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Presence of Negative and Positive *cis*-Acting RNA Splicing Elements within and Flanking the First *tat* Coding Exon of Human Immunodeficiency Virus Type 1

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The human immunodeficiency virus type 1 (HIV-1) RNA follows a complex splicing pathway in which a single primary transcript either remains unspliced or is alternatively spliced to more than 30 different singly and multiply spliced mRNAs. We have used an in vitro splicing assay to identify *cis* elements within the viral genome that regulate HIV-1 RNA splicing. A novel splicing regulatory element (SRE) within the first *tat* coding exon has been detected. This element specifically inhibits splicing at the upstream 3′ splice site flanking this *tat* exon. The element only functions when in the sense orientation and is position dependent when inserted downstream of a heterologous 3′ splice site. In vivo, an HIV-1 SRE mutant demonstrated a decrease in unspliced viral RNA, increased levels of single- and double-spliced *tat* mRNA, and reduced levels of *env* and *rev* mRNAs. In addition to the negative *cis*-acting SRE, the flanking 5′ splice site downstream of the first *tat* coding exon acts positively to increase splicing at the upstream 3′ splice sites. These results are consistent with hypotheses of bridging interactions between cellular factors that bind to the 5′ splice site and those that bind at the upstream 3′ splice site.

Most eukaryotic mRNAs are synthesized as precursor RNAs containing introns which are removed by splicing to generate mature mRNAs. Pre-mRNAs can be alternatively spliced to multiple mRNA species which are used to express different isoforms of the same gene. Regulation of the efficiency of splicing is determined by a variety of *cis*-acting signals, including the sequences within introns just upstream and downstream of the 5′ and 3′ splice sites, respectively, the lariat branch point sequence, and the polypyrimidine tract between the branch point and the 3′ splice site (for a review, see reference 22). In addition, evidence is accumulating that exon sequences can also play a role in determining splicing efficiency (6–9, 13, 18, 26, 28, 34–36, 40, 44, 49, 61, 64, 66, 68–73). In some cases, these exon sequences have been shown to represent binding sites for cellular factors (9, 53, 66, 67, 71).

Retroviruses, whose RNA is transcribed from integrated proviral genomes, utilize the host cell splicing machinery to generate their mRNAs (for a review, see reference 10). The human immunodeficiency virus type 1 (HIV-1) RNA undergoes a complex alternative splicing pathway in which a single 9.2-kb primary transcript, which is capped and polyadenylated similarly to host cell pre-mRNA, is spliced to more than 30 different singly and multiply spliced mRNAs (15, 43, 51, 58). The group of multiply spliced mRNAs with approximate molecular sizes of 2 kb encode the regulatory proteins Tat, Rev, and Nef (Fig. 1A). Single-spliced mRNAs with molecular sizes of approximately 4 kb encode the *env* glycoprotein as well as the Vif, Vpr, and Vpu proteins (19, 59). Approximately half of the HIV-1 RNA remains unspliced to be packaged into progeny virions and used as message for *gag* and *pol* gene products (Fig. 1A). This complex splicing pattern is maintained in different virus strains, which indicates that it plays an important role in the virus life cycle. The steady-state levels of the different viral mRNAs are determined by both splicing efficiency and mRNA stabilities. In addition, the virus-coded RNA-binding protein Rev acts to stabilize unspliced and partially spliced HIV-1 mRNAs and to facilitate the transport of these RNA species from the nucleus to the cytoplasm (14, 16, 25, 30, 38, 39).

Little is known about how RNA splicing of the complex retrovirus HIV-1 is regulated and how inefficient splicing is maintained to provide the nuclear pools of unspliced and partially spliced RNA species required for Rev-dependent transport. Such information is important not only for understanding HIV-1 gene expression but also for understanding regulation of cellular pre-mRNA splicing. The simple retrovirus avian sarcoma virus has been shown to contain several negative *cis*-acting signals within and upstream of its two 3′ splice sites (4, 18, 31) as well as a *cis*-acting site within the *gag* gene distant from both the major 5′ splice site and the 3′ splice sites (2, 62). These elements act to allow only partial splicing of the avian sarcoma virus genome and to ensure balanced splicing of the two spliced viral mRNA species.

To investigate HIV-1 RNA alternative splicing and to localize *cis*-acting elements regulating splicing, we have used an in vitro splicing system with HIV-1 RNA substrates. We show that a novel negative *cis*-acting splicing regulatory element (SRE) is present within the first *tat* coding exon. This element acts selectively to inhibit splicing at the upstream 3′ splice site flanking the *tat* exon. In addition, we show that the 5′ splice site flanking the *tat* exon at its 3′ boundary (5′ splice site 4) acts positively to facilitate splicing at the upstream alternative 3′ splice sites. These results are consistent with the exon definition hypothesis which proposes bridging interaction between factors that bind to the 5′ splice site and those that bind to the 3′ splice site (20, 50).

MATERIALS AND METHODS

Plasmid construction and synthesis of RNA substrates. DNA templates for preparation of splicing substrates were

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derived by restriction enzyme digestion of plasmid pHS1. (This plasmid is diagrammed in Fig. 2A.) It was constructed by deletion of the infectious HIV-1 clone pNL4-3 (1) (obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases) between nucleotides (nt) 909 and 5743 and placing the deleted sequence between nt 679 and 6026 downstream of the T3 promoter in Bluescript SK$^+$ (Stratagene, La Jolla, Calif.). pHS1 was cleaved with *Sal*I, *Bsu36*I, and *Hin*dIII, respectively, at nt 5785, 5954, and 6026 to create linearized templates (HS1-5785, HS1-5954, and HS1-6026) for transcription of RNA splicing substrates (Fig. 2A). The capped, [^{32}P]UTP-labeled RNA substrates were transcribed with T3 polymerase (Promega Co., Madison, Wis.) essentially as described previously (41) in the presence of the cap analog G(5′)ppp(5′)G (Pharmacia Co., Piscataway, N.J.). Substrate HS1-6055I (Fig. 2A) was synthesized by transcription from a plasmid containing an inversion of the *Sal*I-*Sal*I fragment in pHS1 (nt 5785) to a *Sal*I site in the Bluescript polylinker sequences. The plasmid was linearized with *Xho*I for RNA synthesis. Substrate HS1-5934 (Fig. 2A) was transcribed from a template in which an *Nco*I site (underlined below) was created by PCR mutagenesis at nt 5934 in pHS1 by using a sense primer, 5′CTTGGGCAGG AGTGG3′ (nt 5716 to 5730), and an antisense primer containing the *Nco*I site, 5′CCTAAGGACGCACGAAGATCCAT GGTTGGCAATGAAAGC3′ (nt 5921 to 5960). The region corresponding to the PCR product was sequenced to confirm its structure. This plasmid was linearized with *Nco*I for RNA synthesis. Plasmid pNL4-tatA, which contains two base changes at nt 5854 (G to T) and 5857 (C to G) in clone pNL4-3 to create a *Stu*I site at this position, was cleaved with *Eco*RI and *Hin*dIII, and the 283-nt fragment was cloned into pHS1. This plasmid was linearized with *Stu*I and used as a template for synthesis of substrate HS1-5857 (Fig. 2A). Plasmid pNL4-tatC was constructed by insertion of a 41-nt heterologous linker containing a *Sma*I site between nt 5831 and 5894 and was cleaved with *Eco*RI and *Hin*dIII, and the 261-nt fragment was cloned into pHS1 (pHS1-P3). This plasmid was linearized with *Sma*I and used as a template for synthesis of substrate HS1-5860ΔSRE (Fig. 2A).

To create pHS1-X (see Fig. 3A), plasmid pHS1 was cleaved with *Hin*dIII and *Xho*I. The large fragment (3.4 kb) was isolated and ligated with two oligonucleotides (5′AGCT TCTCTATCAAAGCAGTAAGTAGTAC3′ [sense] and 5′TC GAGTACTACTTACTGCTTTGAT3′ [antisense]) which had been annealed to restore the HIV-1 sequence from nt 6031 to 6055. To cause a mutation at 5′ splice site 3 and create pHS1-XΔ5′ss, the cloning procedure was similar to that used for pHS1-X except that oligonucleotides were used, causing G-to-C and T-to-G base changes at the splice site (5′AGCT TCTCTATCAAAGCACGAAGTAGTAC3′ [sense] and 5′TC GAGTACTACTTCGTGCTTTGATAGAGA3′ [antisense]). Both plasmids were linearized with *Xho*I and used as templates for RNA transcription to synthesize substrates HS1-6055 and HS1-6055Δ5′ss (Fig. 3A). HS1-6026ΔSRE (Fig. 3A) was transcribed from pHS1-P3 which was linearized with *Hin*dIII to include 3′ splice sites 4c, 4a, 4b, and 5. Plasmid pHS1-P3X was created by cloning the 261-nt *Eco*RI and *Hin*dIII fragment from pNL4-tatC into pHS1-X. HS1-6055ΔSRE (Fig. 3A) was transcribed from pHS1-P3X linearized with *Xho*I.

The sequence of the Rous sarcoma virus (RSV) infectious provirus clone (Prague A strain) pJD100 (obtained from J. T. Parsons, University of Virginia) was deleted from nt 543 to 6983 (on the basis of the Prague C RSV sequence in reference 57), and the region from nt 255 to 9238 was cloned downstream of the SP6 promoter in pSPT18 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) to create pRSV-7169. PCR products with antisense or sense oligonucleotides containing the HIV-1 sequence from nt 5821 to 5860 were inserted immediately upstream of the RSV *Nco*I site at nt 7127 to create pRSV-7169SRE$^+$ and pRSV-7169SRE$^-$, respectively. In these two constructs, the RSV sequence between nt 7098 and 7127 was deleted. Both plasmids were sequenced within the region of the PCR insert to confirm that no inadvertant mutations had occurred. The plasmids were linearized with *Nae*I (nt 7169) and used as templates for RNA synthesis with SP6 polymerase (Promega) (see Fig. 4A). In these templates, the HIV-1 sequence was at the same distance downstream of the RSV *src* 3′ splice site as from HIV-1 3′ splice site 3 in the HIV-1 genome. Construction of pRSV-7169SRE$^+$D (Fig. 4A) was carried out with an antisense or sense oligonucleotide containing the HIV-1 sequence from nt 5821 to 5860 and containing *Nco*I sticky ends. These were annealed and ligated into pRSV-7169 which was digested with *Nco*I. This placed the SRE at a distance of 73 nt from the *src* 3′ splice site. Since this plasmid does not contain a deletion of RSV sequence, spliced products derived from transcripts with this clone as a template are 29 nt longer than those derived from pRSV7169$^+$ or pRSV7169$^-$ (Fig. 4).

Plasmid p601CC, which spanned the *tat*, *rev*, and *env* 3′ splice sites, was constructed by insertion of the *Eco*RI-*Kpn*I fragment (nt 5743 to 6343) into Bluescript SK$^+$. Plasmid p601CC-tatC was constructed by insertion of the corresponding *Eco*RI-*Kpn*I fragment of pNL4-tatC. These plasmids were linearized with *Eco*RI and used as templates to construct a probe for RNase protection assays.

Plasmid pCMVtat was constructed by insertion of a *Bgl*II-*Sal*I fragment containing the HIV-1 (SF2 strain) two-exon *tat* gene from pSV/TAT-1 (47) into pCMV5 (obtained from M. F. Stinski, University of Iowa) which was digested with *Bgl*II and *Sal*I.

In vitro splicing and gel electrophoresis. HeLa cell nuclear extracts were prepared as described by Dignam et al. (12). Maximal splicing activity was obtained at a nuclear extract concentration of 60% (vol/vol), as described by Krainer et al. (33). The splicing reaction was carried out in a total volume of 25 μl, with a final concentration of 60% (vol/vol) HeLa cell nuclear extract in buffer D (12), 2.6% polyvinyl alcohol, 3 mM $MgCl_2$, 0.5 mM ATP, 20 mM creatine phosphate, and ~5 ng of pre-mRNA by methods essentially as described previously (33). The reaction mixtures were incubated at 30°C for 2 h, and the RNAs were subsequently analyzed on 4% polyacrylamide gels containing 7 M urea.

Cell culture, transfection assays, and RNA isolation from whole cells, nuclei, and cytoplasm. HeLa cells were grown in Eagle's minimal medium containing 10% heat-inactivated newborn calf serum. Transfection of HeLa cells with plasmid DNAs was carried out by a modification of the calcium phosphate coprecipitation method (21). Plasmid DNA (25 to 50 μg) and salmon sperm DNA (5 μg) in 1 ml of 1× HEPES-buffered saline (140 mM NaCl, 0.8 mM Na_2HPO_4, 5 mM KCl, 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.1) and 125 mM calcium chloride were added to 100-mm-diameter culture dishes of HeLa cells (approximately 80% confluent) and incubated at 37°C. The tissue culture dishes were rocked every 20 min to prevent the cells from drying. After 2 h, complete medium (5 ml per dish) was added and the culture dishes were incubated for an additional 2 h. At this time, the medium was removed and the cells were shocked for 9 min with 33% (vol/vol) dimethyl sulfoxide. The cells were then washed one time, and the cultures were further incubated for 48 h. Total RNA was

isolated from the cells by the guanidine hydrochloride method of Strohman et al. (65).

RNase protection assays. [^{32}P]UTP-labeled RNA probes were synthesized with T7 RNA polymerase (Promega). RNA synthesis and RNase protection assays were carried out essentially by the procedures of Melton et al. (41) as modified by Berberich and Stoltzfus (3). Hybridizations of labeled probes with RNA were carried out in 80% formamide at 57°C overnight. RNase digestion of hybridized RNA was carried out with 1,700 U of T1 RNase (Boehringer Mannheim Biochemicals) in a volume of 300 μl. The samples were proteinase K treated, phenol-chloroform extracted, and precipitated. The dried precipitates were redissolved in electrophoresis loading buffer. The samples were denatured at 85°C for 10 min, quenched on ice, and loaded onto 7 M urea–polyacrylamide gels.

RESULTS

The first *tat* coding exon contains a region that acts to inhibit splicing at the *tat* 3′ splice site. The steady-state level of double-spliced *tat* mRNA is considerably lower than the levels of the double-spliced *rev* and single-spliced *env/vpu* mRNAs in both lymphoid and nonlymphoid cells (11a, 23, 48, 51). One possible explanation for this result is that splicing at 3′ splice site 3 (Fig. 1) upstream of *tat* exon 2 may be less efficient than splicing at the other downstream 3′ splice sites in this region. Alternatively, the *tat* mRNA may be less stable than the *rev* and *env* mRNAs. Comparison of the sequences upstream of the alternative 3′ splice sites (Fig. 1B) indicates that all of the 3′ splice sites are nonconsensus in that their pyrimidine tracts are interspersed with purines. Thus, it appears that HIV-1 3′ splice sites are suboptimal, as has been shown for other retroviruses (4, 18, 31, 63). The 3′ splice site (site 3) flanking the first *tat* coding exon possesses a pyrimidine tract which is closer to consensus than any of the other 3′ splice sites. Its pyrimidine tract is also more uridine rich than any of the other 3′ splice sites in the central region of the HIV-1 genome; this property has been correlated with increased splicing efficiency of an adenovirus type 2 precursor (52). Therefore, this analysis of the splice site sequences did not explain why 3′ splice site 3 is apparently used so much more inefficiently in vivo than other alternative 3′ splice sites in this region.

The HIV-1 splicing patterns in HeLa cells and in human T-cell lines are very similar, and splicing from 5′ splice site 1 to alternative 3′ splice sites 3 to 5 within the central region of the HIV-1 genome includes major splicing events occurring in both cell types (48). Thus, to study regulation of splicing at these alternative 3′ splice sites, we tested in vitro splicing of HIV RNA substrates in HeLa cell nuclear extracts, using the precursor substrates shown in Fig. 2A. Splicing of substrate HS1-5785 (which contained the major 5′ splice site 1 at nt 743, a large deletion from nt 909 to 5745, the 3′ splice site 3, and 11 nt of *tat* exon 2) was relatively efficient, as evidenced by the amounts of the 128-nt spliced product and released 206-nt intron (Fig. 2B, lane 2). In contrast, as shown in Fig. 2B, lane 3, substrate HS1-6026, which contained the 3′ splice sites upstream of the first *rev* coding exon (3′ splice sites 4a, 4b, and 4c) and the *env* gene (3′ splice site 5) in addition to 3′ splice site 3, was spliced at 3′ splice site 3, with a sixfold decrease in efficiency relative to HS1-5785 as determined from the amount of 370-nt spliced product (Fig. 2C). Very small amounts of more-rapidly-migrating final products spliced at the downstream 3′ splice sites 4a to 4c and 3′ splice site 5 were detected with substrate HS1-6026 (Fig. 2B, lane 3). Three prominent bands, migrating more slowly than the nonreacting substrate

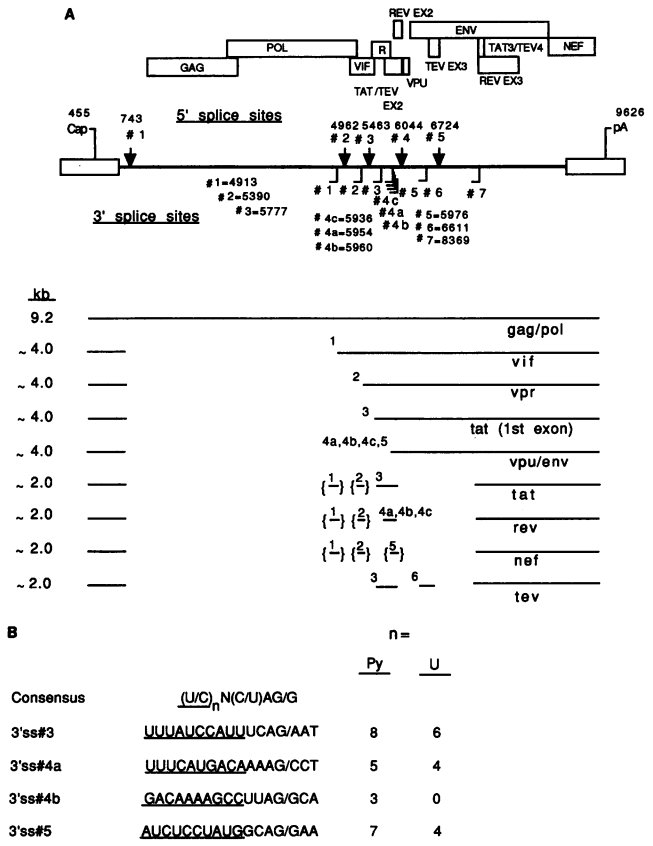
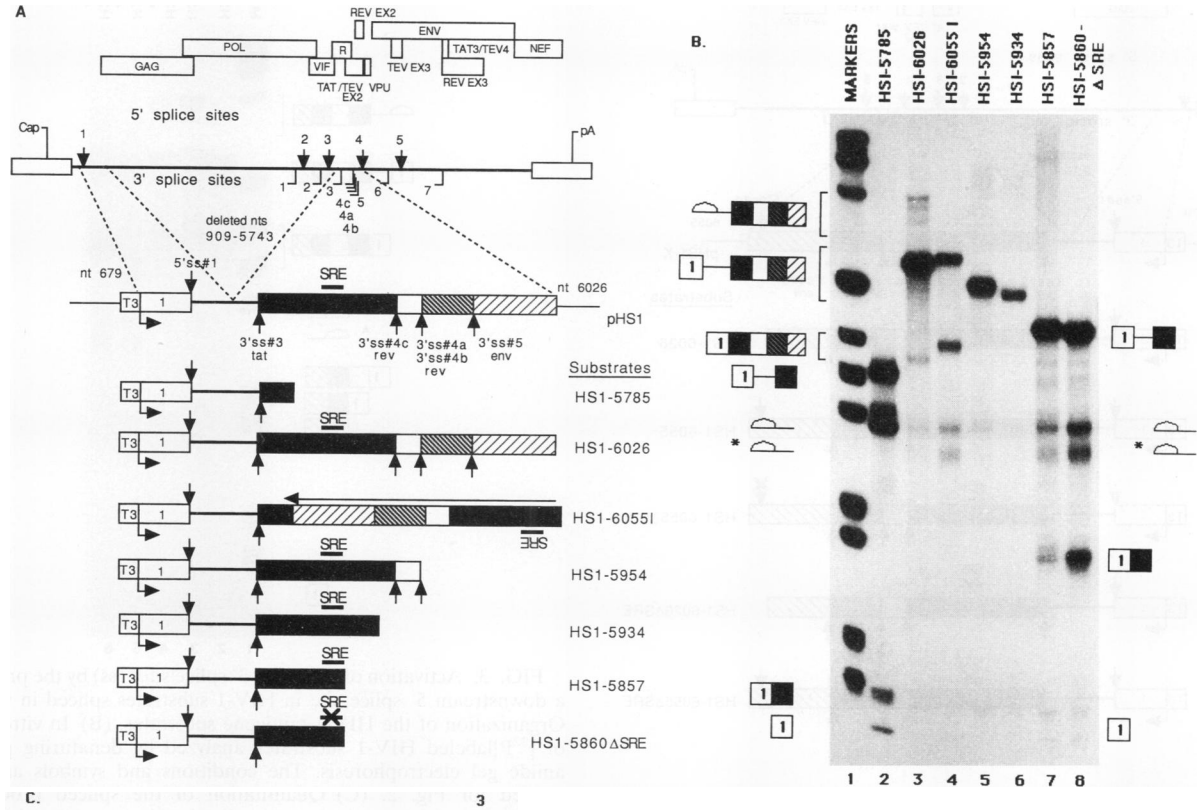


FIG. 1. (A) HIV-1 mRNAs generated from the multiple 3′ (angled lines) and 5′ (down arrows) splice sites. Alternative exons (EX) which are present in only some of the mRNAs are shown in braces. The nucleotide numbers of the splice sites are shown. The approximate sizes of the mRNAs are also shown. (B) Diagram of sequences of alternative 3′ splice sites in the central region of the HIV-1 genome. Nomenclature of splice sites is similar to that used for panel A. Shown are the number (n) of pyrimidines (Py) in the pyrimidine tract (underlined) and the number of uridines. ss, splice site.

band, which have the properties of lariat intermediates formed in the first step of the splicing reaction, were present (for a review, see reference 22). Thus, there was evidence for a significant inhibition of splicing of substrate HS1-6026 at the upstream 3′ splice site 3 accompanied by a very inefficient splicing at the downstream 3′ splice sites. The inhibitory region downstream of 3′ splice site 3 only functions in the sense orientation, since a substrate transcribed from a template with an inversion of the sequence between the 3′ ends of substrates HS1-6026 and HS1-5785 (substrate HS1-6055I in Fig. 2B, lane 4) was spliced at 3′ splice site 3, with an approximately threefold increase in efficiency over HS1-6026, as determined from the amount of 394-nt spliced product (Fig. 2C).

To locate the inhibitory sequences downstream of 3′ splice site 3, we tested additional RNA substrates which contained 3′ splice site 3 and which were truncated at several different sites downstream of 3′ splice site 3 (Fig. 2A). Splicing of substrates HS1-5954 and HS1-5934 was almost completely inhibited (Fig. 2B, lanes 5 and 6, and Fig. 2C). This was indicated by the lack of both expected spliced products (298 and 274 nt, respectively) and the absence of lariat intermediates at the expected



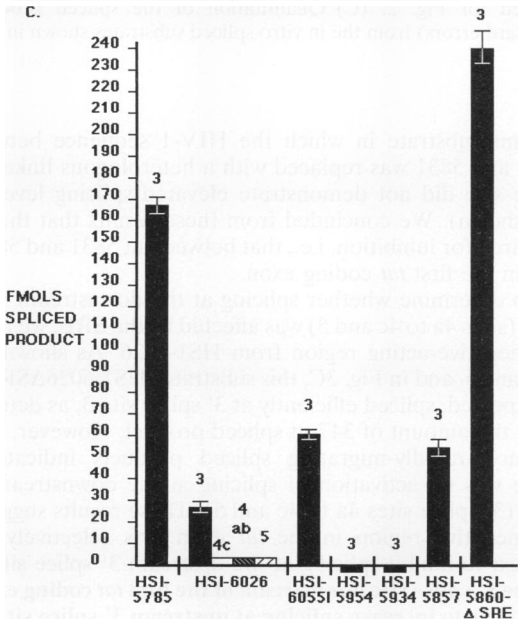
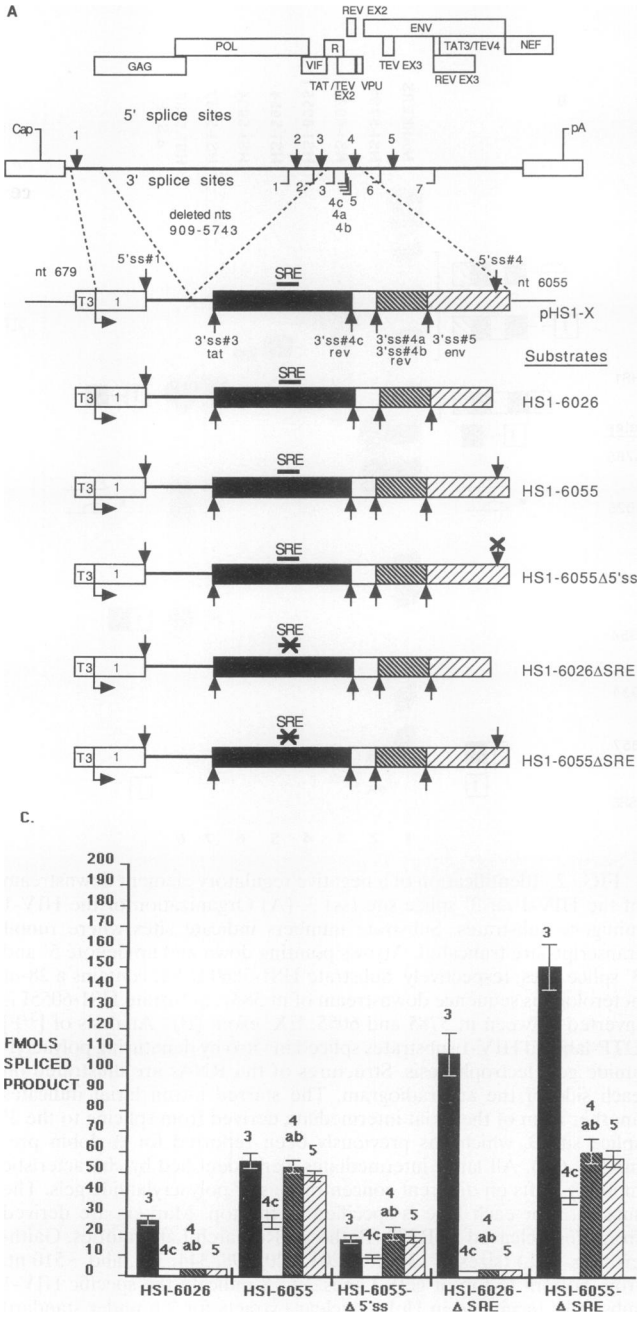


FIG. 2. Identification of a negative regulatory element downstream of the HIV-1 *tat* 3′ splice site (ss) 3. (A) Organization of the HIV-1 minigene substrates. Substrate numbers indicate sites where runoff transcripts are truncated. Arrows pointing down and up denote 5′ and 3′ splice sites, respectively. Substrate HS1-5860ΔSRE contains a 28-nt heterologous sequence downstream of nt 5831. Substrate HS1-6055I is inverted between nt 5785 and 6055. EX, exon. (B) Analysis of [^{32}P]UTP-labeled HIV-1 substrates spliced in vitro by denaturing polyacrylamide gel electrophoresis. Structures of the RNAs are illustrated on each side of the autoradiogram. The starred intron lariat indicates another form of the lariat intermediate derived from splicing to the 3′ splice site 3, which has previously been reported for β-globin pre-mRNA (55). All lariat intermediates were identified by characteristic mobility shifts on different concentrations of polyacrylamide gels. The substrate for each lane is specified at the top. Markers are derived from *Hin*fI-cleaved pBR322 (Bethesda Research Laboratories, Gaithersburg, Md.) (sizes of 134, 154, 201, 220, 298, 344, 396, and ~510 nt, from bottom to top of gel). Lanes 2 to 8 indicate the specific HIV-1 substrates incubated in HeLa nuclear extracts for 2 h under standard splicing reaction conditions. Substrates were spliced in parallel and electrophoresed on a single 4% polyacrylamide–7 M urea gel. (C) Quantitation of the spliced products from the in vitro-spliced substrates shown in panel A. Multiple gels were scanned and quantitated on the AMBIS phosphoimager. The amount of product (± standard errors) was calculated on the basis of the uridine content of the RNA species and is expressed as femtomoles of spliced product. The 3′ splice site used to generate the specific spliced product is shown above each bar. The spliced products resulting from the use of 3′ splice sites 4c, 4a, 4b, and 5 in substrate HS1-6026 are detected only on longer exposures. The two Rev 3′ splice sites, 4a and 4b, appear as one band because of the close proximity of their 3′ splice sites (23, 56). Splice site 4c is used infrequently in vivo and is detected on longer exposures (48). The amount of products after a 2-h reaction did not change significantly when the amount of substrate was increased threefold.



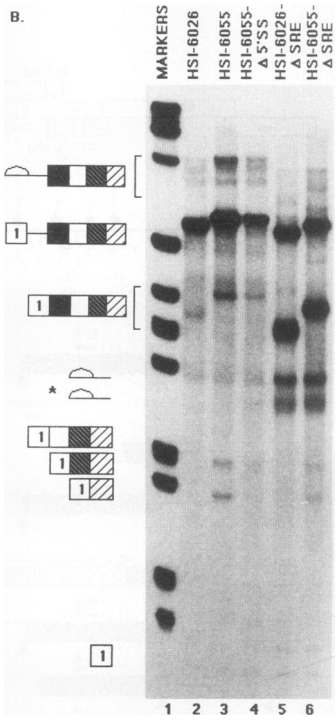


FIG. 3. Activation of upstream 3′ splice sites (ss) by the presence of a downstream 5′ splice site in HIV-1 substrates spliced in vitro. (A) Organization of the HIV-1 minigene substrates. (B) In vitro splicing of [^{32}P]labeled HIV-1 substrates analyzed by denaturing polyacrylamide gel electrophoresis. The conditions and symbols are as described for Fig. 2. (C) Quantitation of the spliced products (± standard errors) from the in vitro spliced substrates shown in panel A.

positions. These results showed that removal of the downstream 3′ splice sites 4 through 5 did not relieve the inhibition of splicing at 3′ splice site 3, and thus, the splicing inhibition was not caused by these inefficient downstream splice sites competing for the single upstream 5′ splice site. In fact, splicing was more inhibited, suggesting that the downstream 3′ splice sites may partially reverse the negative effect (see Discussion). As shown in Fig. 2B, lanes 7 and 8, the splicing of substrate HS1-5857, determined by the amounts of 196-nt spliced product (Fig. 2C), was somewhat less inhibited, whereas the splicing of substrate HS1-5860ΔSRE, in which the viral sequence was truncated at nt 5831, was performed with an efficiency comparable to that of the HS1-5785 substrate. A mutant substrate in which the HIV-1 sequence between nt 5821 and 5831 was replaced with a heterologous linker of the same size did not demonstrate elevated splicing levels (data not shown). We concluded from these results that the region required for inhibition, i.e., that between nt 5831 and 5860, was within the first *tat* coding exon.

To determine whether splicing at the downstream 3′ splice sites (sites 4a to 4c and 5) was affected by the SRE, we removed the negative-acting region from HS1-6026. As shown in Fig. 3B, lane 5, and in Fig. 3C, this substrate (HS1-6026ΔSRE) was, as expected, spliced efficiently at 3′ splice site 3, as determined from the amount of 347-nt spliced product. However, the lack of more-rapidly-migrating spliced products indicated that there was no activation of splicing at the downstream splice sites (3′ splice sites 4a to 4c and 5). These results suggest that the negative region in the *tat* exon acts selectively in this context to inhibit splicing at the upstream 3′ splice site 3.

The 5′ splice site downstream of the first *tat* coding exon acts positively to increase splicing at upstream 3′ splice sites (sites 3 to 5). We next compared the splicing of substrates containing additional HIV-1 sequence downstream of nt 6026. Substrate HS1-6055 (Fig. 3A), which contained 29 nt of sequence downstream of 3′ splice site 5, was spliced with a striking increase in efficiency at the downstream 3′ splice sites (sites 4a to c and 5) relative to splicing at 3′ splice site 3 (Fig. 3B, lane 3). This was quantitated (Fig. 3C) by measuring the amounts of products spliced at 3′ splice sites 3 (395 nt), 4a and 4b (212 nt), 4c (237 nt), and 5 (196 nt). Contained within this additional 29-nt sequence is 5′ splice site 4, which is normally used to

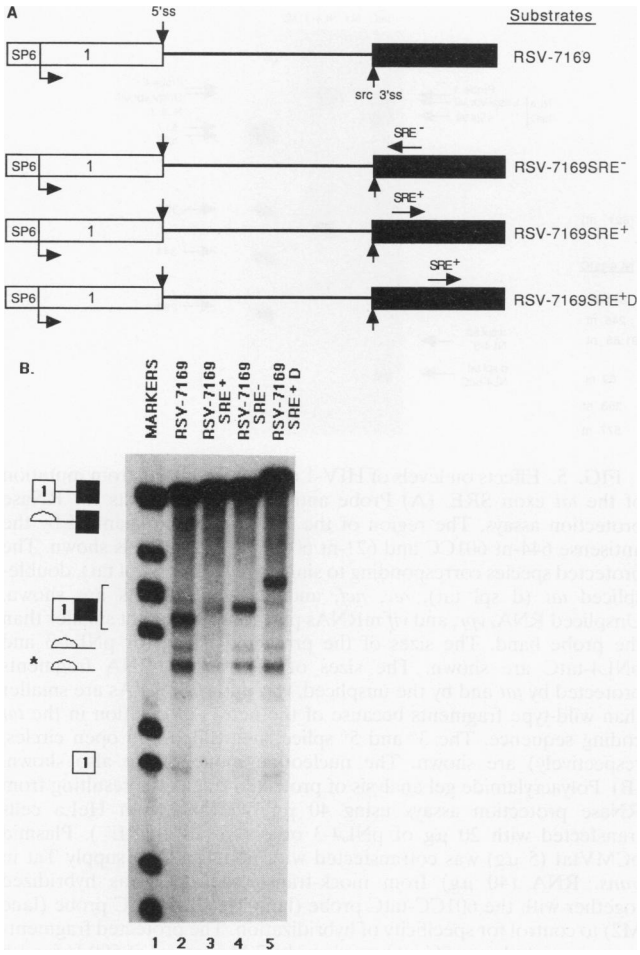


FIG. 4. Negative regulatory element inhibits RNA splicing of a heterologous substrate. (A) Organization of RSV substrates with HIV-1 SRE present in both orientations downstream of the *src* 3′ splice site (ss). 5′ ss is the major RSV 5′ splice site (see Materials and Methods). (B) In vitro splicing of ^{32}P-labeled *src* RSV substrates analyzed by denaturing polyacrylamide gel electrophoresis. The conditions and symbols are as described for Fig. 2.

form double-spliced *tat*, *rev*, and *nef* mRNAs by splicing to 3′ splice site 7 (Fig. 1A). To determine whether the presence of this unused 5′ splice site would account for the increased splicing efficiency at the downstream 3′ splice sites 4a to 4c and 5, we tested a substrate in which the consensus GU within 5′ splice site 4 was mutated to CG (Fig. 3B, lane 4). This substrate (HS1-6055Δ5′ss) was spliced with a lower efficiency (approximately threefold [Fig. 3C]) at all of the 3′ splice sites (i.e., 3′ splice sites 3 to 5) than was substrate HS1-6055. We also determined the effect of deleting the negative region downstream of 3′ splice site 3 in the context of a substrate containing the positive region downstream of 3′ splice site 5. As expected, this substrate (HS1-6055ΔSRE), which has the negatively acting region deleted, was efficiently spliced at 3′ splice site 3, in contrast to substrate HS1-6055, in which this region is present (Fig. 3B, compare lane 6 with lane 3). However, the level of splicing at the downstream 3′ splice sites 4 through 5, determined by measuring the amounts of 212-nt (3′ splice site 4a and 4b), 237-nt (3′ splice site 4c), and 196-nt (3′ splice site 5) products, was not significantly different from that of HS1-6055 (Fig. 3C). These results indicate that in the in vitro system, increased splicing at 3′ splice site 3 does not preclude maintenance of normal amounts of splicing at the downstream 3′ splice sites 4 through 5. Thus, these data further suggest that the negative region in the *tat* exon acts selectively to inhibit splicing at 3′ splice site 3.

The negative region in the *tat* exon acts only in the positive-strand sense and is a position-dependent element when placed downstream of a heterologous 3′ splice site. We next tested whether the negative signal in *tat* was sufficient to cause inhibition of splicing when inserted at the same distance downstream of another heterologous 3′ splice site. To this end, the HIV-1 sequence from nt 5821 to 5860 was placed in both the sense and antisense orientations at a distance of 44 nt downstream of the avian retrovirus RSV *src* gene 3′ splice site. These plasmids were used to prepare RNA transcripts whose splicing could be compared with that of a substrate lacking inserts (Fig. 4A). We compared the levels of spliced products migrating at approximately 310 nt. The data indicated that splicing at the *src* 3′ splice site was inhibited more than 10-fold when the HIV-1 sequence was present in the sense orientation (compare RSV-7169SRE$^+$ in Fig. 4B, lane 3, with RSV-7169 in Fig. 4B, lane 2). When the HIV-1 sequence was in the antisense orientation (RSV-7169SRE$^-$ in Fig. 4B, lane 4), the *src* substrate was spliced with efficiency comparable to that of substrate RSV-7169, which has no insertion. These data show that the *tat* sequence has the properties of a negative-acting SRE. We also placed the SRE at a greater distance (73 nt) from the *src* 3′ splice site (RSV-7169SRE$^+$D). The results, shown in Fig. 4, lane 5, indicated that the level of the 348-nt spliced product was more elevated than the level of the ~310-nt spliced product of RSV-7169SRE$^+$ (Fig. 4B, lane 3). This indicates that the SRE exerts its effect only when placed close to the 3′ splice site and, therefore, that the negative effect of the SRE is position dependent.

Mutation of the SRE in the context of the HIV-1 provirus results in increased splicing at the *tat* 3′ splice site. Having defined the location and activity of the *tat* gene SRE by using in vitro splicing assays, we studied the effect of an SRE mutation on levels of HIV-1 RNA species in vivo. This mutant (pNL4-tatC) at the N terminus of the *tat* gene was constructed by deleting 63 nt between nt 5831 and 5893 and inserting a heterologous 41-nt polylinker sequence in the infectious HIV-1 proviral clone pNL4-3 (5a). In addition to the mutation in *tat*, this insertion restored the 3′ end of the *vpr* reading frame but changed its nucleotide sequence. Mutant and wild-type proviral plasmid DNAs were transfected into HeLa cells. Since the mutant was defective in expression of the Tat transactivator, cotransfection with cytomegalovirus-driven Tat expression plasmid was required for the accumulation of significant levels of HIV-1 RNA. After 48 h, total RNA was isolated from these cells and the levels of RNA species alternatively spliced at the *tat* and *env* 3′ splice sites were determined by RNase protection assays spanning 3′ splice sites 3 to 5 using homologous RNA probes (Fig. 5A). The level of double-spliced *tat* mRNA was increased, and that of single-spliced *env* mRNA was decreased in the mutant-transfected cells (compare lanes tatC and NL4-3 in Fig. 5B). In addition, a larger amount of single-spliced *tat* mRNA was present than for the wild type, where a negligible amount of single-spliced *tat* mRNA was found (48). It was possible to visualize the smaller protected band arising from the *rev* mRNA on gels electrophoresed for shorter times. An approximately 90-nt band protected by double-spliced *rev* mRNA was also significantly reduced in the mutant-transfected cells (Fig. 5C). These results are consistent with the in vitro splicing data and suggest that

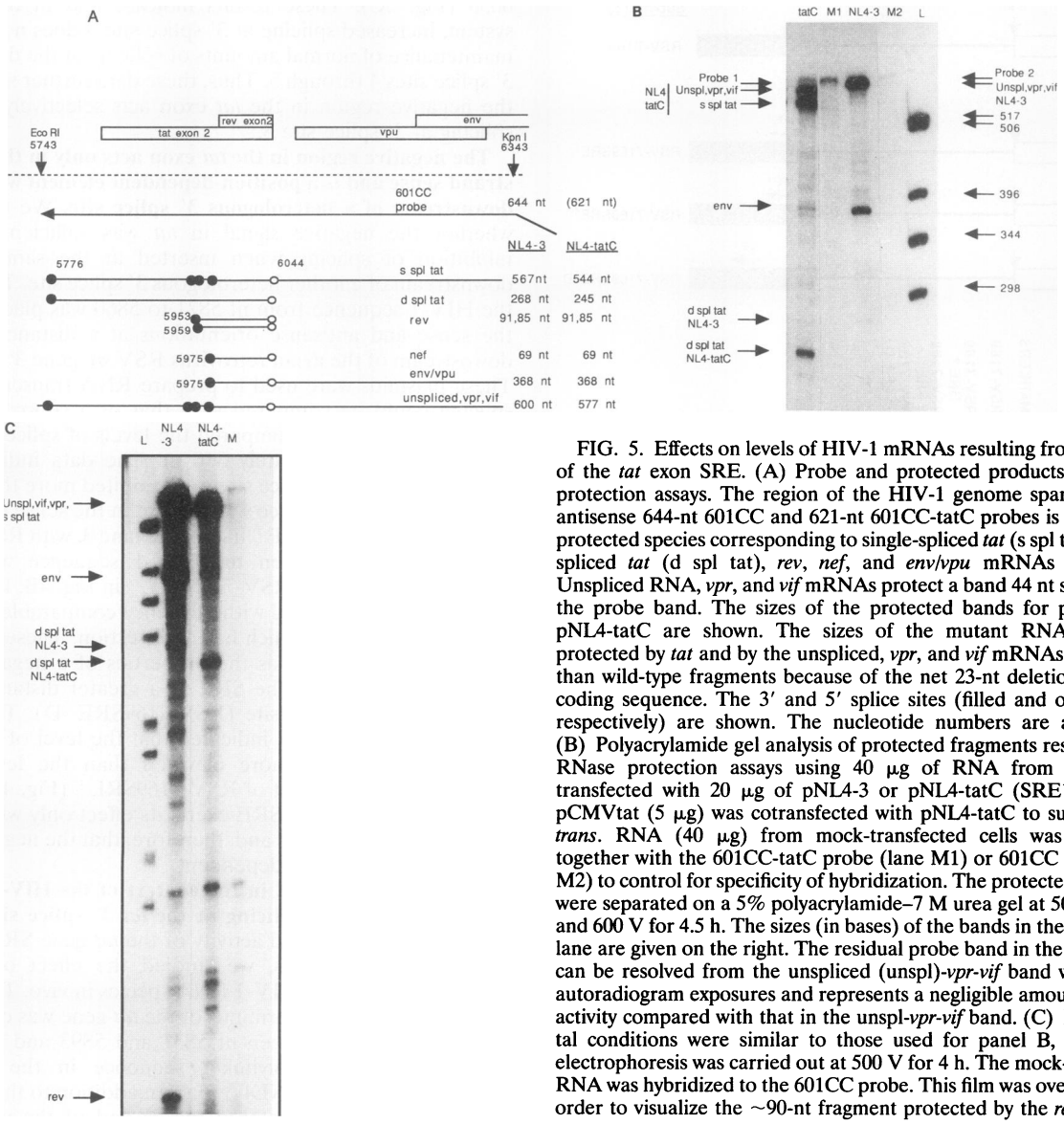


FIG. 5. Effects on levels of HIV-1 mRNAs resulting from mutation of the *tat* exon SRE. (A) Probe and protected products for RNase protection assays. The region of the HIV-1 genome spanned by the antisense 644-nt 601CC and 621-nt 601CC-tatC probes is shown. The protected species corresponding to single-spliced *tat* (s spl tat), double-spliced *tat* (d spl tat), *rev*, *nef*, and *env/vpu* mRNAs are shown. Unspliced RNA, *vpr*, and *vif* mRNAs protect a band 44 nt smaller than the probe band. The sizes of the protected bands for pNL4-3 and pNL4-tatC are shown. The sizes of the mutant RNA fragments protected by *tat* and by the unspliced, *vpr*, and *vif* mRNAs are smaller than wild-type fragments because of the net 23-nt deletion in the *tat* coding sequence. The 3′ and 5′ splice sites (filled and open circles, respectively) are shown. The nucleotide numbers are also shown. (B) Polyacrylamide gel analysis of protected fragments resulting from RNase protection assays using 40 μg of RNA from HeLa cells transfected with 20 μg of pNL4-3 or pNL4-tatC (SRE^-). Plasmid pCMVtat (5 μg) was cotransfected with pNL4-tatC to supply Tat in *trans*. RNA (40 μg) from mock-transfected cells was hybridized together with the 601CC-tatC probe (lane M1) or 601CC probe (lane M2) to control for specificity of hybridization. The protected fragments were separated on a 5% polyacrylamide–7 M urea gel at 500 V for 1 h and 600 V for 4.5 h. The sizes (in bases) of the bands in the marker (L) lane are given on the right. The residual probe band in the NL4-3 lane can be resolved from the unspliced (unspl)-*vpr-vif* band with shorter autoradiogram exposures and represents a negligible amount of radioactivity compared with that in the unspl-*vpr-vif* band. (C) Experimental conditions were similar to those used for panel B, except that electrophoresis was carried out at 500 V for 4 h. The mock-transfected RNA was hybridized to the 601CC probe. This film was overexposed in order to visualize the ~90-nt fragment protected by the *rev* mRNA.

removal of the SRE results in the selective increased usage of 3′ splice site 3 upstream of the *tat* exon. Concomitant with this is the decreased usage of alternative 3′ splice sites 5 upstream of *env* and 4 upstream of *rev*. It can also be seen in Fig. 5B that the level of the RNA fragment protected by the unspliced RNA relative to the levels of fragments protected by single- and double-spliced RNA species was reduced in the SRE mutant. The fragment protected by unspliced RNA is also protected by single-spliced *vif* and *vpr* mRNAs, but the levels of these spliced mRNAs have been shown to be very low in comparison with unspliced RNA levels (48). To confirm the reduction in unspliced RNA in the mutant-transfected cells, we carried out RNA protection analyses, using a probe which spanned the major 5′ splice site (5′ splice site 1). This probe allowed us to measure the total amounts of spliced and unspliced HIV-1 RNAs. These results indicated that the level of unspliced RNA was reduced two- to threefold in the mutant-transfected cells. Similar results were obtained by Northern (RNA) blot analysis (data not shown).

DISCUSSION

Other laboratories have previously demonstrated splicing in vitro of HIV-1 RNA with different RNA substrates (17, 32). In this report, we describe conditions and substrates that permit studies on the regulation of alternative HIV-1 RNA splicing of *tat*, *rev*, and *env/nef* mRNAs in nuclear extracts. Previous studies have relied on measurements of steady-state levels of these different HIV-1 RNA species in infected cells as a measure of alternative splice site usage (23, 48, 51). The interpretation of such experiments is complicated because the steady-state levels of different viral RNA species in the cell are determined not only by splicing but also by RNA stabilities. Using the in vitro system, we have identified two different *cis* elements that play direct roles in determining the splicing efficiencies in the region of the first coding exons of *tat* and *rev* and upstream of the *env* gene. Since the HIV-1 splicing patterns in HeLa cells and in human T-cell lines are very

similar (48), use of HeLa cell nuclear extracts is a relevant system for studying regulation of HIV-1 splicing.

A negative SRE is present within the first *tat* coding exon. Current data support the hypothesis that HIV-1 Rev-dependent transport requires inefficient HIV-1 RNA splicing (5, 38). Significant pools of unspliced RNA in the nucleus are present even in the absence of Rev (16, 25, 39). Such inefficient splicing allows the accumulation of unspliced and partially spliced HIV-1 RNAs that are stabilized in the nucleus and transported to the cytoplasm. This process is mediated by the Rev protein, which binds to a sequence within the viral *env* gene referred to as the Rev-responsive element (11, 24, 27, 46, 74). Inefficient splicing of HIV-1 RNA results from the presence of negative *cis*-acting signals in the viral genome.

We have demonstrated that one of the *cis* signals responsible for inefficient HIV-1 RNA splicing in vitro resides in the N-terminal part of the first *tat* coding exon. This sequence is highly conserved in different HIV-1 strains (42). The same results were observed in transiently transfected HeLa cells with an HIV-1 infectious proviral clone from which the SRE was removed (Fig. 5). The NL4-tatC mutant also demonstrated an over-splicing phenotype in chronically infected T cells (5b). In transfected cells, we showed an increase in both single- and double-spliced *tat* mRNAs and this increase in *tat* splicing was concomitant with an overall decrease in the level of unspliced RNA as well as a decrease in *env* mRNA spliced at the major *env* 3′ splice site (site 5) and *rev* mRNA spliced at 3′ splice sites 4a and 4b. These differences in the splicing pattern of the mutant cannot be explained by a deficiency of Rev, since the levels of splicing and transport of unspliced RNA from the nucleus to the cytoplasm were not affected by cotransfection with a cytomegalovirus-driven Rev expression plasmid (61a).

What is the mechanism of the splicing inhibition resulting from the presence of the *tat* SRE? Previous studies of alternatively spliced cellular pre-mRNAs have identified sequences within exons that play roles in determining splicing efficiencies of adjacent splice sites. Most of these exon sequences act positively to facilitate splicing at these splice sites (6–9, 13, 18, 26, 28, 34, 36, 40, 44, 49, 61, 66, 69, 71–73). In some cases, positive exon sequences have been shown to be binding sites for both constitutive and tissue-specific factors (9, 53, 66, 67, 71). Sequences within exons have also been shown to act negatively. For some pre-mRNAs, such as for the β tropomyosin pre-mRNA, this has been attributed to the formation of a secondary structure involving the splice site (35). This secondary structure is thought to inhibit splice site recognition and prevent access to necessary splicing factors. In other cases, such as that of the leukocyte common antigen (CD45) pre-mRNA, tissue-specific splicing of the alternative exons in B and T cells is regulated by exon sequences. Mutations within the exon region resulted in the loss of tissue specificity and constitutive splicing of these exons in both B and T cells (64, 68). Experiments to localize *cis* elements within one of the CD45 alternatively spliced exons (exon 4) indicated that at least three different *cis* elements spanning a large portion of the exon were required to maintain the tissue-specific alternative splicing (64).

The HIV-1 *tat* SRE has the properties of a negative element which only functions in the sense orientation. It acts as an inhibitor of splicing when placed within the exon of a heterologous avian sarcoma virus *src* substrate at the same distance from the upstream 3′ splice site as it is in its natural context in the HIV-1 genome. It is also position dependent and has a reduced effect when it is placed farther from the heterologous 3′ splice site. Since the SRE functions in a heterologous context, it seems unlikely that the results can be explained by a model in which secondary structure interactions between the SRE and the upstream 3′ splice site act to partially "hide" this 3′ splice site, similarly to the model proposed for the chicken β-tropomyosin pre-mRNA (34). It is more likely that the element represents a binding site for a cellular factor or factors. We have found that the level of splicing upstream of the first *tat* coding exon (i.e., at 3′ splice site 3) varies in different human cell lines (11a), suggesting that splicing at this site may be responsive to different levels of cellular factors, one or more of which may bind to the SRE. It is possible that such factors could modulate the level of *tat* mRNA and thus the amount of Tat protein produced by HIV-1.

We noted above that splicing of RNA substrate HS1-6026 at 3′ splice site 3 occurs to some extent, whereas splicing of substrates truncated at nt 5954 and 5934 was almost completely inhibited (Fig. 2). All of these substrates contain the SRE sequence. This result may indicate the presence of a positive-acting region in HS1-6026 which can overcome, to some extent, the inhibitory effect of the SRE. This corresponds to the region of 3′ splice sites 4a, 4b, and 5. Although we do not yet fully understand this phenomenon, we speculate that the binding of splicing factors to these 3′ splice sites may partially negate the effect of the putative SRE binding factor(s). This may be necessary for virus propagation since, by itself, the SRE would exert an overwhelming inhibitory effect on the production of *tat* mRNA.

The 5′ splice site downstream of the first *tat* coding exon acts as a positive splicing signal. We have also shown that the 5′ splice site (site 4) downstream of the first *tat* coding exon acts positively to increase splicing at the upstream 3′ splice sites. When the consensus GU in this splice site was mutated to CG, splicing at all the upstream 3′ splice sites (i.e., 3′ splice sites 3 to 5) was reduced. This suggests that splicing at all the upstream 3′ splice sites (i.e., *tat*, *rev*, and *env*) is facilitated by the presence of splicing factors bound to the 5′ splice site (e.g., U1 small nuclear ribonucleoprotein). It has been proposed that interactions between splicing factors bound at the 3′ splice site and at the downstream flanking 5′ splice site are necessary to define exons in cellular pre-mRNAs (29, 50). A recent study suggests that this definition is caused by stabilizing the binding of the splicing factor U2 auxiliary factor to relatively weak 3′ splice sites (29). We have attempted to study the in vivo behavior of 5′ splice site 4 mutants in the context of the HIV-1 genome. Our preliminary data have indicated that a cryptic 5′ splice site downstream of this mutated 5′ splice site is activated; this has complicated the analysis and precluded direct comparisons of the activities of 5′ splice site 4 in the in vitro and in vivo systems.

Presence of additional splicing elements in the HIV-1 genome. It is very likely that additional SREs are present in the viral genome. First, the steady-state level of *tat* mRNA in vivo is approximately fivefold lower than that of either the *rev* or *env* mRNA (11a). However, the in vitro substrate HS1-6055 was spliced at 3′ splice sites 3, 4, and 5, with comparable efficiencies at each splice site (Fig. 3C). These results may indicate an effect of additional sequences in the HIV-1 genome which are not in the in vitro splicing substrates and which act to regulate splicing in vivo. Alternatively, this result may indicate that in vivo the *tat* mRNA is less stable than the *rev* and *env* mRNAs and that this reason its steady-state level is reduced.

Second, unspliced HIV-1 RNA, although reduced in level, was still present in the *tat* SRE mutant-transfected cells (Fig. 5). In addition, the presence of high levels of single-spliced *tat*

mRNA after inactivation of the *tat* SRE suggests an inefficient removal of the intron between the 5′ splice site flanking *tat* exon 1 (5′ splice site 4) and the 3′ splice site flanking *tat* exon 2 (3′ splice site 7). We have found that extensive deletions in the *gag*, *pol*, and *env* genes have little or no qualitative or quantitative effect on HIV-1 RNA splicing in vivo (11a). These results suggest that HIV-1 may not contain distant elements analogous to the negative splicing element (NRS) present in the *gag* gene of the simple retrovirus, avian sarcoma virus (2, 62), since by analogy with the avian retrovirus RNA, an increase in splicing would be expected if such elements were removed. Elements that cause Rev-responsive expression when inserted into heterologous constructs may correspond to binding sites for splicing factors (37, 45, 54, 60). However, no evidence for NRS activity was obtained when such negative *cis*-repressive elements in *gag* and *pol* were inserted into the intron of a heterologous c-*myc* construct (40a). We suspect that additional *cis*-acting SREs are present in the HIV-1 genome near other 3′ splice sites. Application of the methods described here to other HIV-1 substrates and comparisons of splicing in vivo and in vitro should be useful tools in the identification and characterization of such elements.

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