, and D. Derse. 1991. Identification of lentivirus Tat functional domains through generation of equine infectious anemia virus/human immunodeficiency virus type 1 *tat* gene chimeras. *J. Virol.* **65**:3460-3467.
- Carvalho, M., and D. Derse. 1991. Mutational analysis of the equine infectious anemia virus Tat-responsive element. *J. Virol.* **65**:3468-3474.
- Chang, D. D., and P. A. Sharp. 1989. Regulation by HIV Rev depends upon recognition of splice sites. *Cell* **59**:789-795.
- Cheevers, W. P., and T. C. McGuire. 1985. Equine infectious anemia virus: immunopathogenesis and persistence. *Rev. Infect. Dis.* **7**:83-88.
- Cheevers, W. P., and T. C. McGuire. 1988. The lentiviruses: Maedi/visna, caprine arthritis-encephalitis, and equine infectious anemia. *Adv. Virus Res.* **34**:189-215.
- 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.
- Cullen, B. R. 1992. Mechanism of action of regulatory proteins encoded by complex retroviruses. *Microbiol. Rev.* **56**:375-394.
- 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.
- Derse, D., M. Carvalho, R. Carroll, and B. M. Peterlin. 1991. A minimal lentivirus Tat. *J. Virol.* **65**:7012-7015.
- Dorn, P., L. DaSilva, L. Martarano, and D. Derse. 1990. Equine infectious anemia virus *tat*: insights into the structure, function, and evolution of lentivirus *trans*-activator proteins. *J. Virol.* **64**:1616-1624.
- Dorn, P. L., and D. Derse. 1988. *cis*- and *trans*-acting regulation of gene expression of equine infectious anemia virus. *J. Virol.* **62**:3522-3526.
- Dunn, J. J., B. Krippl, K. E. Bernstein, H. Westphal, and F. W. Studier. 1988. Targeting bacteriophage T7 RNA polymerase to the mammalian cell nucleus. *Gene* **68**:259-266.
- 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.
- Eperon, I. C., D. C. Ireland, R. A. Smith, A. Mayeda, and A. R. Krainer. 1993. Pathways for selection of 5' splice sites by U1 snRNPs and SF2/ASF. *EMBO J.* **12**:3607-3617.
- 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.
- Fischer, U., J. Huber, W. C. Boelens, I. W. Mattaj, and R. Lührmann. 1995. The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell* **82**:475-483.

29. Fischer, U., S. Meyer, M. Teufel, C. Heckel, R. Lührman, and G. Rautmann. 1994. Evidence that HIV-1 Rev directly promotes the nuclear export of unspliced RNA. *EMBO J.* **13**:4105–4112.
30. Franchini, G., K. A. Fargnoli, F. Giombini, L. Jagodzinski, A. De Rossi, M. Bosch, G. Biberfeld, E. M. Fenyö, J. Albert, R. C. Gallo, and F. Wong-Staal. 1989. Molecular and biological characterization of a replication competent human immunodeficiency type 2 (HIV-2) proviral clone. *Proc. Natl. Acad. Sci. USA* **86**:2433–2437.
31. Fritz, C. C., M. L. Zapp, and M. R. Green. 1995. A human nucleoporin-like protein that specifically interacts with HIV Rev. *Nature (London)* **376**:530–533.
32. Fu, X.-D., R. A. Katz, A. M. Skalka, and T. Maniatis. 1991. The role of the branchpoint and 3'-exon sequences in the control of balanced splicing of avian retrovirus RNA. *Genes Dev.* **5**:211–220.
33. Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss. 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* **83**:8122–8126.
34. 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.
35. Hadzopoulou-Cladaras, M., B. K. Felber, C. Cladaras, A. Athanassopoulos, A. Tse, and G. N. Pavlakis. 1989. The *rev* (*trslart*) 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.
36. Hammarström, M. L., J. Heimer, B. Hammarström, I. Sangwan, L. Albert, and D. Rekosh. 1989. Regulation of human immunodeficiency virus *env* expression by the *rev* gene product. *J. Virol.* **63**:1959–1966.
37. Henderson, L. E., R. C. Sowder, G. W. Smythers, and S. Oroszlan. 1987. Chemical and immunological characterization of equine infectious anemia virus *gag*-encoded proteins. *J. Virol.* **61**:1116–1124.
38. Hope, T., and R. J. Pomerantz. 1995. The human immunodeficiency virus type 1 Rev protein: a pivotal protein in the viral life cycle, p. 91–105. *In* I. S. Y. Chen, H. Koprowski, A. Srinivasan, and P. K. Vogt (ed.), *Transacting functions of human retroviruses*, vol. 193. Springer-Verlag, Berlin.
39. Horowitz, D. S., and A. R. Krainer. 1994. Mechanisms for selecting 5' splice sites in mammalian pre-mRNA splicing. *Trends Genet.* **10**:100–106.
40. Kandels-Lewis, S., and B. Séraphin. 1993. Role of U6 snRNA in 5' splice site selection. *Science* **262**:2035–2039.
41. Kjems, J., M. Brown, D. D. Chang, and P. A. Sharp. 1991. Structural analysis of the interaction between the human immunodeficiency virus Rev protein and the Rev response element. *Proc. Natl. Acad. Sci. USA* **88**:683–687.
42. Kohtz, J. D., S. F. Jamison, C. L. Will, P. Zuo, R. Luhrmann, M. A. Garcia-Blanco, and J. L. Manley. 1994. Protein-protein interactions and 5' splice-site recognition in mammalian mRNA precursors. *Nature (London)* **368**:119–124.
43. Konforti, B. B., M. J. Koziolkiewicz, and M. M. Konarska. 1993. Disruption of base pairing between the 5' splice site and the 5' end of U1 snRNA is required for spliceosome assembly. *Cell* **75**:863–873.
44. Lavigne, H. L., H. L. Branche, A. R. Kornblihtt, and B. Chabot. 1993. A splicing enhancer in the human fibronectin alternate ED1 exon interacts with SR proteins and stimulates U2 snRNP binding. *Genes Dev.* **7**:2405–2417.
45. Lawrence, J., A. Cochrane, C. Johnson, A. Perkins, and C. Rosen. 1991. The HIV-1 Rev protein: a model system for coupled RNA transport and translation. *New Biol.* **3**:1220–1232.
46. Lesser, C. F., and C. Guthrie. 1993. Mutations in U6 snRNA that alter splice site specificity: implications for the active site. *Science* **262**:1982–1988.
47. Lu, X., J. Heimer, D. Rekosh, and M.-L. Hammarström. 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.
48. Luukkainen, B. G. M., W. Tan, and S. Schwartz. 1995. Efficiency of reinitiation of translation on human immunodeficiency virus type 1 mRNAs is determined by the length of the upstream open reading frame and by intercistronic distance. *J. Virol.* **69**:4086–4094.
49. Maldarelli, F., M. A. Martin, and K. Strebel. 1991. Identification of post-transcriptionally active inhibitory sequences in human immunodeficiency virus type 1 RNA: novel level of gene regulation. *J. Virol.* **65**:5732–5743.
50. Malim, M. H., and B. R. Cullen. 1993. Rev and the fate of pre-mRNA in the nucleus: implications for the regulation of RNA processing in eukaryotes. *Mol. Cell. Biol.* **13**:6180–6189.
51. Malim, M. H., J. Hauber, S. Le, J. V. Maizel, and B. R. Cullen. 1989. The HIV-1 rev transactivator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. *Nature (London)* **338**:254–257.
52. Martarano, L., R. Stephens, N. Rice, and D. Derse. 1994. Equine infectious anemia virus *trans*-regulatory protein Rev controls viral mRNA stability, accumulation, and alternative splicing. *J. Virol.* **68**:3102–3111.
53. Mount, S. M. 1982. A catalogue of splice junction sequences. *Nucleic Acids Res.* **10**:459–472.
54. Nasioulas, G., A. S. Zolotukhin, C. Tabernero, L. Solomin, C. P. Cunningham, G. N. Pavlakis, and B. K. Felber. 1994. Elements distinct from human immunodeficiency virus type 1 splice site are responsible for the Rev dependence of *env* mRNA. *J. Virol.* **68**:2986–2993.
55. Newman, A. J., and C. Norman. 1992. U5 snRNA interacts with exon sequences at 5' and 3' splice sites. *Cell* **68**:743–754.
56. Noiman, S., A. Yaniv, T. Tsach, L. Sherman, T. Miki, S. R. Tronick, and A. Gazit. 1991. The tat protein of equine infectious anemia virus is encoded by at least three types of transcripts. *Virology* **184**:521–530.
57. Pedersen, N. C. 1989. Animal virus infections that defy vaccination: equine infectious anemia, caprine arthritis-encephalitis, maedi-visna, and feline infectious peritonitis. *Adv. Vet. Sci. Comp. Med.* **33**:413–428.
58. Reddy, R. 1985. Compilation of small RNA sequences. *Nucleic Acids Res.* **13**:155–163.
59. Rimsky, L., J. Hauber, M. Dukovich, M. H. Malim, A. Langlois, B. R. Cullen, and W. C. Greene. 1988. Functional replacement of the HIV-1 rev protein by the HTLV-1 rex protein. *Nature (London)* **335**:738–740.
60. 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.
61. Rosin-Arbesfeld, R., M. Rivlin, S. Noiman, P. Mashiah, A. Yaniv, T. Miki, S. R. Tronick, and A. Gazit. 1993. Structural and functional characterization of *rev*-like transcripts of equine infectious anemia virus. *J. Virol.* **67**:5640–5646.
62. Schalling, M., K. Friberg, K. Seroogy, P. Riederer, E. Bird, S. N. Schiffmann, P. Mailloux, J.-J. Vanderhaeghen, S. Kuga, M. Goldstein, K. Kitahama, P. H. Luppi, M. Jouvett, and T. Hökfelt. 1990. Analysis of expression of cholecystokinin in dopamine cells in the ventral mesencephalon of several species and in humans with schizophrenia. *Proc. Natl. Acad. Sci. USA* **87**:8427–8431.
63. 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.
64. 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.
65. 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.
66. Seiki, M., A. Hikikoshi, and M. Yoshida. 1990. The U5 sequence is a *cis*-acting repressive element for genomic RNA expression of human T cell leukemia virus type 1. *Virology* **176**:81–86.
67. Sherman, L., A. Gazit, A. Yaniv, T. Kawakami, J. E. Dahlberg, and S. R. Tronick. 1988. Localization of sequences responsible for *trans*-activation of the equine infectious anemia virus long terminal repeat. *J. Virol.* **62**:120–126.
68. Sherman, L., A. Yaniv, H. Lichtman-Pleban, S. R. Tronick, and A. Gazit. 1989. Analysis of regulatory elements of the equine infectious anemia virus and caprine arthritis-encephalitis virus long terminal repeats. *J. Virol.* **63**:4925–4931.
69. Sontheimer, E. J., and J. A. Steitz. 1993. The U5 and U6 small nuclear RNAs as active site components of the spliceosome. *Science* **262**:1989–1996.
70. Staffa, A., and A. Cochrane. 1995. Identification of positive and negative splicing regulatory elements within the terminal *tat*-*rev* exon of human immunodeficiency virus type 1. *Mol. Cell. Biol.* **15**:4597–4605.
71. Staffa, A., and A. Cochrane. 1994. The *tat/rev* intron of human immunodeficiency virus type 1 is inefficiently spliced because of suboptimal signals in the 3' splice site. *J. Virol.* **68**:3071–3079.
72. Staknis, D., and R. Reed. 1994. SR proteins promote the first specific recognition of pre-mRNA and are together with the U1 small nuclear ribonucleoprotein particle in a general splicing enhancer complex. *Mol. Cell. Biol.* **11**:7670–7682.
73. Stephens, R. M., J. W. Casey, and N. R. Rice. 1986. Equine infectious anemia virus *gag* and *pol* genes: relatedness to visna and AIDS virus. *Science* **231**:589–594.
74. Stephens, R. M., D. Derse, and N. R. Rice. 1990. Cloning and characterization of cDNAs encoding equine infectious anemia virus Tat and putative Rev proteins. *J. Virol.* **64**:3716–3725.
75. Striebeck, R., and M. W. Hentze. 1992. Bacteriophage and spliceosomal proteins function as position-dependent *cis/trans* repressors of mRNA translation in vitro. *Nucleic Acids Res.* **20**:5555–5564.
76. Tan, W., B. K. Felber, A. S. Zolotukhin, G. N. Pavlakis, and S. Schwartz. 1995. Efficient expression of the human papillomavirus type 16 L1 protein in epithelial cells by using Rev and the Rev responsive element of human immunodeficiency virus or the *cis*-acting transactivation element of simian retrovirus type 1. *J. Virol.* **69**:5607–5620.
77. Tan, W., and S. Schwartz. 1995. The Rev protein of human immunodeficiency virus type 1 counteracts the effect of an AU-rich negative element in the human papillomavirus type 1 late 3' untranslated region. *J. Virol.* **69**:2932–2945.
78. Tanaka, K., A. Watakabe, and Y. Shimura. 1994. Polypurine sequences within a downstream exon function as a splicing enhancer. *Mol. Cell. Biol.* **14**:1347–1354.
79. Temin, H. M. 1993. A proposal for a new approach to a preventive vaccine

- against human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **90**:4419–4420.
80. **Unge, D., L. Solomin, M. Mellini, D. Derse, B. K. Felber, and G. N. Pavlakis.** 1991. The Rex regulatory protein of human T-cell lymphotropic virus type I binds specifically to its target within the viral RNA. *Proc. Natl. Acad. Sci. USA* **88**:7145–7149.
 81. **Watakabe, A., K. Tanaka, and Y. Shimura.** 1993. The role of exon sequences in splice site selection. *Genes Dev.* **7**:407–418.
 82. **Xu, R., J. Teng, and T. A. Cooper.** 1993. The cardiac troponin T alternative exon contains a novel purine-rich positive splicing element. *Mol. Cell. Biol.* **13**:3660–3674.
 83. **Zhuang, Y., H. Leung, and A. M. Weiner.** 1987. The natural 5' splice site of simian virus 40 large T antigen can be improved by increasing the base complementarity to U1 RNA. *Mol. Cell. Biol.* **7**:3018–3020.
 84. **Zhuang, Y., and A. M. Weiner.** 1986. A compensatory base change in U1 snRNA suppresses a 5' splice site mutation. *Cell* **46**:827–835.
 85. **Zolotukhin, A. S., A. Valentin, G. N. Pavlakis, and B. K. Felber.** 1994. Continuous propagation of RRE(–) and Rev(–)RRE(–) human immunodeficiency virus type 1 molecular clones containing a *cis*-acting element of simian retrovirus type 1 in human peripheral blood lymphocytes. *J. Virol.* **68**:7944–7952.
 86. **Zuo, P., and J. L. Manley.** 1994. The human splicing factor ASF/SF2 can specifically recognize pre-mRNA 5' splice sites. *Proc. Natl. Acad. Sci. USA* **91**:3363–3367.