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Presence of Exon Splicing Silencers within Human Immunodeficiency Virus Type 1 *tat* Exon 2 and *tat-rev* Exon 3: Evidence for Inhibition Mediated by Cellular Factors

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Human immunodeficiency virus type 1 (HIV-1)pre-mRNA splicing is regulated in order to maintain pools of unspliced and partially spliced viral RNAs as well as the appropriate levels of multiply spliced mRNAs during virus infection. We have previously described an element in *tat* exon 2 that negatively regulates splicing at the upstream *tat* 3 splice site 3 (B. A. Amendt, D. Hesslein, L.-J. Chang, and C. M. Stoltzfus, Mol. Cell. Biol. 14:3960–3970, 1994). In this study, we further defined the element to a 20-nucleotide (nt) region which spans the C-terminal *vpr* and N-terminal *tat* coding sequences. By analogy with exon splicing enhancer (ESE) elements, we have termed this element an exon splicing silencer (ESS). We show evidence for another negative *cis*-acting region within *tat-rev* exon 3 of HIV-1 RNA that has sequence motifs in common with a 20-nt ESS element in *tat* exon 2. This sequence is juxtaposed to a purine-rich ESE element to form a bipartite element regulating splicing at the upstream *tat-rev* 3 splice site. Inhibition of the splicing of substrates containing the ESS element in *tat* exon 2 occurs at an early stage of spliceosome assembly. The inhibition of splicing mediated by the ESS can be specifically abrogated by the addition of competitor RNA. Our results suggest that HIV-1 RNA splicing is regulated by cellular factors that bind to positive and negative *cis* elements in *tat* exon 2 and *tat-rev* exon 3.

Alternative splicing of mRNA precursors plays a critical role in the regulation of gene expression. In metazoan cells, splicing of pre-mRNA is mediated by *cis*-acting signals which include 5′ and 3′ splice sites, branchpoint sequences, and polypyrimidine tracts preceding 3′ splice sites (for a review, see reference 19). However, the mechanisms by which alternative splice site selection is regulated are not well understood. There are numerous examples of sequences within introns that act to either enhance or inhibit splicing (3, 7, 10, 16, 21, 25, 35, 40, 43, 65). Some of these intron sequences have been shown to bind cellular factors (21, 35, 40, 43). Exon sequences have also been shown to play a role in alternative splicing. Positive-acting exon sequences and purine-rich regions or exon splicing enhancer (ESE) elements have been reported for a number of different cellular and viral genes (4, 5, 8, 23, 30, 36, 50, 51, 53, 56, 58–60). Some of these positive-acting exon sequences are binding sites for cellular factors (5, 23, 30, 50, 58). A family of factors called SR proteins are required for splicing and, in some cases, have been shown to regulate alternative splice site selection in a concentration-dependent manner (14, 17, 28, 33, 61). Recent reports have shown that the SR proteins selectively bind to purine-rich splicing elements present in cellular exons (30, 49, 50). There are also several examples of negative-acting exon splicing elements (2, 4, 18, 45, 55). To date, factors interacting with negative-acting exon splicing elements affecting alternative 3′ splice site usage in metazoan cells have not yet been reported.

Human immunodeficiency virus type 1 (HIV-1), a complex retrovirus, transcribes its RNA from an integrated proviral genome and utilizes the host cell splicing machinery to produce its mRNAs (for a review, see reference 6). To generate the more than 30 different singly and multiply spliced mRNAs, the HIV-1 9.2-kb primary transcript undergoes splicing by a complex pathway (11, 34, 39, 44) (Fig. 1). The regulatory proteins Tat, Rev, and Nef are encoded by the multiply spliced mRNAs with approximate molecular sizes of 2 kb, whereas the Env, Vif, Vpr, and Vpu proteins are encoded by the singly spliced mRNAs with approximate molecular sizes of 4 kb (Fig. 1). In addition, approximately half of the HIV-1 RNA remains unspliced and is used as the message for *gag* and *pol* gene products. This unspliced RNA is also packaged into progeny virions. The HIV-1 Rev protein, which binds to the *rev*-responsive element located in the *env* gene, ensures that a pool of unspliced and partially spliced RNAs are present in the cytoplasm by facilitating their stabilization and nuclear transport (9, 12, 13, 22, 31, 32). However, prior to the action of Rev, the efficiency of viral RNA splicing at alternative splice sites must be regulated. These regulatory events are only beginning to be understood.

Using both in vitro and in vivo splicing assays, we have shown that a *cis*-acting negative splicing regulatory element present within HIV-1 *tat* exon 2, which we term an exon splicing silencer (ESS), acts to inhibit splicing at the flanking upstream 3′ splice site (2). In this study we localized the element to a 20-nucleotide (nt) sequence in the C-terminal region of the HIV-1 *vpr* and the N-terminal *tat* coding sequences. We present evidence for another ESS element in *tat-rev* exon 3 which is juxtaposed to an element with the properties of an ESE. Finally, we show evidence for a cellular factor or factors that bind to the ESS element and that inhibit splicing at an early step in the splicing reaction.

MATERIALS AND METHODS

Plasmid construction and synthesis of RNA substrates. Plasmid pHS1-X was used as the DNA template for the preparation of splicing substrates and was constructed by the deletion of the infectious HIV-1 clone pNL4-3 (1). Plasmid pHS1-X was cleaved with *Sal*I, *Bsu*36I, *Hin*dIII, and *Xho*I at nt 5785, 5954, 6026, and 6055, respectively, to create linearized templates (HS1-5785, HS1-5954,

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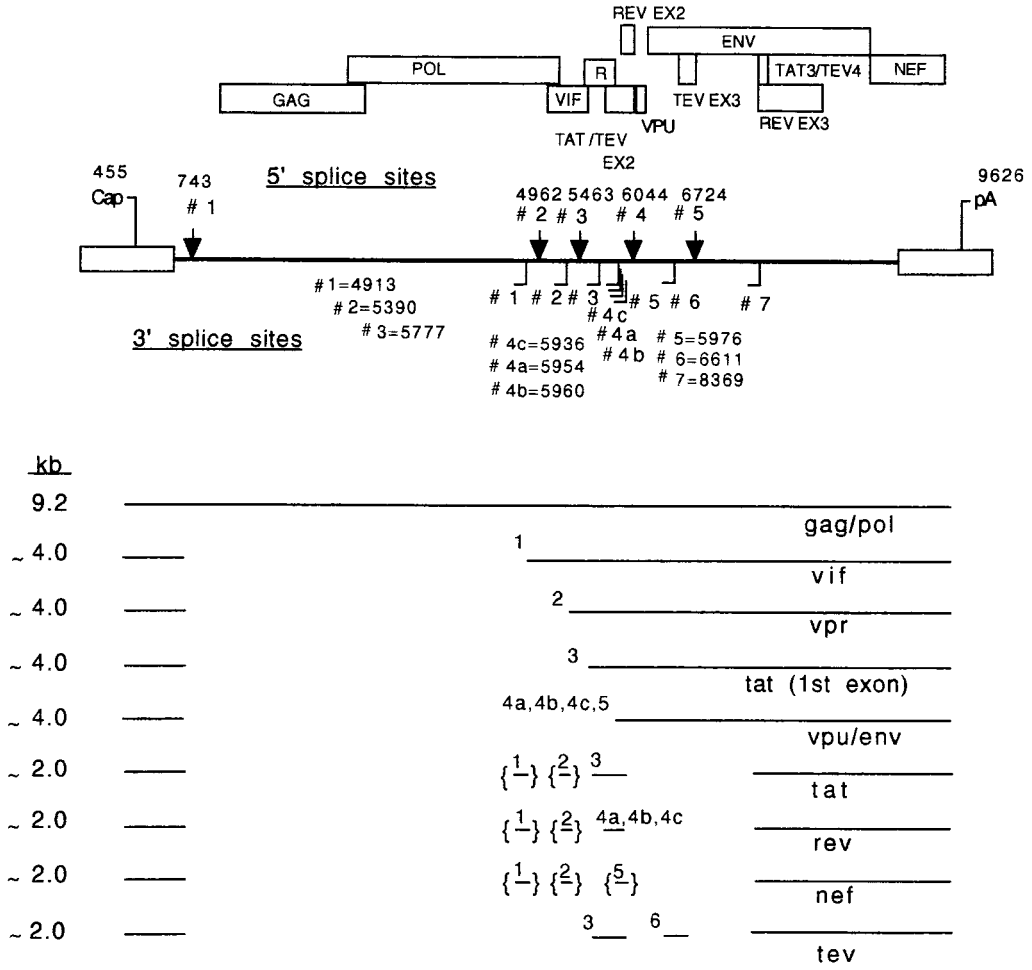
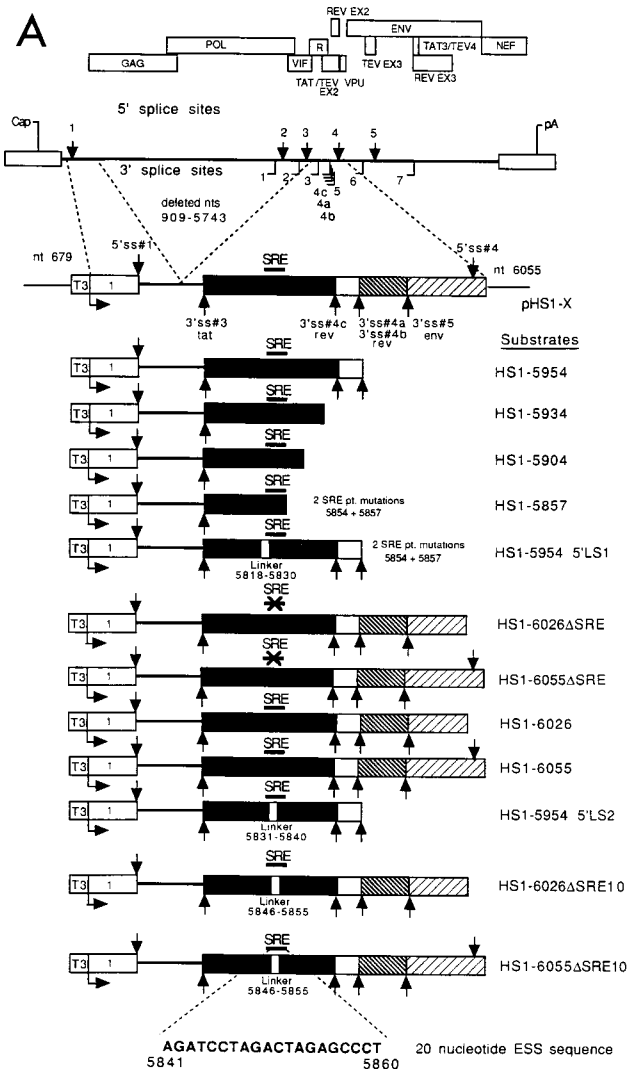


FIG. 1. HIV-1 mRNAs generated from the multiple 5′ and 3′ splice sites. pA, poly(A); R, *vpr*. The nucleotide numbers of the splice sites are shown. In the lower diagram, alternative exons which are present in only some of the mRNAs are shown in braces.

HS1-6026, and HS1-6055) for transcription of RNA splicing substrates (Fig. 2). Substrates HS1-5857 and HS1-5934 were synthesized as described previously (2). Clone pHS1-P3 (2) was cleaved with *Hin*dIII and *Xho*I at nt 6026 and 6055 to make templates for the transcription of substrates HS1-6026ΔSRE and HS1-6055ΔSRE. The capped, [^{32}P]UTP-labeled substrates were synthesized by transcription of the templates with T3 polymerase as previously described (2). Substrate HS1-5954 5′LS1 (Fig. 2A) was synthesized by transcription from a plasmid containing a heterologous DNA linker at nt 5818 to 5830. This plasmid was created by PCR mutagenesis of the plasmid pNL4-tatA (obtained from L.-J. Chang, University of Alberta), which contains two point mutations at nt 5854 (G to T) and nt 5857 (C to G) to create a *Stu*I site (2). The following primers were used: sense, 5′AATTCTGCAACAACTGC3′ (nt 5744 to 5760), and antisense, 5′CCAGGCCTATAGTCTAGGATCTACTGGCTCCAGCAGCTAGCTGAATCTGTCGAGTAACGCC3′ (nt 5862 to 5802). The heterologous DNA linker is underlined at nt 5818 to 5830. This plasmid was linearized with *Bsu*36I at nt 5954. The regions amplified by PCR were confirmed by sequence analysis. Substrate HS1-5954 5′LS2 (Fig. 2A) was linearized with *Bsu*36I at nt 5954 from plasmid pH1-X but contains a heterologous DNA linker at nt 5831 to 5840. This substrate was derived from pHS1-X by PCR mutagenesis with the sense primer 5′TGGGTGTCGACATAGCAGAATAGGCGTTACTCGACAGAGGAGAGCAAGAAACACGCGTGCCAGATCCTAGACTAGA3′ (nt 5780 to 5855) and antisense primer 5′AGAGAAGCTTGATGAG3′ (nt 6035 to 6020). The heterologous DNA linker is underlined at nt 5831 to 5840. Substrates HS1-6026ΔSRE10 and HS1-6055ΔSRE10 were transcribed from plasmid pHS1-X10 with T3 polymerase, which contained a heterologous linker between nt 5846 to 5855, by a two-step megaprimer PCR mutagenesis technique. The first 139-bp PCR product was synthesized with the sense primer 5′CTTGGGCAGGAGTGG3′ (nt 5716 to 5730) and the mutagenic antisense primer 5′GCGTACTTACGATCTACTGGCTCCAT3′ (the base changes deviating from the pNL4-3 sequence are underlined). The first product was denatured and used as the sense primer for the second PCR with the antisense primer 5′ATGGACCACACAAC3′ (nt 6131 to 6118). The second product was cleaved with *Eco*RI (nt 5743) and *Hin*dIII (nt 6026) and cloned into pHS-X, which was also cleaved with *Eco*RI and *Hin*dIII.

Plasmid pHS2, which was used to study regulation of the downstream splicing of 5′ splice site 4 to 3′ splice site 7, was created by the following methods. Two oligonucleotide primers were used to amplify the pNL4-3 sequence from nt 5716 to 6133: sense primer, nt 5716 to 5730, and antisense primer, nt 6133 to 6118. The antisense primer contained a *Pst*I site at its 5′ end. The PCR product was cleaved with *Sac*I and *Pst*I to produce a fragment spanning nt 5999 to 6133. To generate plasmid pHS-SP, this fragment was ligated into BluescriptSK$^+$ (Stratagene, La Jolla, Calif.), which was cleaved with *Sac*I and *Pst*I. To create plasmid pHS-HX, the *Hin*dIII-*Xho*I fragment of pNL4-3 spanning nt 8131 to 8887 was cloned into BluescriptSK$^+$, which was cleaved with *Hin*dIII and *Xho*I. Plasmid pHS-HX was cleaved to generate a 760-nt *Pst*I-*Xho*I fragment. To create pHS2, this fragment was ligated with plasmid pHS-SP, which was cleaved with *Pst*I and *Xho*I.

The competitor RNA used in the splicing competition assays was transcribed from a template (T7TAT118) containing a 118-nt fragment spanning nt 5786 to 5904; the fragment was derived from plasmid pHS1-LN. Plasmid pHS1-LN contains a 30-nt heterologous linker which replaces the HIV-1 sequence from nt 5905 to 5934. DNA containing this linker was synthesized by PCR mutagenesis to create an *Nhe*I site at nt 5904. The antisense oligonucleotide spanned nt 5940 to 5890, 5′ATCCATGG[ACTGAGACTCCATACGATGCGGACGCTAGC]GCAATTGGTACAAGC3′, and the sense oligonucleotide spanned nt 5744 to 5760, 5′AATTCTGCAACAACTGC3′ (the heterologous linker is bracketed and the new *Nhe*I site is underlined). These two oligonucleotides were used as primers for PCR amplification. The PCR product was cleaved with *Eco*RI and *Nco*I and ligated into HS1-5934 (2), previously cleaved with *Eco*RI and *Nco*I to create pHS1-LN. This plasmid was cleaved with *Sal*I at nt 5785 and *Nhe*I at nt 5903 to obtain the 118-nt fragment containing the ESS element. This fragment was ligated into BluescriptSK$^+$, which was cleaved at the *Sal*I and *Xho*I sites in the multiple cloning site. This plasmid (T7TAT118) was linearized with *Eag*I,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nt 679
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HS1-5904
HS1-5857
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Linker
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HS1-6026ΔSRE
HS1-6055ΔSRE
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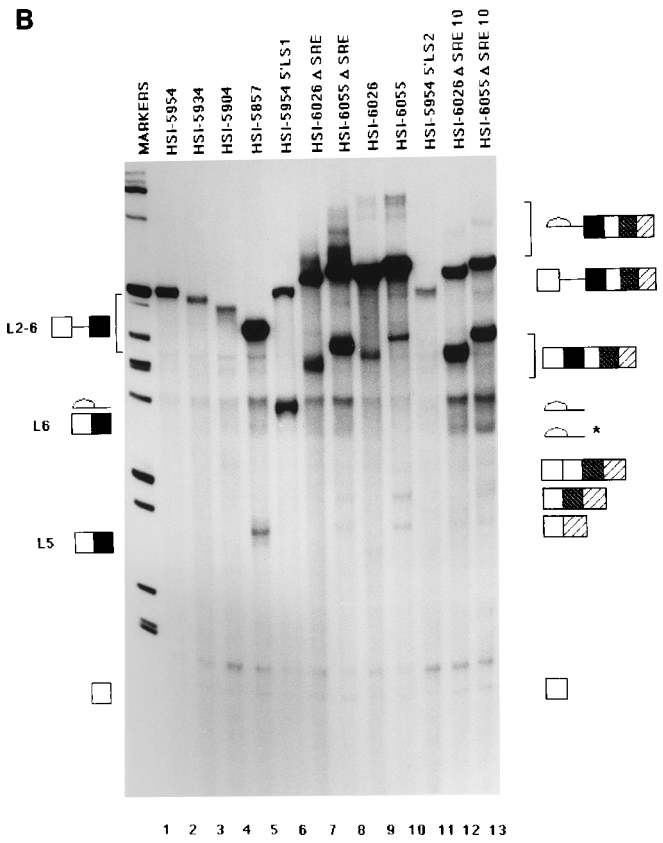
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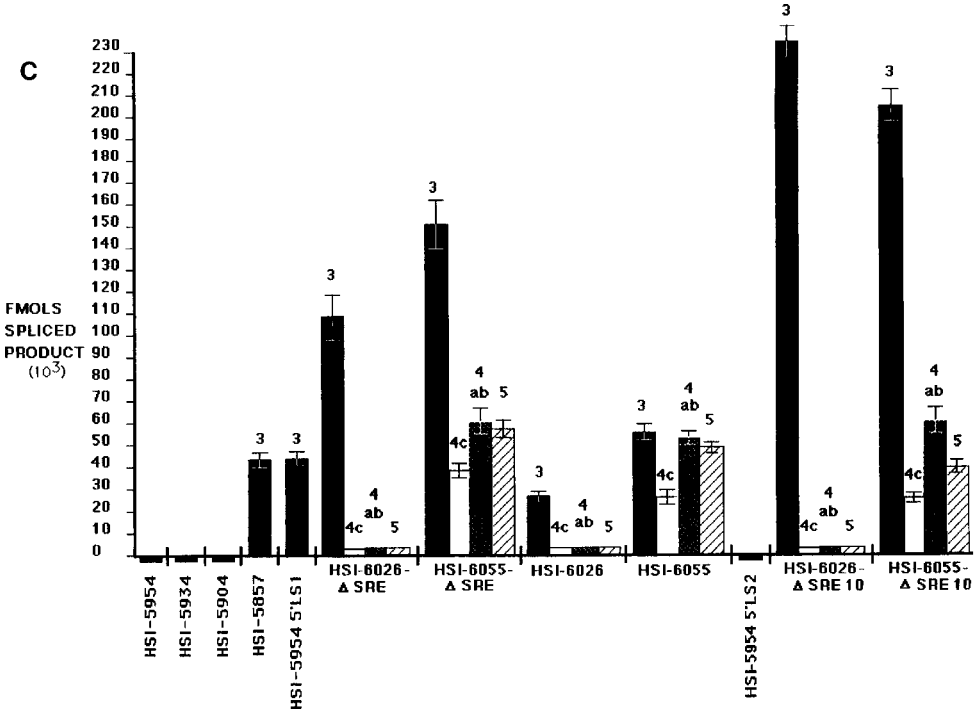
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Δ SRE 10

and the competitor RNA was transcribed with T7 RNA polymerase. The control RNA used in the splicing competition assays was derived by transcription of a plasmid which contained the HIV-1 sequence from nt 5821 to 5860 in which the HIV-1 sequence from 5821 to 5860 was replaced with a 10-bp heterologous linker. This was carried out by annealing two oligonucleotides: 5′CATGGGAG CAAGAAATGGAGCCAGTAGATCACTCGTACGCGCCCTA3′ and 5′CA TGTAGGGCGCGTACGAGTGATCTACTGGCTCCATTTCTTGCTCC3′ (the heterologous linkers are underlined). The hybridized oligonucleotides, containing sticky *Nco*I ends, were ligated into pGEM5Zf$^+$ (Pro-mega, Madison, Wis.), which was cleaved with *Nco*I. This plasmid, T7TAT46Δ, was linearized with *Eco*RV, and the 90-nt RNA was transcribed with T7 polymerase.

In vitro RNA splicing and gel electrophoresis of splicing reaction products. Preparation of HeLa cell nuclear extracts and RNA splicing reactions were performed as previously described (2). The in vitro splicing competition assays were modified from this procedure with 13 μl of nuclear extract and 1 μl of nonradioactive competitor or control RNA at various concentrations. The splicing reaction mixtures were preincubated at for 15 min at 30°C in the presence of cold competitor or control RNAs. After 15 min, labeled substrates were added and the splicing reaction mixture was incubated at 30° for 2 h.

Analysis of splicing complexes. Analysis of splicing complexes was carried out essentially as described by Konarska (27). The reaction mixtures were incubated under splicing conditions for various times and then placed on ice prior to separation of complexes on 4% polyacrylamide gels.

RESULTS

The HIV-1 *tat* exon 2 contains a 20-nt element that acts to inhibit splicing at the 3 splice site flanking *tat* exon 2. We previously detected a *cis*-acting negative splicing regulatory element in *tat* exon 2. To further define the element affecting splicing within this region, we synthesized substrates with linker-scanner mutations as well as substrates with various 3′-end truncations (Fig. 2A). Truncated substrates HS1-5954, HS1-5934, and HS1-5904 were spliced in vitro at 3′ splice site 3 very inefficiently (Fig. 2B, lanes 2 to 4). Substrate HS1-5857 contains two point mutations at nt 5854 and 5857 which caused a small activation of splicing (Fig. 2B, lane 5, and Fig. 2C). These experiments suggested that the 3′ end of the splicing element was upstream of nt 5860. This is also consistent with our previous data showing that the insertion of a region from nt 5821 to 5860 into a heterologous exon was sufficient to inhibit splicing at the upstream 3′ splice site (2). The necessity of this region was confirmed in our study with substrates HS1-6026ΔSRE and HS1-6055ΔSRE, which have deletions between nt 5821 and 5860 and which contain a heterologous linker insertion (Fig. 2B, lanes 7 and 8). Compared with the wild-type substrates HS1-6026 and HS1-6055, these substrates demonstrated a three- to fourfold increase in splicing at the *tat* 3′ splice site (Fig. 2B, lanes 9 and 10, and Fig. 2C). To more precisely define the 5′ endpoint of the splicing element, we constructed two linker-scanner mutations in this region. Substrate HS1-5954 5′LS1 contains a linker between nt 5818 and 5830 and has the same two point mutations at nt 5854 and 5857 as HS1-5857 (Fig. 2A). (The latter two mutations created a *Stu*I site and were present to facilitate cloning of the template.) Substrate HS1-5954 5′LS1 produced a molar amount of spliced *tat* product comparable to that of HS1-5857 (compare Fig. 2B, lanes 5 and 6, and Fig. 2C). Thus, we concluded that the linker in HS1-5954 5′LS1 did not impinge upon the splicing element. Substrate HS1-5954 5′LS2 contains a heterologous linker between nt 5831 and 5840 (Fig. 2A). This substrate does not contain the two point mutations at nt 5854 and 5857. Splicing of this substrate at the *tat* exon 2 3′ splice site was almost completely inhibited (Fig. 2B, lane 11). Thus, the 5′ endpoint of the element required for the inhibition of splicing appeared to be distal to the linker in HS1-5954 5′LS2, i.e., downstream of nt 5840. On the basis of these experiments, we concluded that the sequence necessary and sufficient for inhibition of splicing was within the 20-nt region between nt 5841 and 5860. By analogy with current nomenclature for exon splicing elements, we have termed this region an ESS element. This is a highly conserved region of the HIV-1 genome which spans the C-terminal *vpr* and N-terminal *tat* coding sequence (Fig. 3A).

To confirm that the core of the ESS element was necessary for inhibition, the region between nt 5846 and 5855 was deleted and replaced with a heterologous sequence (HS1-6026ΔSRE10 and HS1-6055ΔSRE10) (Fig. 2A). These substrates were tested for splicing activity (Fig. 2B, lanes 12 and 13, and Fig. 2C). Consistent with removal of the ESS element, the level of splicing at the *tat* exon 2 3′ splice site was four- to eightfold greater than that of wild-type substrates HS1-6026 and HS1-6055 (Fig. 2C).

Using substrate HS1-6055ΔSRE10, we also observed spliced products arising from the use of 3′ splice sites 4c, 4ab, and 5. These splice sites are used to generate messages for *rev*, *nef*, and *env* gene products (Fig. 1). The amounts of spliced products arising from the use of these 3′ splice sites were similar to those arising from substrates HS1-6055 and HS1-6055ΔSRE (Fig. 2B, lanes 8, 10, and 13, and Fig. 2C). This was not surprising since these substrates all contain the naturally occurring downstream 5′ splice site 4, which we have previously shown acts as a positive *cis* element to facilitate splicing at the upstream 3′ splice sites 4c, 4ab, and 5 (2). These data were consistent with the exon definition model, as proposed by other investigators (24, 38).

Evidence for an ESS element in *tat-rev* exon 3 juxtaposed to an ESE element. Having defined the ESS element within HIV-1 *tat* exon 2 to a 20-nt sequence, we searched the HIV-1 genome for regions with similar sequence motifs (Fig. 3B). Interestingly, these motifs were also present downstream of other HIV-1 3′ splice sites. A region with sequence homology to *tat* exon 2 was located approximately 70 nt downstream of 3′

FIG. 2. The negative regulatory element downstream of HIV-1 *tat* 3′ splice site 3 consists of a defined 20-nt ESS. (A) Organization of the HIV-1 minigene substrates. Substrate numbers indicate sites where runoff transcripts are truncated. R, *vpr*; pA, poly(A); ss, splice site; T3, T3 polymerase. SRE defines the region (nt 5821 to 5860) which was previously shown to contain a splicing regulatory element (2). Substrates HS1-5857 and HS1-5954 5′LS1 contain two point mutations at nt 5854 and 5857 located at the 3′ end of the ESS. Substrate HS1-5954 5′LS1 also has a heterologous linker replacing HIV-1 nt 5818 to 5830, and HS1-5954 5′LS2 contains a linker replacing nt 5831 to 5840. Substrates HS1-6026ΔSRE and HS1-6055ΔSRE have been described previously (2). Substrates HS1-6026ΔSRE10 and HS1-6055ΔSRE10 contain a 10-nt heterologous linker replacing the HIV-1 sequence between nt 5846 and 5855. (B) Analysis by denaturing polyacrylamide gel electrophoresis of [^{32}P]UTP-labeled HIV-1 substrates spliced in vitro. Structures of the RNAs are illustrated on each side of the autoradiogram (L, lane). The starred intron lariat indicates another form of the lariat intermediate derived from splicing to 3′ splice site 3, which has previously been reported for beta-globin pre-mRNA (41). All lariat intermediates were identified by characteristic mobility shifts on gels containing different concentrations of polyacrylamide. Markers are derived from *Hin*fI-cleaved pBR322 (Bethesda Research Laboratories, Gaithersburg, Md.) (sizes, 134, 154, 201, 220, 298, 344, 396, and 510 nt, from bottom to top). Lanes 2 through 13 contain the HIV-1 substrates (indicated at the top) incubated in HeLa cell nuclear extracts for 2 h under standard splicing conditions. This figure shows the results of an experiment in which 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 an AMBIS phosphoImager. The amounts of products were calculated on the basis of the uridine content of the RNA species. The individual 3′ splice site used to generated 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 with 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 (20, 42). Splice site 4c is used infrequently in vivo (37) and is detected upon longer autoradiographic exposures. The amounts of products after a 2-h reaction did not change significantly when the amounts of substrate were increased threefold.

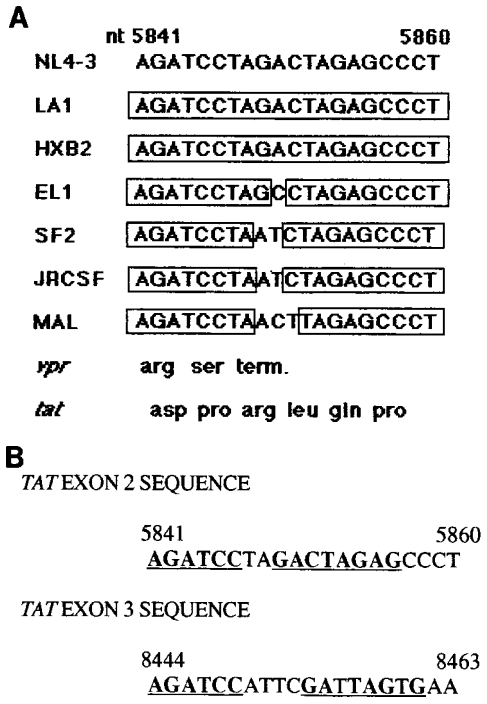

FIG. 3. (A) Sequence homologies of the ESS element in *tat* exon 2 in several divergent HIV-1 strains. The boxed sequences indicate homology to HIV-1 strain pNL4-3; the amino acids encoded by *vpr* and *tat* are shown in lowercase letters. term., termination codon. (B) Nucleotide sequence of a potential ESS element in *tat* exon 3. This is compared with the ESS sequence in *tat* exon 2. The nucleotide numbers of the pNL4-3 sequence are shown. The underlined sequences are regions of homology between the two elements (identical residues are in boldface).

splice site 7 within *tat-rev* exon 3 (Fig. 3B). The ESS element in *tat* exon 2 is approximately 65 nt downstream of 3′ splice site 3. Thus, the homologous sequence is similarly positioned downstream of 3′ splice site 7. To test whether this exon sequence regulated the upstream 3′ splice site, appropriate substrates containing 5′ splice site 4, a large deletion of the HIV-1 sequence, 3′ splice site 7, and exon sequences truncated at various sites were synthesized (Fig. 4A and Materials and Methods). Substrate HS2-8383, which was truncated 15 nt downstream of 3′ splice site 7, was spliced very inefficiently (Fig. 4B), and little or none of the expected 70-nt spliced product was seen in the exposure shown in Fig. 4B. A small amount of lariat intermediate was detected, indicating that the splicing of HS2-8383 was occurring at low levels (Fig. 4B, lane 2). Upon longer autoradiographic exposures, we could detect a small amount of the 70-nt spliced product. The inefficient splicing of this substrate is unlikely to be a consequence of the small size of the exon (15 nt) downstream of the 3′ splice site, since an HIV-1 substrate, HS1-5785, which was truncated in *tat* exon 2 only 9 nt downstream of *tat* 3′ splice site 3, was spliced efficiently under the same conditions (2). Furthermore, we found that a substrate which was truncated 97 nt downstream of 3′ splice site 7 and in which heterologous plasmid sequence was substituted downstream of nt 8383 was spliced with the same low efficiency as that of HS2-8383 (data not shown). In addition to showing that the low efficiency of splicing at 3′ splice site 7 was not due to the small size of the exon, these results also indicated that *tat* 3′ splice site 3 is an inherently stronger splice site than *tat-rev* 3′ splice site 7.

We next tested a substrate truncated at nt 8465 (substrate HS2-8465). This substrate was spliced approximately 10-fold more efficiently than HS2-8383 (Fig. 4B, lane 3, and Fig. 4C). Interestingly, the sequence between nt 8383 and 8365 contains a purine-rich region that is immediately upstream of the putative ESS element. Such polypurine regions have been implicated as ESE elements affecting upstream 3′ splice sites (Fig. 4 and Discussion). To separate the effect of the purine-rich region from the ESS element, we synthesized substrate HS2-8444, which was truncated upstream of the putative ESS element (Fig. 4A). This substrate contains the ESE element but lacks the ESS region. The splicing of this substrate was further increased at least 20-fold compared with the level of splicing of HS2-8383, which lacks both elements, and was increased approximately 2- to 3-fold compared with the level of splicing of HS2-8465, which contains the ESS element (Fig. 4B, lane 4, and Fig. 4C). To confirm this, we tested an HIV-1 substrate with a length similar to that of HS2-8444 but which had the ESE element deleted as defined in vivo by the studies of Staffa and Cochrane (48). It was spliced with the same low efficiency as HS2-8383 (data not shown). Our data therefore suggest that for efficient splicing to occur at 3′ splice site 7, the purine-rich or ESE element is required. However, the positive effect of this element is attenuated by the sequence immediately downstream of the ESE (Fig. 4A). These data are therefore consistent with the presence of an ESS in *tat-rev* exon 3 which is juxtaposed to an ESE to form a bipartite splicing element.

Inhibition of splicing at HIV-1 *tat* 3 splice site 3 occurs before the formation of a functional spliceosome. To elucidate the mechanism by which the ESS elements act to inhibit splicing, we determined at which step spliceosome assembly is inhibited by the ESS in *tat* exon 2. For this study, two substrates were compared: HS1-5785, which lacks the ESS, and HS1-5934, which contains the ESS. As we have previously shown (2), HS1-5785 was spliced efficiently at 3′ splice site 3 but HS1-5934 was not detectably spliced. (Fig. 2B, lane 3). The lack of splicing intermediates which accumulate in reaction mixtures with substrates containing *tat* 3′ splice site 3 and the downstream ESS element suggested that their splicing may be blocked at an early step, before 5′ exon cleavage and lariat formation. To obtain further information about the step of the splicing reaction which was inhibited, the two substrates were assayed for splicing complex formation on native polyacrylamide gels by the method of Konarska (27) (Fig. 5). With substrate HS1-5785, prespliceosome complex A was apparent after 15 min and spliceosome complex B was detectable after 30 min of reaction (Fig. 5, lanes 1 to 7). On the other hand, substrate HS1-5934 did not form functional spliceosome complexes (Fig. 5, lanes 8 to 14). The amount of substrate in complexes also decreased with increasing times of reaction. Thus, the inhibition of splicing caused by the presence of the ESS appeared to take place at an early step, prior to complex B formation in the splicing reaction. The rapid disappearance of RNA substrates containing the ESS suggests that if the pre-mRNA transcript is not recognized by the splicing machinery and engaged in splicing, it undergoes degradation.

Restoration of efficient splicing of HIV-1 substrates by addition of competitor RNA containing the ESS element. We next determined whether the inhibition of splicing mediated by the ESS was caused by the binding of a negative-acting cellular factor or factors. We reasoned that, if this was the case, the inhibition should be specifically abrogated by the addition of competitor RNA containing the ESS. The efficiency of splicing at *tat* 3′ splice site 3 after the addition of competitor RNA was analyzed with substrate HS1-6026, which contains the ESS element. This substrate, even though it contains the ESS, is spliced with low but detectable efficiency at *tat* 3′ splice site 3

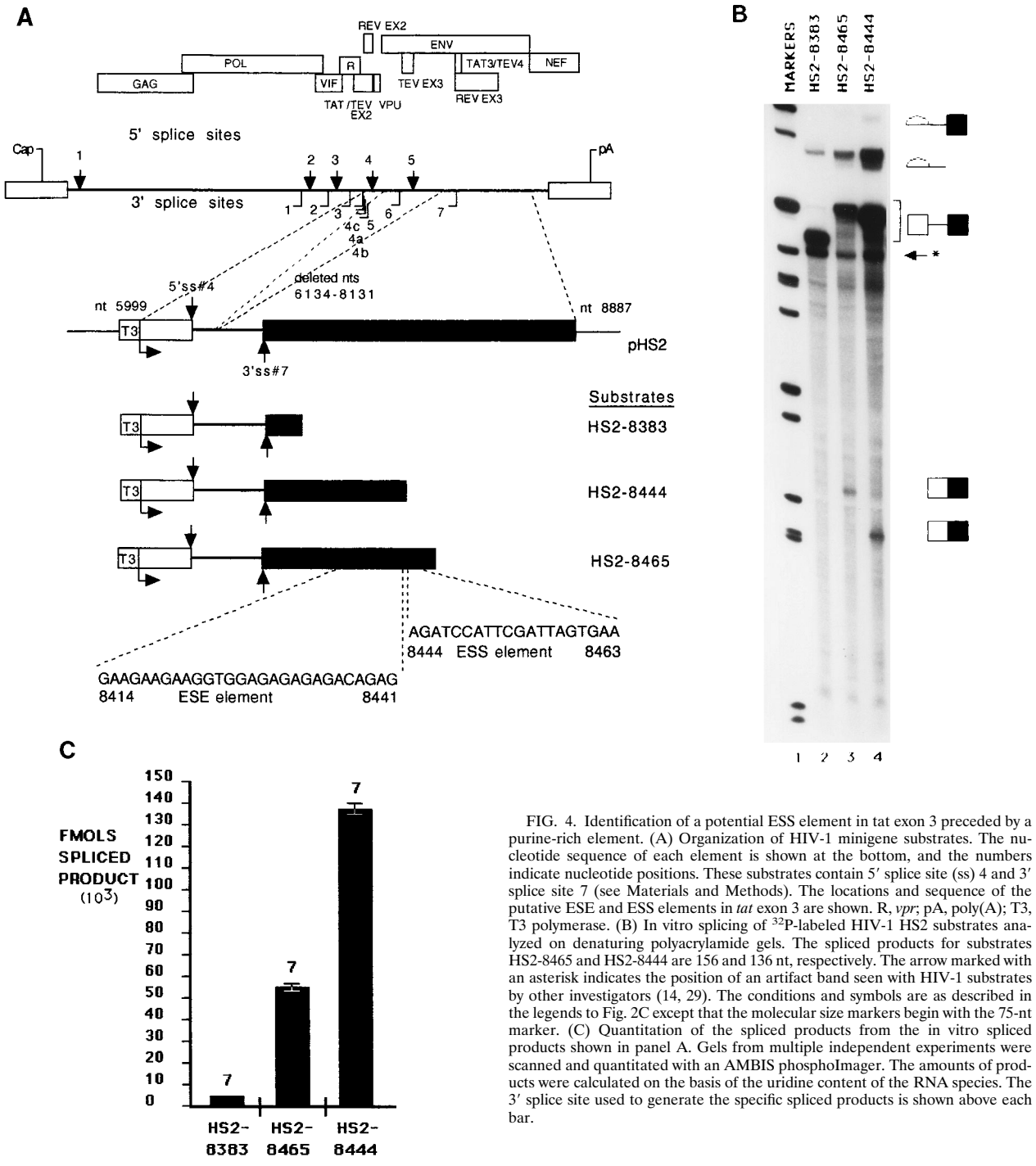

FIG. 4. Identification of a potential ESS element in tat exon 3 preceded by a purine-rich element. (A) Organization of HIV-1 minigene substrates. The nucleotide sequence of each element is shown at the bottom, and the numbers indicate nucleotide positions. These substrates contain 5′ splice site (ss) 4 and 3′ splice site 7 (see Materials and Methods). The locations and sequence of the putative ESE and ESS elements in *tat* exon 3 are shown. R, *vpr*; pA, poly(A); T3, T3 polymerase. (B) In vitro splicing of ^{32}P-labeled HIV-1 HS2 substrates analyzed on denaturing polyacrylamide gels. The spliced products for substrates HS2-8465 and HS2-8444 are 156 and 136 nt, respectively. The arrow marked with an asterisk indicates the position of an artifact band seen with HIV-1 substrates by other investigators (14, 29). The conditions and symbols are as described in the legends to Fig. 2C except that the molecular size markers begin with the 75-nt marker. (C) Quantitation of the spliced products from the in vitro spliced products shown in panel A. Gels from multiple independent experiments were scanned and quantitated with an AMBIS phosphoImager. The amounts of products were calculated on the basis of the uridine content of the RNA species. The 3′ splice site used to generate the specific spliced products is shown above each bar.

(Fig. 2B). We have previously attributed this to the presence of the downstream *rev* and *env* 3′ splice sites in this substrate, which partially reverse the negative effect of the ESS (2). The splicing efficiency of HS1-6026, as determined by amounts of spliced product, was significantly increased by the addition of 100- to 700-fold molar excesses of cold competitor RNA (Fig. 6, lanes 9 to 13). In contrast, the addition of control RNA at the same concentration had no effect on the splicing of this substrate (Fig. 6, lanes 14 and 15). The amount of product spliced at *tat* 3′ splice site 3 in substrate HS1-6026ΔESS, which lacks the ESS element, was unaffected by the addition of either competitor or control RNA (Fig. 6, lanes 1 to 8). In addition to the increase in spliced product obtained upon the addition of competitor RNA to substrate HS1-6026, an increase in the level of *tat* 3′ splice site 3-specific exon 2-intron lariat intermediate was also observed in the presence of excess competitor RNA (Fig. 6, lanes 12 and 13). This lariat intermediate is the most rapidly migrating of the lariat exon intermediate species detectable with mobilities lower than that of the unreacted substrate band. The more slowly migrating lariat exon species

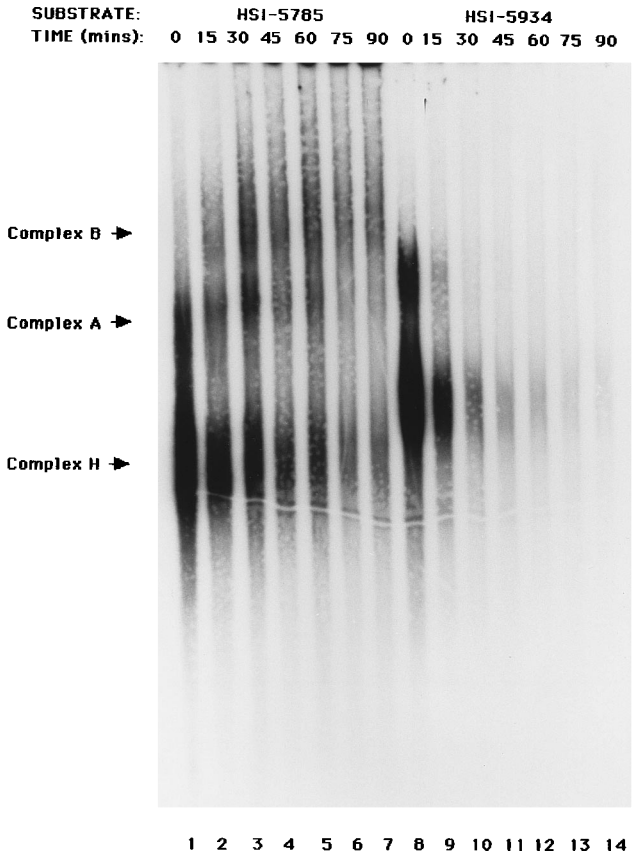

FIG. 5. Inhibition of spliceosome complex formation by the HIV-1 ESS element. Substrates were incubated for the indicated times under previously reported splicing conditions (2). The complexes were analyzed on a 4% native polyacrylamide gel. HS1-5785 does not contain the ESS element (lanes 1 to 7), and HS1-5934 does contain the ESS element (lanes 8 to 14).

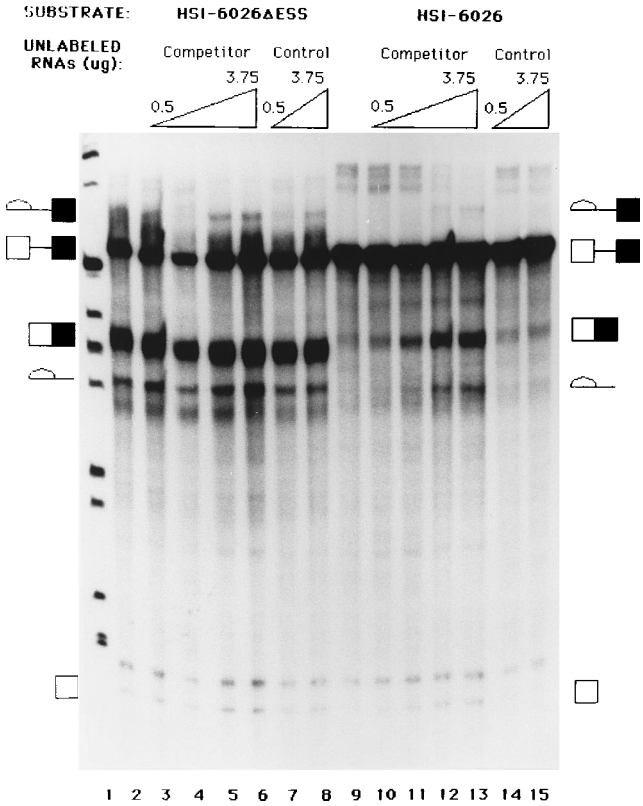

FIG. 6. The increase in splicing of substrate HS1-6026 at 3′ splice site 3 shows that the HIV-1 ESS element interacts with a cellular factor. In vitro splicing assays were carried out in the presence of either a competitor RNA (T7TAT118) containing the ESS or control RNAs (T7TAT46Δ) without the ESS. Substrate HS1-6026ΔESS does not contain the ESS, and HS1-6026 does contain the ESS. The substrates were spliced for 2 h after a 15-min preincubation with the indicated concentrations of nonradioactive competitor or control RNA at 30°C. The spliced products were analyzed on a 4% denaturing polyacrylamide gel.

correspond to products of first-step splicing reactions at the downstream 3′ splice sites 4a, 4b, 4c, and 5. The faster-migrating intron-exon 2 lariat RNA was the most prominent intermediate in the products of substrate HS1-6026ΔESS, and this correlates with the increase in splicing at *tat* 3′ splice site 3 when this substrate is used (Fig. 6, lanes 2 to 5).

The change in the amounts of spliced product obtained in the presence of increasing amounts of competitor RNA was quantitated by beta scanning of the gels (Table 1). These data showed that the ESS-containing substrate was spliced with an approximate threefold increase in splicing at the *tat* 3′ splice site when assayed in the presence of excess competitor RNA. However, the large molar excesses of competitor RNA required to restore splicing suggest either that the ESS factor binds with relatively low affinity or that it is present at a relatively high concentration. Table 1 shows that the efficiency of splicing of the substrate without the ESS was not significantly affected by the addition of increasing amounts of competitor RNA. The results suggested that the HIV-1 *tat* exon 2 ESS element specifically interacts with a cellular factor or factors and that this is responsible for the negative regulation of HIV-1 *tat* pre-mRNA splicing.

DISCUSSION

cis-acting splicing regulatory elements in HIV *tat* exons 2 and 3: evidence for a bipartite splicing element in *tat* exon 3. HIV-1 RNA splicing has been shown to be regulated by several mechanisms. First, splicing is negatively regulated by the presence of suboptimal 3′ splice sites. Staffa and Cochrane have reported that 3′ splice site 7, which flanks the *tat-rev* exon 3, has both a suboptimal branchpoint sequence and the polypyrimidine tract (47). Our unpublished results have indicated that 3′ splice site 4, which flanks *tat* exon 2, also has a suboptimal polypyrimidine tract and that splicing at this site can be increased by mutations of interspersed purines in the tract to pyrimidines (2a). Regulation by inefficient splice sites in HIV-1 appears to be analogous to the mechanism reported previously

TABLE 1. Quantitation of spliced *tat* RNA from the in vitro splicing competition assays

Substrate	fmol (10^3) of spliced product from assays with[a]:							
	No RNA	Competitor RNA (μg)					Control RNA (μg)	
		0.5	1.0	1.5	2.5	3.75	0.5	3.75
HS1-6026ΔESS	164	180	ND[b]	150	175	162	149	174
HS1-6026	33	52	ND	62	81	93	28	34

[a] The spliced *tat* RNA band was quantitated and expressed as described in the legend to Fig. 2C.
[b] ND, not determined.

to inhibit splicing at the Rous sarcoma virus *env* and *src* gene 3′ splice sites (26, 64).

In addition to the presence of inefficient splice sites, HIV-1 RNA splicing is regulated by the presence of positive and negative exon elements. We have previously demonstrated by both in vivo and in vitro splicing assays the presence of an element within HIV-1 *tat* exon 2 that acts to inhibit splicing at the flanking upstream 3′ splice site (3′ splice site 3). In this study, we have further localized the *cis*-acting negative element to a 20-nt ESS element that contains the C-terminal coding sequence of the HIV-1 *vpr* gene and the N-terminal sequence of the *tat* gene. A sequence comparison of a number of divergent strains of HIV-1 revealed that this region of the genome contains highly conserved nucleotide sequences (Fig. 3A). A similar ESS element may be present downstream of 3′ splice site 7 within the second *tat-rev* coding exon (*tat-rev* exon 3). It has sequence motifs in common with the *tat* exon 2 element and is approximately the same distance downstream of the 3′ splice site. The AGATCC motif in the ESS within *tat* exon 2, which is conserved in all the HIV-1 strains, is also highly conserved in the putative ESS element within *tat-rev* exon 3 (Fig. 3B). The downstream motif of the *tat* exon 2 ESS (GATAGAG) is less conserved in the *tat-rev* exon 3. Consistent with its role as a splicing silencer, the removal of the region containing the ESS element in *tat-rev* exon 3 resulted in a two- to threefold enhancement of splicing at 3′ splice site 7.

In addition to the putative ESS element within *tat-rev* exon 3, the exon also contains a purine-rich sequence with several GAR sequence motifs (Fig. 4A). These elements have been shown to be present in several alternatively spliced cellular exons (4, 8, 30, 58, 59) and in the Rous sarcoma virus *env* gene (15, 26, 51). They have been shown to act as ESE elements. Consistent with a role as a splicing enhancer, removal of the region containing the purine-rich sequence from *tat-rev* exon 3 resulted in an approximate 10-fold decrease in splicing efficiency. Thus, it appears from these data that HIV-1 *tat-rev* exon 3 contains contiguous positive and negative elements that form a bipartite splicing element. Results showing the existence of contiguous positive purine-rich and negative splicing elements in the *tat-rev* exon 3 have also been obtained in vivo with chimeric constructs containing a beta-globin 5′ splice site, the HIV-1 *tat-rev* 3′ splice site, and part of exon 3 (48). Recently, the EDA exon, which is skipped during splicing of human fibronectin RNA in some cell types, has also been shown to contain a bipartite element in which a negative regulatory sequence is preceded by a splicing enhancer (4). The sequence of the negative modulator in the EDA exon is CAAGG, which does not appear to have any homology to the HIV-1 ESS elements (Fig. 3). As we showed for the HIV-1 *tat* exon 3 element, the activity of the negative modulator in the EDA exon element is recessive to the activity of the positive element (4). Thus, the arrangement of contiguous positive and negative elements may be common to a number of alternatively spliced genes. The significance of such an arrangement for the regulation of splicing of HIV-1 RNA is not yet clear.

The activities of the HIV-1 exon elements may require suboptimal signals at the regulated upstream 3′ splice sites. Staffa and Cochrane have shown that the positive activity of the *tat-rev* exon 3 ESE can be compensated for by mutations that improve the branchpoint or polypyrimidine tract of the upstream *tat-rev* 3′ splice site 7. In addition, the negative activity of the exon 3 ESS requires a suboptimal branchpoint sequence at the upstream 3′ splice site, but mutants in which the polypyrimidine tract is optimized are still regulated (48). Consistent with these data, we have found that inhibition by the *tat* exon 2 ESS element still occurs, albeit to a somewhat lesser extent, when the polypyrimidine tract of the upstream *tat* 3′ splice site 3 is mutated to consensus (2a).

Mechanism of inhibition by the ESS element in *tat* exon 2. We have previously shown that the ESS sequence in *tat* exon 2 functions as a splicing inhibitor when placed downstream of a heterologous 3′ splice site (2). On the basis of these data, we hypothesized that this element represents a binding site for a negative-acting cellular factor(s) in HeLa cell nuclear extracts. We have shown additional evidence in this report for the existence of such factors. The addition of competitor RNA containing the ESS to splicing reaction mixtures with HIV-1 RNA substrates containing the ESS caused a specific increase in splicing at the upstream 3′ splice site 3. It was found, however, that even at the highest concentration of the specific competitor RNA, splicing was not fully restored to the levels of control substrates lacking the ESS even after preincubation at the highest levels of competitor RNA (Table 1). This suggests either that this factor(s) is bound with relatively low affinity to the ESS-containing RNA or that it is present in relatively high concentrations in the extract.

Only a few other examples of sequences within exons that bind factors which negatively regulate splicing have been reported. One such factor is the ribosomal protein L32 of *Saccharomyces cerevisiae*, which binds to the first exon of its own gene. Splicing of the gene is inhibited at an early step subsequent to U1 small nuclear ribonucleoprotein (snRNP) binding but prior to the association with U2 snRNP, whose binding is necessary to form a complete spliceosome (57). Another example is the regulation of *Drosophila* P-element transposase, in which somatic factors bind to the exon upstream of the 5′ splice site. These factors act to block U1 snRNP binding by stabilizing U1 snRNP binding to an inactive pseudo-5′ splice site (46). The HIV-1 *tat* exon 2 ESS element-binding factor(s) that we have described in this report is, to our knowledge, the first example of negative regulation by factors binding to exons downstream of the 3′ splice site.

Evidence is accumulating for a number of sequences within exons downstream of 3′ splice sites that act to enhance splicing by binding cellular factors. First, positive regulation of *doublesex* (*dsx*) female-specific pre-mRNA splicing is mediated by the binding of Tra and Tra-2 factors to an element (*dsx* responsive element [RE]) within the exon downstream of the female-specific 3′ splice site (23, 36, 52). It has been proposed that this effect is caused by stabilizing the binding of SR proteins to the *dsx* RE (54). The purine-rich splicing enhancers, which we have described above, have been found to be present in the exons of a number of other alternatively spliced genes (4, 8, 30, 58, 59). These elements have also been shown to preferentially bind to the SR proteins (30, 49, 50). It has been proposed, in the cases of both *dsx* RE and the purine-rich element, that binding of SR proteins enhances prespliceosome complex A formation by facilitating binding of the essential splicing factor U2AF (49, 50, 54).

Interestingly, the ESS elements share a number of properties with ESE elements. Both the ESE and the *tat* ESS elements are functional within exons only in the sense orientation, and they work more effectively when positioned close to the 3′ splice site (2, 30, 54). As discussed above, the activities of both types of elements appear to require suboptimal 3′ splice sites. Both types of elements can be transferred to heterologous exons, where they regulate the splicing of the upstream heterologous 3′ splice sites (2, 51, 58). Both elements also bind cellular factors; the ESE elements preferentially bind SR proteins (30, 49, 50, 54). Our preliminary data from UV crosslinking assays have indicated that at least one nuclear protein is selectively bound to ESS-containing RNA; whether this pro-

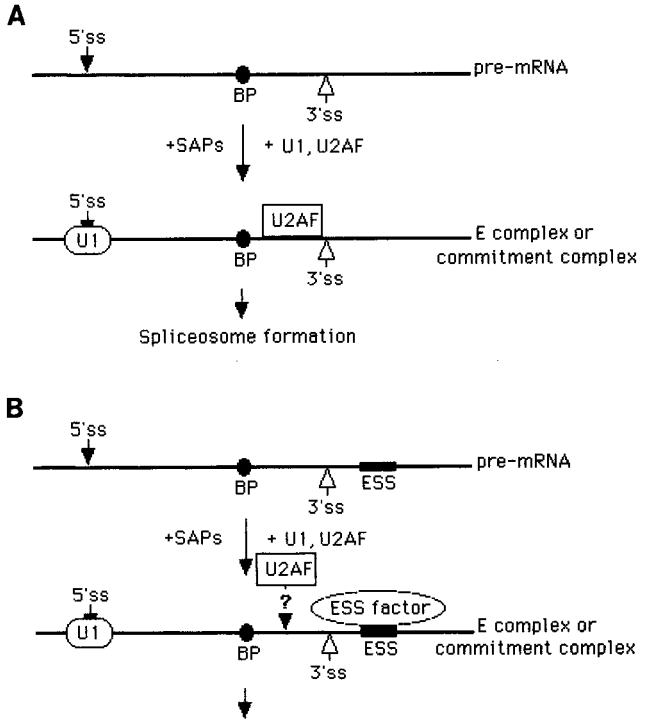

FIG. 7. Model for the inhibition of early events in pre-mRNA splicing by binding of the ESS factor to the HIV-1 *tat* exon 2 ESS element. BP, branchpoint; ss, splice site; SAPs, spliceosome-associated proteins. (A) Early spliceosome assembly of a pre-mRNA transcript. (B) Inhibition of early spliceosome assembly in an ESS-containing pre-mRNA transcript. The model proposes that the ESS factor binds to the ESS, prevents formation of a mature spliceosome, and leads to the rapid degradation of ESS-containing transcripts.

tein corresponds to the inhibitory factor is currently under investigation.

How would such factors act negatively to inhibit splicing at the 3′ splice site early in the splicing reaction? It has been proposed that binding factors which enhance splicing act by increasing the affinity of essential splicing factors early in spliceosome assembly (30, 49, 54). These models have proposed increased U2AF binding, since binding of this factor to the polypyrimidine tract upstream of the 3′ splice site is essential for the formation of the earliest ATP-dependent prespliceosome, complex A (62, 63). As a working model, we propose the mechanism shown in Fig. 7. In this model, the ESS-binding factor binds to the exon ESS element and inhibits the formation of functional spliceosomes. The ESS-containing RNA substrates are rapidly degraded (Fig. 5). The interaction between the ESS and the 3′ splice site may be facilitated by RNA secondary structure. Moving the ESS farther from the 3′ splice site would be expected to negate its effect as we have previously shown (2). Further studies of the composition of the splicing complexes formed with substrates containing the ESS will be required to test this hypothesis and to define the step where inhibition occurs.

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