SC35 and Heterogeneous Nuclear Ribonucleoprotein A/B Proteins Bind to a Juxtaposed Exonic Splicing Enhancer/Exonic Splicing Silencer Element to Regulate HIV-1 tat Exon 2 Splicing*

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Splicing of the human immunodeficiency virus, type 1, primary transcript is highly regulated. Maintaining the proper equilibrium among spliced, unspliced, and partially spliced isoforms is essential for the replication of the virus. Here we characterize a complex cis-acting element located in tat exon 2 that is required for the splicing regulation of the upstream intron. An exonic splicing enhancer (ESE) and an exonic splicing silencer (ESS) are both located within the regulatory element. Heterogeneous nuclear ribonucleoprotein (hnRNP) A/B proteins bind the ESS to repress splicing, whereas the SR protein SC35 binds the ESE to activate it. We show that the SC35 and the hnRNP A1 binding sites overlap within the juxtaposed ESE/ESS. We propose that hnRNP A1 binding to the ESS inhibits splicing of the upstream intron by directly masking the SC35 binding site.

The human immunodeficiency virus, type 1 (HIV-1), primary RNA transcript is alternatively spliced into more than 40 different mRNAs, which can be divided into three main classes. The 2-kb class of multiply spliced messages encodes the regulatory proteins Tat, Rev, and Nef; the partially spliced 4-kb class of mRNAs encodes the Env, Vpu, Vif, and Vpr proteins; and the 9.2-kb unspliced transcript encodes the Gag and Pol proteins and provides RNA genomes for packaging into virions (1, 2). Alteration of the complex splicing pattern generating the viral mRNAs can dramatically affect HIV-1 infectivity and pathogenesis (1, 3–5). The study of HIV-1 splicing gives us helpful insights into the general mechanisms regulating the alternative splicing of cellular and viral messages. HIV-1 utilizes the cellular splicing machinery to properly regulate production of its primary transcripts through at least four alternative 5′ splice sites (ss) and eight alternative 3′ ss.

Several of the HIV-1 viral sequences that regulate splicing have been characterized. These include suboptimal 3′ ss sequences characterized by weak polypyrimidine tracts, non-consensus branch points (6–9), exonic splicing enhancers (ESEs) (10–13), and exonic splicing silencers (ESS) (3, 10, 14–19). ESEs and ESS are splicing regulatory sequences found in exons (15, 20–24). These regulatory sequences often bind trans-acting factors that interact with the basal splicing machinery. The factors that have been characterized as binding to HIV-1 regulatory elements belong to either the arginine-serine-rich (SR) protein family (25) or the heterogeneous nuclear ribonucleoprotein (hnRNP) family (26). SR proteins mainly participate in positive regulation of splicing, whereas members of the hnRNP family are often involved in negative regulation. However, proteins from both families have been implicated in positive as well as negative splicing regulation (26, 27). The mechanisms by which ESEs, ESS, and the factors they bind modulate splicing are still elusive.

The binding properties of hnRNP A1 to different nucleic acid substrates have been thoroughly investigated (26). Although a minimal recognition sequence (UAGG) has been found (28, 29), it has become evident that hnRNP A1 interacts with a broad range of RNA substrates. Similarly, SR proteins have a range of preferential binding sequences (25). SELEX experiments and functional studies have characterized consensus binding sequences for several SR proteins (30–32). Nevertheless, a fine mapping of the precise site of interactions between SR proteins and their target ESEs has rarely been carried out (33).

Five splicing inhibitory sequences in the HIV-1 pre-mRNA have been identified. ESS2 and ESS2p are located within tat exon 2 (3, 14). ESS3 is located within tat exon 3 (10, 15–18). ESSV is located in the vif coding region (19), and an intronic splicing silencer (ISS) is located in the intron upstream of tat exon 3 (13). In a previous study we showed that hnRNP A/B family bind ESS2 to repress splicing of the upstream intron (34). Similar studies have extended these results to the ESS3, ESSV, and ISS elements (13, 16, 19). This binding to the pre-mRNA is necessary for the inhibitory effect of these splicing silencers (13, 16–19, 34). ESS2p has been shown to be a binding site for hnRNP H, and this binding is thought to be important for inhibition of usage of the upstream 3′ splice site (14).

ESS sequences that bind SR proteins and promote the usage of both 3′ and 5′ splice sites have been found in tat exons 2 and 3 (10–12). Proper splicing of the intron upstream tat exon 3 is regulated by a combination of the ESS binding hnRNP A/B proteins and ESEs binding the SR proteins SC35 and SF2/ASF (13, 16, 35). hnRNP A1 appears to bind cooperatively to the
ESS3, a UAGNNAUg motif in the ESE, and to an ISS that overlaps with the branch point (17, 18). This may in turn block U2 small nuclear ribonucleoprotein (snRNP) from binding to the branch point and prevent positive splicing factors from binding to the ESE (13, 16). Binding of SF2/ASF to the ESE located upstream of ESS3 appears to interfere with binding of hnRNP A1 to the transcript, thus activating splicing (13, 16). Alternative 3′ splice sites necessary for the generation of the tat, rev, env, vpu, and nef gene products are located within tat exon 2 (see Figs. 1A and 6A). The characterization of the viral sequences and cellular proteins involved in this complex splicing regulatory system is essential for our understanding of the viral replication process. In particular, repression of the tat-specific 3′ splice site 4 is key for the production of rev, env, vpu, and nef gene products. Our previous functional characterization of ESS2 and its interacting factors, the hnRNP A/B proteins, demonstrated that these factors are essential for ESS2 function without giving us a clear understanding of the mechanism by which these factors repress splicing of the upstream intron (34). In this study, we investigate the exact viral sequences that interact with hnRNP A/B proteins. Because ESS elements are often associated with ESE sequences (10, 22), we explored the possibility that an SR protein-dependent ESE is localized in close proximity to ESS2. Utilizing biochemical and functional assays, we describe a novel SC35-dependent ESE that overlaps an hnRNP A1 binding site. We propose a model for HIV-1 splicing inhibition in which SC35 and hnRNP A/B proteins compete for binding the same viral sequences regulating the splicing of an upstream intron.

**EXPERIMENTAL PROCEDURES**

In Vitro Pre-mRNA Splicing Assays and Protein Preparations—Capped, 32P-labeled run-off transcripts were synthesized by in vitro transcription using T7 RNA polymerase. HeLa cell and S100 extracts were prepared as described (35). Splicing reactions were performed in a total volume of 25 l, containing 15 l of HeLa cell nuclear extract or S100 as described (35). The reaction mixtures were incubated at 30 °C for 2 h. RNAs recovered from the splicing reaction mixtures were separated on an 8 M urea-6% polyacrylamide gel and visualized with an S100 as described (35). The reaction mixtures were incubated at 30 °C for 15 min. Each sample was treated with either 1 l of double-distilled H2O (negative control) or 1 l of RNase T1 (5 units/ml, 0.5 l of RNase T2 (2 units/ml), or 0.5 l of RNase V1 (5 units/ml). One reaction without RNase was included as a control for nonspecific background. After incubation for 15 min, reactions were terminated by adding 180 l of 300 mM sodium acetate, pH 6.0, and 2 l of EDTA, and reactions were phenol-extracted twice and the RNA was precipitated. The RNA pellets were re-dissolved in 10 l of 95% formamide-loading buffer and analyzed on a 6% denaturing polyacrylamide gel. Markers were generated as described previously (17).

**RESULTS**

**ESS2 Inhibits Binding of SC35 to the Viral Substrate**—To determine whether ESS2 functions like other ESS as a modulator of an SR protein-dependent ESE, we utilized an RNA affinity chromatography assay to determine the effects of ESS2 mutation on SR protein recruitment to the exon. Short RNA substrates (Fig. 1A) were covalently linked to agarose beads and incubated in HeLa cell nuclear extract. Proteins that bound specifically to the beads were separated on SDS-PAGE, transferred to nitrocellulose, and probed with antibodies specific for hnRNP A1, SF2/ASF, SC35, and all the SR proteins (mAb 104) (Fig. 1B). Analysis of the proteins bound to the wild type substrate (ESS2 WT, Fig. 1B, lane 1) showed that no SR protein can bind specifically to the substrate, as demonstrated by comparison to a random control RNA sequence (RNA Cont, Fig. 1B, lane 3). Analysis of the proteins bound by the ESS2 mutant substrate showed an overall increase in SR protein binding. In particular an SR protein of 30 kDa was predominantly bound to the ESS2 mutant substrate. Because the mAb 104 recognizes the phosphoserine/arginine domain of SR protein family members, and SC35, SF2, and 9G8 have similar gel mobility, we utilized SC35, SF2/ASF, and 9G8-specific antibodies to determine which of these SR proteins is the main 30-kDa SR protein that binds to the mutant ESS2 substrate. From this analysis we determined that SC35 specifically binds to the ESS2 mutant substrate (Fig. 1B, lane 2). hnRNP A1 binds efficiently to the wild type substrate but not the ESS2 mutated or the control RNA sequence. Because recruitment of the SC35 is dependent on the ESS2 mutation, it is possible that the mutation introduced into ESS2 introduced a new SC35 binding site. However, different nucleotide substitutions in the ESS2 activate tat exon 2 splicing with equal efficiency (7), and we have tested several of these in an RNA affinity chromatography assay similar to that shown in Fig. 1B. All that we tested promoted SC35 recruitment (data not shown).

hnRNP A1 Inhibits Binding of SC35 to the ESE2—Based on the increased ability of SC35 to bind to the tat exon 2 substrate when ESS2 is mutated, we hypothesized that the function of hnRNP A1 binding to this ESS is to inhibit binding of SC35 to nuclear extract. 4 mM MgCl2, 4 mM ATP, and 5 mM creatine phosphate in buffer D (20 mM Hepes, pH 7.9, 5% glycerol, 0.1 M KCl, 0.2 M EDTA, 0.5 mM dithiothreitol) was incubated two consecutive times with the RNA-bound beads to deplete >95% of hnRNP A/B proteins.

**Substrate RNA Synthesis, Immobilization of RNA on Agarose Beads, and RNA Affinity Chromatography Assays**—Substrate RNAs for bead immobilization were synthesized by in vitro transcription using T7 RNA polymerase and DNA oligonucleotide templates.

Substrate RNA synthesis from oligonucleotides, covalent linkage to adipic acid dihydrazide-agarose beads, and RNA affinity chromatography assays were performed as described previously (34). The HIV-1 genomic location of the HIV substrates are indicated in Fig. 1, the ESS2 mutation we introduced is indicated in Fig. 5A, and the control RNA substrate has been described previously (34).

**RNA Footprinting Analysis**—RNA footprinting was essentially done as described previously (17). 9 l containing 42 ng of 5′ end-labeled tat exon 2 RNA and 1 l of rRNA in probing buffer (10 mM Hepes/KOH, pH 7.6, 100 mM KCl, 2 mM MgCl2, 0.5 mM EDTA, 1.0 mM DTT, and 10% glycerol, v/v) were incubated with either 1 l of buffer D or increasing amounts of either recombinant GST-hnRNP A1 (150 or 300 ng) or purified SC35 (30, 100, or 250 ng) at room temperature for 15 min. Each sample was treated with either 1 l of double-distilled H2O (negative control) or 0.5 l of RNase T1 (5 units/ml, 0.5 l of RNase T2 (2 units/ml), or 0.5 l of RNase V1 (5 units/ml). One reaction without RNase was included as a control for nonspecific background. After incubation for 15 min, reactions were terminated by adding 180 l of 300 mM sodium acetate, pH 6.0, and 2 l of EDTA, and reactions were phenol-extracted twice and the RNA was precipitated. The RNA pellets were re-dissolved in 10 l of 95% formamide-loading buffer and analyzed on a 6% denaturing polyacrylamide gel. Markers were generated as described previously (17).
the exon. If this is the case, then it is predicted that SC35 binding to the wild type substrate should be increased by depletion of hnRNP A/B proteins from the nuclear extract. We have previously shown that all the members of the hnRNP A/B protein family (A1, A1b, A2, and B1) share common activities and binding specificities for ESS2 (34). Therefore, to test if hnRNP A/B proteins are important for inhibiting SC35 recruitment to tat exon 2, nuclear extract must be functionally depleted of all hnRNP A/B proteins. To generate an hnRNP A/B protein-depleted extract, we incubated HeLa cell nuclear extract with beads linked to RNA whose sequence contains several high affinity binding sites for hnRNP A/B proteins in tandem. After two consecutive incubations of the extract with these beads, >95% of hnRNP A/B proteins were removed from the extract (Fig. 2, compare nuclear extract samples in lanes 1 versus 2). Using untreated, hnRNP A/B protein-depleted and mock-depleted extracts, we tested the ability of SC35 and hnRNP A1 to bind to RNA substrates from tat exon 2 containing wild type sequence or a mutation in ESS2 (Fig. 2). Depletion of hnRNP A/B proteins from HeLa nuclear extract resulted in a dramatic increase in SC35 binding to the wild type substrate (Fig. 2, ESS2 WT, lanes 4 and 5), whereas no change in the ability of SC35 to bind to the ESS2 Mut substrate was detected. This is consistent with a model in which binding of hnRNP A/B proteins to ESS2 results in a block in SC35 association with tat exon 2. This blockage can be reversed by mutating ESS2 so that it no longer binds hnRNP A/B proteins or by depleting hnRNP A/B proteins from the extract.

**tat Exon 2 Contains a Functional SC35-dependent ESE**—The presence of an SC35 binding site in tat exon 2 that is regulated by hnRNP A/B proteins binding to ESS2 is suggestive of a functional SC35-responsive ESE. To investigate whether a functional ESE is present between HIV-1 3’s 3’s and 3a, we inserted this portion of tat exon 2 into the downstream exon of an enhancer-dependent splicing reporter substrate derived from the Drosophila melanogaster dsex gene (Fig. 3A). The intron in the dsex system is efficiently spliced in HeLa cell nuclear extract only when an SR-dependent splicing enhancer is present.
ent in the downstream exon (38, 41, 42). Splicing of the parent-
tal dsx substrate (dsx-ΔE) is extremely weak (Fig. 3B, lane 1)
because of a non-consensus 3′ as (7). Insertion of an SR pro-
tein-dependent splicing enhancer activates splicing of the SR pro-
teins, stabilizing the interaction of the splicing factor
U2AF65/35 with the weak 3′ splice site (38, 42). A control, an
SR protein-dependent splicing enhancer derived from the avian
sarcoma-leukosis virus, was inserted into the downstream exon
of the dsx substrate, generating the substrate dsx-avian sarco-
ma-leukosis virus, which was spliced efficiently (Fig. 3B, lane 2).
Insertion of the wild type viral sequence of tat exon 2 to
generate the dsx-tat WT substrate did not increase splicing
efficiency over the enhancer-less control (Fig. 3B, lanes 1 and
3). On the contrary, when ESS2 was mutated in the tat-con-
taining substrate, splicing efficiency increased to a level simi-
lar to that obtained with the avian sarcoma-leukosis virus
enhancer (Fig. 3B, lane 4). This demonstrated that there is an
ESE in tat exon 2 that can function when ESS2 is mutated.

The RNA affinity chromatography results in Figs. 1 and 2
indicated that SC35 was the main SR protein interacting with the
tat exon 2 fragment. To determine which SR proteins can
function to promote splicing of the dsx-tat exon 2 transcripts,
we complemented splicing deficient HeLa S100 cytoplasmic
extracts with single SR proteins. SR proteins are absent from
the S100 extracts, which leads to loss of splicing activity.
Addition of SR proteins can complement this deficiency, allowing
for in vitro splicing to occur (43). We tested the ability
of individual SR proteins isolated from calf thymus to com-
plement the S100 deficient extract and promote splicing of the
dsx-tat ESS2 mutant splicing substrate. Only the total SR
protein preparation from calf thymus and purified SC35 was
able to efficiently complement the S100 extract to activate
splicing of the dsx-tat ESS2 Mut substrate (Fig. 3B, lanes 7
and 8). SRp70, SRp55, and SRp40 were unable to stimulate the splicing of the dsx-tat ESS2 Mut substrate (Fig. 3B, lanes 9–11).
The amount of SR protein added in lanes 7–11 were first normalized
so that they had equal activity in promoting splicing of a pre-mRNA
substrate containing the first intron of the human
β-globin gene (data not shown). These results indicate that the
tat exon 2 fragment contains a functional SC35-dependent ESE
that activates splicing of a weak upstream 3′ splice site when
hnRNP A/B proteins cannot bind to the pre-mRNA at ESS2.

hnRNP A1 and SC35 Binding Sites Are Juxtaposed—Antag-
onism between hnRNP A/B proteins and SR proteins, binding
closely associated ESS and ESEs, is a splicing regulatory mecha-
anism found in several cellular systems (20). Nevertheless,
little is known about how these proteins antagonize each other.
Do the binding sites for hnRNP A/B and SR proteins overlap? If
so, does binding of hnRNP A/B proteins to the substrate di-
rectly prevent SR proteins from binding the same sequence,
thus preventing splicing? Does this competition between
hnRNPs and SR protein involve a more complex set of interac-
tions? To analyze this further, we wanted to determine the
position of the SC35-dependent ESE relative to the hnRNP A1
binding site at ESS2.

To map the precise binding sites for hnRNP A1 and SC35 on
tat exon 2, we performed an RNA footprinting experiment on
5′-end-labeled tat exon 2 substrates in the presence of increasing
concentrations of either recombinant hnRNP A1 or purified
SC35 (Fig. 4, B and C; the results are summarized in Fig. 4A).
We used RNase T1, which cleaves downstream of unpaired G
nucleotides, RNase T2, which cleaves downstream of any un-
paired nucleotide, and RNase V1, which predominantly cleaves
base-paired nucleotides. Analysis of the sequences protected
from digestion by RNase T1, T2, and V1 indicates that SC35
and hnRNP A1 protect the same sequence, which is just upstream of ESS2. The sequence protected by hnRNP A1 extends further into the ESS2 element than that protected by SC35, but we are unable to determine whether hnRNP A1 protects the UAG motif in the 3′ part of the ESS2 sequence because of lack of cleavages in this region in the absence of added proteins. Previous results demonstrated that the UAG at the 3′ end of ESS2 is required for splicing silencing function, but sequences downstream of this point are not (7). This suggests that hnRNP A1 interaction is likely to extend to the UAG at the 3′ end of ESS2. A secondary site of interaction for both hnRNP A1 and SC35 with the viral substrate is located in a purine-rich region just upstream of the primary interacting region. At this site SC35 protection extends further 3′ than the sequence protected by hnRNP A1. Interestingly, none of the RNase V1 cleavages becomes protected by either protein. This indicates a high degree of specificity toward single-stranded regions. The increased cleavage by V1 nuclease of the substrate downstream of ESS2 may reflect minor changes in the secondary structure upon hnRNP A1 binding. From the footprinting data we conclude that hnRNP A1 binds to a discrete site in the viral substrate that overlaps with the characterized ESS2 and extends upstream into a secondary site. Two regions of SC35 protection are found overlapping the hnRNP A1 binding sites, although the one immediately adjacent to ESS2 does not extend into the characterized splicing silencer. The fact that the SC35 and hnRNP A1 binding sequences overlap suggests a mechanism for hnRNP A1 splicing inhibition in which binding to ESS2 prevents SC35 binding (see Fig. 6A).

The SC35 Binding Site Corresponds to ESE2—The results obtained with the doublesex splicing reporter construct indicate the presence of an SC35-dependent ESE in the region adjacent to ESS2 (Fig. 3). RNA footprinting experiments show an SC35 binding site overlapping the ESS2 hnRNP A1 binding site. To test if this SC35 binding site is the functional tat exon 2 ESE (ESE2), we utilized a 2 exon/1 intron HIV-1 construct consisting of tat exons 1 and 2 and a shortened intron (Fig. 5A). The wild type substrate (pHSIX-WT) is inefficiently spliced (Fig. 5B, lane 1) (3). Substrate pH1X-ESS2 Mut carries a mutation in 7 of 10 nucleotides of ESS2 and is efficiently spliced (3). The nucleotide substitutions in the pH1X-ESS2 Mut substrate disrupt the ESS2 hnRNP A1 binding site with-
out modifying the overlapping SC35 binding site. We tested the splicing efficiency of a substrate containing a mutation in ESS2 and a mutation of 2 of 3 nucleotides in the middle of the SC35 binding site (substrate pHS1X-ESS2/ESE2 Mut). This substrate with both ESE2 and ESS2 mutated has markedly reduced splicing efficiency relative to the substrate with only ESS2 mutated (Fig. 5B, compare lanes 2 and 3). This new mutation functionally defines a novel ESE (ESE2). Functionality of ESE2 was also confirmed in the dsx-tat exon 2 enhancer-dependent splicing reporter system where splicing efficiency of the dsx-tat ESS2 Mut substrate is decreased by this same mutation in the ESE2 sequence (dsx-tat ESS2/ESE2 Mut substrate, Fig. 5C, lane 3). To show that the ESE2 mutation inhibits binding of SC35, we tested the ability of SC35 to bind an RNA substrate containing a mutation in both ESS2 and ESE2. As predicted, the binding of SC35 to the double mutant (Fig. 5D, lane 3) was decreased when compared with the substrate carrying only the ESS2 mutation (Fig. 5D, lane 2). To determine whether the region of ESE2 is required for hnRNPA1 binding to tat exon 2, we tested a 10-nucleotide deletion of ESE2 that did not overlap ESS2, the ∆ESE2 substrate (Fig. 5D, lane 4). Results with this substrate indicate that the binding of hnRNPA1 to ESS2 is independent of the upstream sequence that functions as an ESE.

DISCUSSION

We studied the mechanism by which hnRNP A/B proteins repress splicing of HIV-1 tat exon 2. We identified a novel ESE, named ESE2, that is activated by the binding of the SR protein SC35. ESE2 activates splicing in vitro of a tat exon 1–2 viral splicing construct and a heterologous enhancer-dependent doublesex splicing reporter substrate. RNA footprinting analysis identified hnRNPA1 and SC35 binding sites as partially overlapping sequences. These binding sites correlate with the functional ESE2 and ESS2. In HeLa cell extracts, the binding of hnRNPA/B proteins to the viral substrate is dominant over SC35 binding, leading to repression of ESE2 function and thus inhibiting splicing of the upstream intron. Although there are at least four distinct members of the hnRNPA/B family, hnRNPs A1, A1b, A2, and B1, they appear to share similar functions and RNA binding specificities (34). Therefore results obtained characterizing the activity and RNA binding specificity of hnRNPA1 can be extended to the other members of the family.

Previous studies on the mechanism regulating splicing of HIV-1 tat exon 3 suggest that cooperative binding of hnRNPs A/B starts at a high affinity binding site in the ESS3 and the ISS that overlaps with the major branch point. The binding
inhibits splicing in part by blocking U2 snRNP binding to the branch point but also by stimulating binding of hnRNP A/B proteins to a UAG motif that overlaps with the ESE positioned upstream of the ESS3 (17). The cooperative binding of hnRNP A1 seems to involve discrete binding sites as suggested by Damgaard et al. (17), although a linear nucleation mechanism starting from the ESS3 has been proposed (16). In this model, the SR protein SF2/ASF binds to an ESE upstream of ESS3 and appears to antagonize the effects of hnRNP A/B proteins. ESE-bound SR proteins are thought to activate weak 3's by increasing the recruitment of U2AF65/35 through bridging interactions between SR protein and the U2AF35 RS domains (44). However, in the case of tat exon 3 activation, the ability of SF2/ASF to counteract the nearby splicing repressor does not require its RS domain (13, 16). Thus, the function of the SR-dependent ESE in tat exon 3 may be to counterbalance the inhibitory effect on splicing exerted by the ISS and ESS through a prevention of cooperative binding of hnRNP A/B proteins to the pre-mRNA that includes a functional branch point. Our results suggest that the function of hnRNP A/B proteins in tat exon 2 splicing inhibition is to inhibit the binding of SC35 to an overlapping binding site. In substrates in which both the ESE and ESS of tat exon 2 are mutated, precluding both SC35 and hnRNP A/B protein binding, splicing does not occur efficiently (Fig. 5). This indicates that SC35 binding to ESE2 is essential for splicing of the upstream intron. In this model, the function of the ESE2 is to recruit an SR protein (SC35) that in turn will promote splicing through bridging interactions with other factors such as U2AF65/35 and U1 snRNP. From our footprinting data, it is not clear if hnRNP A/B proteins stimulate spreading or cooperative binding of hnRNP A/B proteins to sites upstream from ESS2 to cover the SC35 binding site, or whether the effect is because of the primary binding site for hnRNP A1 alone covering the SC35 binding region. From x-ray crystallography studies, it is likely that hnRNP A1 contacts 10 nucleotides of nucleic acid directly (29). Accordingly, ESS2 alone appears to be sufficient and essential for efficient hnRNP A1 binding to tat exon 2 (Fig. 5D, lane 4). Non-denaturing gel mobility retardation assays indicate that multiple hnRNP A1 proteins assemble onto the tat exon 2 substrate (data not shown). These experiments also indicate that ESS2 is required for any significant binding of hnRNP A1 to this substrate. The sizes of the footprints we obtained may be consistent with binding of multiple hnRNP A1 proteins, but the functional significance of the hnRNP A1 multimerization is unclear. hnRNP A1 multimerization could function by masking the binding sites for SR proteins that are not directly juxtaposed to the primary hnRNP A1 binding site as appears to be the case in tat exon 3 (16). The ability of hnRNP A1 multimerization to spread the binding region from a high affinity seed position can also explain the ability of ESS2 to repress splicing in heterologous substrates (7).

In our model, splicing inhibition does not require the cooperative binding of multiple hnRNPs A/B along the exon after the initial interaction with the high affinity ESS2 binding site. Nevertheless, cooperative binding and propagation of hnRNP A/B upstream from ESS2 are likely. The mechanisms regulating HIV-1 splicing appear to be highly redundant to ensure efficient viral replication despite a high mutation rate. It is conceivable that the viral transcript contains several suboptimal hnRNP A/B binding sites that can assist in the cooperative binding of additional hnRNP A/B proteins to the substrate once it initiates at a high affinity binding site, such as ESS2. Random mutations could disrupt the high affinity binding site, but this can be balanced by mutations activating lower affinity ones.

Within tat exon 2 are located 3’ splice sites specific for the tat, rev, env, vpu, and nef gene products. The model in Fig. 6B shows the complex set of interactions between ESEs, ESS, and cellular splicing factors regulating these splice products. The relative strength of each regulatory element varies, and their reciprocal interactions have not been completely elucidated. For example ESS2 appears to be dominant in cis toward ESS2p. Splicing efficiency is greatly increased by mutating ESS2 but only marginally improved by the ESS2p mutation. If a substrate with a shortened tat exon 2 lacking the ESS2/ESE2

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**Fig. 6.** A, schematic model of the inhibitory effect on SC35 recruitment exerted by hnRNP A/B protein binding to ESS2 (left panel). Mutation of the ESS or depletion of hnRNP A/B allows efficient binding of SC35 to the ESE2 (right panel). B, model of the complex network of interactions regulating the splice sites located within tat exon 2. ESEs, ESS, and interacting proteins are indicated together with their inhibitory and enhancing effects on the splice sites present in the region.
region is assayed for \textit{in vitro} splicing activity, mutation of the ESS2p can increase splicing efficiency to levels comparable with the ones obtained with the ESS2 mutation in the \textit{tat} exon 1–2 substrate (14). Furthermore, SC35 binding to ESE2 promotes the recruitment of U1 snRNP to a downstream, non-functional, suboptimal 5′ ss (data not shown). The functional significance of this event is unknown but may be part of a functional splicing enhancer complex. This is consistent with the observation that binding of U1 snRNP to the nearby 5′ ss 4 has been shown to be required for stability and expression of the partially spliced \textit{env} mRNA (12). Splicing regulation of the viral pre-mRNA seems to involve mechanisms utilizing multiple cis elements with redundant functions. Cellular regulatory proteins that are abundant in all cell types recognize these cis elements. This ensures that a proper balance of spliced and unspliced viral messages can be produced.

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