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hnRNP A/B proteins are required for inhibition of HIV-1 pre-mRNA splicing

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Splicing of the human immunodeficiency virus type 1 (HIV-1) pre-mRNA must be inefficient to provide a pool of unspliced messages which encode viral proteins and serve as genomes for new virions. Negative *cis*-regulatory elements (exonic splicing silencers or ESSs) are necessary for HIV-1 splicing inhibition. We demonstrate that heterogeneous nuclear ribonucleoproteins (hnRNPs) of the A and B group are *trans*-acting factors required for the function of the *tat* exon 2 ESS. Depletion of hnRNP A/B proteins from HeLa cell nuclear extract activates splicing of *tat* exon 2 pre-mRNA substrate. Splicing inhibition is restored by addition of recombinant hnRNP A/B proteins to the depleted extract. A high-affinity hnRNP A1-binding sequence can substitute functionally for the ESS in *tat* exon 2. These results demonstrate that hnRNP A/B proteins are required for repression of HIV-1 splicing.
Keywords: HIV-1/hnRNP proteins/pre-mRNA splicing

Introduction

The single human immunodeficiency virus type 1 (HIV-1) pre-mRNA is alternatively spliced into >30 different messages which can be divided into three main classes. The 2 kb class of multiply spliced messages encode the regulatory proteins Tat, Rev and Nef; the partially spliced 4 kb class of messages encode 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 (Purcell and Martin, 1993; Rabson and Graves, 1997). The HIV-1 Rev protein binds the rev response element (RRE) to ensure stabilization and transport of the pool of unspliced and partially spliced RNAs. Unspliced RNA substrates for Rev activity are produced through inefficient splicing of the HIV-1 primary transcript. Weak splicing signals, such as non-consensus branch sites and short polypyrimidine tracts, play a role in the inefficient splicing of the primary transcript of HIV-1 and other retroviruses (Fu *et al*., 1991; Staffa and Cochrane, 1994; O'Reilly *et al*., 1995; Si *et al*., 1997). In addition, exonic splicing silencer (ESSs) sequences have been identified that are required for negative regulation of this splicing (Amendt *et al*., 1994, 1995; Staffa and Cochrane, 1995; Wentz *et al*., 1997).

Two different ESSs have been identified in the HIV-1 pre-mRNA. These ESSs are located in *tat* exons 2 and 3, the coding exons of the *tat* gene. The 3′ splice sites flanking both exons are characterized by suboptimal polypyrimidine tracts and by a non-consensus branch point (Staffa and Cochrane, 1994; Si *et al*., 1997). Mutation of each of these ESSs causes increased splicing efficiency *in vitro* and *in vivo* (Amendt *et al*., 1994; Staffa and Cochrane, 1995). Both sequences block spliceosome assembly at an early stage, after E complex formation but prior to A complex formation, and their insertion in a heterologous gene produces a detectable reduction in splicing efficiency (Amendt *et al*., 1994; Staffa and Cochrane, 1995; Si *et al*., 1998). The ESS in *tat* exon 3 can be substituted for the ESS in *tat* exon 2 and promotes splicing inhibition, implying a similar mechanism of action. Addition of competitor RNA containing the *tat* exon 2 ESS or *tat* exon 3 ESS to an *in vitro* splicing reaction containing a *tat* exon 2 pre-mRNA substrate increases the splicing of the substrate RNA. This finding suggests that *trans*-acting inhibitory factor(s) bind to the competitor and thus allow the substrate RNA to be spliced (Amendt *et al*., 1995; Si *et al*., 1998). Recently, experiments performed with HeLa cell nuclear extracts have identified hnRNP A1 as a protein that can be UV cross-linked to the ESS element in the human fibroblast growth factor (FGF) receptor 2 K-SAM exon and to the ESS element of HIV-1 *tat* exon 2 (del Gatto-Konczak *et al*., 1999). In addition, this work showed that the C-terminal domain of hnRNP A1 is required for the function of the ESS element of the K-SAM exon *in vivo*.

The goal of the experiments described here was to identify the *trans*-acting cellular factors that interact with the *tat* exon 2 ESS sequence to negatively regulate its splicing. To this end, we covalently linked RNAs containing the ESS sequence to agarose beads and used these beads to affinity-purify factors from HeLa cell nuclear extract which assemble onto the ESS. HeLa cells represent a relevant system for the study of HIV-1 splicing regulation since the splicing pattern of the virus in HeLa and human T cells is almost identical (Purcell and Martin, 1993). Using this RNA affinity chromatography approach, we have identified members of the hnRNP A/B family of proteins (Dreyfuss *et al*., 1993; Weighardt *et al*., 1996) as *trans*-acting factors that bind to the exon 2 ESS sequence. Depletion/reconstitution experiments demonstrate that these proteins are required for specific inhibition of splicing of HIV-1 *tat* exon 2. Finally, we show that a high-affinity hnRNP A1-binding sequence can also function as an ESS in *tat* exon 2.

Results

The HIV-1 tat exon 2 ESS binds hnRNP A/B proteins

RNAs containing the ESS sequence, a mutated ESS sequence or a control SV40 early message sequence (Figure 1A) were coupled to agarose beads and used to affinity-purify factors from HeLa cell nuclear extracts. Nuclear proteins which bound to the immobilized RNAs were analyzed by SDS–PAGE. Four proteins were found that bound strongly to the ESS wild-type RNA but weakly to the ESS mutant RNA. These proteins did not bind to the control RNA or to control beads (Figure 1B). The range of apparent molecular weights of these proteins (34–40 kDa) is consistent with that of the core heterogeneous nuclear ribonucleoproteins (hnRNPs) of the A, B and C groups. These hnRNP proteins have been reported to have sequence-specific RNA-binding activity and to be involved in nucleo/cytoplasmatic transport of mRNA and in pre-mRNA processing (Dreyfuss *et al*., 1993). We confirmed the identity of the two higher mobility proteins of ~34 and 35 kDa as hnRNP A1 and hnRNP A1x with the anti-hnRNP A1 monoclonal antibody (mAb) 4B10 (Figure 1B, lane 5) (Piñol-Roma *et al*., 1988). HnRNP A1x was described previously as an hnRNP A1 isoform that arises by an unknown post-translational modification (Wilk *et al*., 1985). The 36 and 38 kDa proteins were identified as hnRNP A2 and hnRNP B1, respectively, using an anti-hnRNP A1 polyclonal antibody that cross-reacts with these two alternatively spliced isoforms of hnRNP A2 (Valentini *et al*., 1985) (Figure 1C, lane 2). None of the proteins which bound to the ESS wild-type RNA were recognized by the anti-hnRNP C mAb 4F4 (Figure 1C, lanes 4 and 5) (Choi and Dreyfuss, 1984).

Depletion of hnRNP A/B proteins from HeLa cell nuclear extract activates tat exon 2 splicing

We next sought to determine whether hnRNP A/B proteins are required for specific inhibition of HIV-1 pre-mRNA splicing. We used bead-immobilized RNAs to deplete these proteins from HeLa cell nuclear extract and tested the ability of the depleted extract to perform splicing of pre-mRNA substrates derived from HIV-1. Nearly complete depletion of hnRNP A1 was achieved by incubating the nuclear extract twice consecutively with beads containing the immobilized ESS wild-type RNA (Figure 2A, lane 2). Comparison of the immunoblot signal of hnRNP A1 between this depleted extract and a titration of known amounts of nuclear extract indicates that at least 90% of hnRNP A1 present in nuclear extract had been removed (data not shown). Control depletions done with immobilized ESS mutant RNA, control RNA or beads with no RNA bound did not result in depletion of hnRNP A1 from the splicing extract (Figure 2A, lanes 4, 6 and 8). Initial tests of the depleted extracts showed a marked reduction in splicing activity, even for the control-depleted extracts. We determined that the loss of splicing activity was due to non-specific depletion of SR proteins, a family of essential splicing factors (Fu, 1995; Manley and Tacke, 1996), from the reaction mixture (data not shown). We determined that more than half of the SR proteins in the HeLa cell extract precipitated under these bead incubation conditions and were therefore removed from the extracts

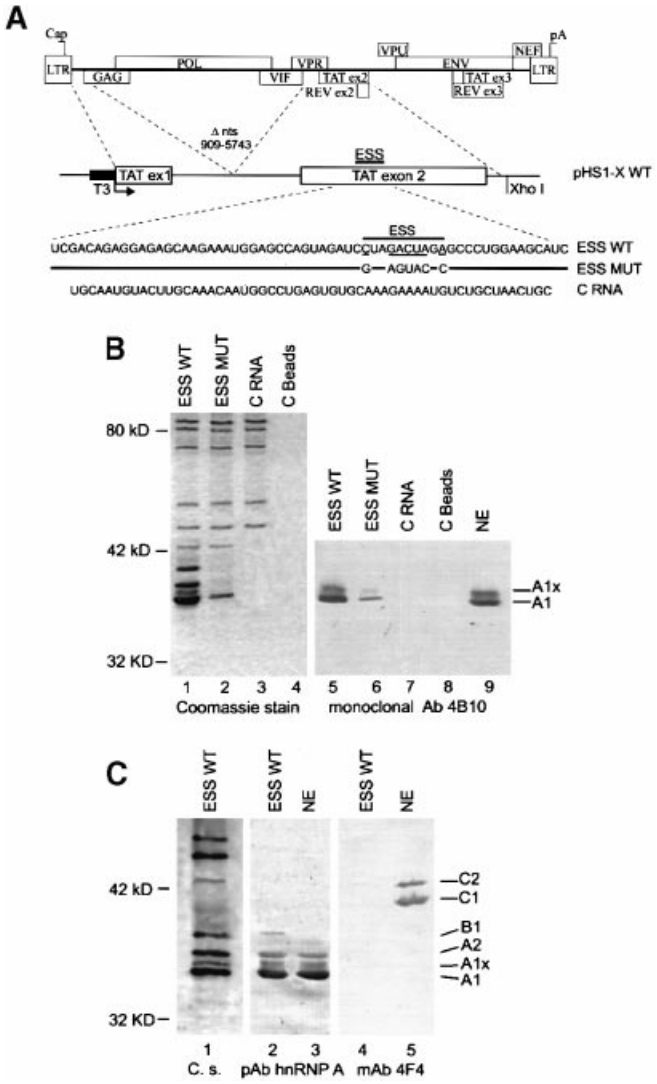

Fig. 1. Identification of *trans*-acting factors that bind to the *tat* exon 2 ESS. (**A**) Schematic representation of *in vitro* splicing substrate pHS1-X and its derivation from the HIV-1 genome (Amendt *et al*., 1994). At the bottom is the sequence of the 60 nucleotide RNA derived from exon 2 that was transcribed and covalently linked to beads. The location of the ESS is indicated. The sequences of the ESS mutant (ESS MUT) and control SV40 early message RNA (C RNA) are also shown. (**B**) Identification of proteins that interact with the ESS sequence. Different RNAs were covalently linked to agarose beads and incubated in HeLa cell nuclear extract under splicing conditions. Proteins that remained bound to the RNAs after washing were separated on SDS–PAGE and detected by Coomassie Blue staining (lanes 1–4) or immunoblotting with mAb 4B10, which is specific for hnRNP A1 (lanes 5–9). ESS WT RNA (lanes 1 and 5), ESS MUT RNA (lanes 2 and 6), C RNA (lanes 3 and 7) and control beads containing no linked RNAs (C Beads) (lanes 4 and 8) were tested. Lane 9 contains 10 μl of HeLa cell nuclear extract proteins. (**C**) Further identification of hnRNP proteins that bind to the HIV *tat* exon 2 ESS. Lane 1, Coomassie Blue staining of proteins that bind to the ESS wild-type RNA. Lanes 2 and 3 show immunostaining with a polyclonal antibody against hnRNP A1, which also cross-reacts with hnRNP A2 and B1. Lane 2, proteins that bind to the ESS wild-type RNA. Lane 3, 10 μl of HeLa cell nuclear extract proteins. Lanes 4 and 5 show immunostaining with mAb 4F4, which is specific for hnRNP C1/C2. Lane 4, proteins that bind to the ESS wild-type RNA. Lane 5, 10 μl of HeLa cell nuclear extract proteins.

whether or not RNA was bound to the beads. These precipitated SR proteins were part of a small visible precipitate of proteins from the splicing reaction mixture

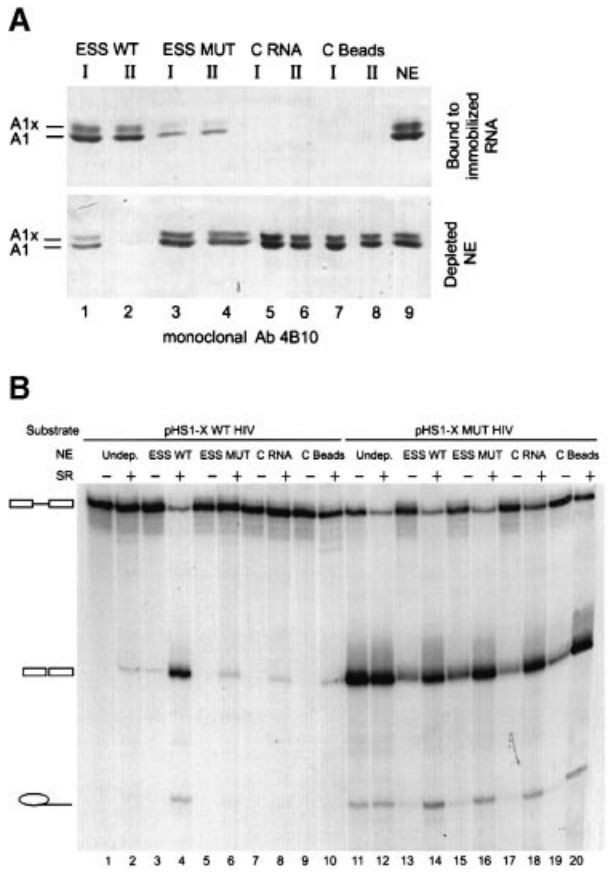

Fig. 2. Depletion of hnRNP A1 from HeLa cell nuclear extract activates splicing of the pHS1-X WT HIV-1 substrate. (**A**) Analysis of hnRNP A1 depletion using bead-linked RNAs. The upper part of the figure shows an mAb 4B10 immunoblot of proteins that remained bound to beads containing covalently linked RNAs. The lower part of the figure shows an mAb 4B10 immunoblot of proteins remaining in the nuclear extract after incubation with RNA bound to beads. Two rounds of depletion (I and II) were performed for each set. RNAs bound to beads are indicated for each set, and control beads containing no bound RNA were also tested (lanes 7 and 8). (**B**) Splicing activity of bead-depleted extracts. Non-depleted nuclear extracts and nuclear extracts depleted twice consecutively with the indicated RNAs linked to beads were tested for splicing activity on pHS1-X WT substrate or pHS1-X MUT substrate (Figure 1A). Splicing reactions were performed with (+) or without (–) the addition of 200 ng of total HeLa cell SR proteins to replace essential splicing factors non-specifically lost during the depletion procedure.

which was visible in the first pellet of beads after incubation. This precipitate subsequently was resolubilized upon bead washes in buffer alone. No specific interaction of SR proteins with the wild-type substrate RNA could be detected (data not shown). Since SR proteins are required for constitutive splicing activity, a preparation of total SR proteins from HeLa cells was added to the depleted extracts to improve splicing efficiency.

The ability of the depleted extracts to splice *tat* exon 2-containing substrates *in vitro* was tested with two pre-mRNAs. HIV-1 substrate pHS1-X WT (Figure 1A), consisting of *tat* exons 1 and 2 and a shortened intron, was described previously (Amendt *et al.*, 1994). This substrate includes the *tat* exon 2 ESS and is spliced inefficiently *in vitro*. Substrate pHS1-X MUT is identical to pHS1-X except for mutation of seven out of 10 nucleotides of the *tat* exon 2 ESS, and is spliced efficiently *in vitro* (Amendt *et al.*, 1994). The addition of SR proteins to the reaction mixtures increased the efficiency of splicing of the pHS1-X MUT substrate in both hnRNP A/B-depleted and control-depleted nuclear extracts (Figure 2B, lanes 13–20). The hnRNP A/B-depleted and control-depleted extracts spliced the pHS1-X MUT substrate with the same efficiency (lanes 14, 16, 18 and 20). The pHS1-X WT substrate was spliced with high efficiency only in the hnRNP A/B-depleted extract (lane 4). Extracts not depleted of hnRNP A/B proteins spliced the pHS1-X WT substrate only weakly (lanes 2, 6, 8 and 10). This result was also seen without the addition of SR proteins (lane 3 versus lanes 1, 5, 7 and 9), although splicing efficiency was lower. Therefore, splicing of the substrate with the *tat* exon 2 ESS was dramatically improved in the absence of hnRNP A/B proteins.

Recombinant hnRNP A/B proteins reconstitute splicing inhibition in depleted HeLa cell nuclear extract

We next tested whether we could reconstitute ESS inhibition of splicing in the hnRNP A/B-depleted extract by addition of hnRNP A/B proteins. First, we determined the concentration of hnRNP A1 present in HeLa cell nuclear extract in order to ascertain the proper range of recombinant protein to add to the depleted extracts. By comparing the mAb 4B10 immunoblot signal of known amounts of recombinant hnRNP A1 with different amounts of HeLa cell nuclear extract, it was estimated that hnRNP A1 is present in our preparation of HeLa cell nuclear extract at ~3 pmol/μl (Figure 3A). An aliquot of nuclear extract was depleted of hnRNP A/B proteins and complemented with SR proteins as described above. Prior to addition of the splicing substrate, 3 or 10 pmol of recombinant hnRNP A1, $A1^B$, A2 or B1 was added to the reaction mixtures, which contained 10 μl of the depleted nuclear extract. HnRNP $A1^B$ is an alternatively spliced isoform of hnRNP A1 that contains an insertion in the C-terminal domain (Buvoli *et al.*, 1990) and is not detected in HeLa cell nuclear extract (Hanamura *et al.*, 1998). The mixtures were tested for the ability to splice the pHS1-X WT and pHS1-X MUT splicing substrates. Addition of 10 pmol of any of the hnRNP proteins tested restored splicing inhibition of the pHS1-X WT substrate (Figure 3B, lanes 3, 5, 7 and 9). Addition of the same amount of hnRNP proteins to the hnRNP A/B-depleted extract did not decrease the splicing efficiency of the pHS1-X MUT substrate (lanes 12, 14, 16 and 18). This control ruled out a role in general inhibition of splicing activity by these hnRNP A/B proteins. The depletion/reconstitution experiment demonstrates that hnRNP A/B proteins are *trans*-acting factors required for the specific splicing silencing function of the *tat* exon 2 ESS.

We next sought to determine whether the effect of hnRNP A/B protein reconstitution of specific splicing inhibition of the wild-type HIV-1 splicing substrate was specific for functional hnRNP proteins. We tested two recombinant mutant forms of the hnRNP A1 protein summarized in Figure 4A. UP1 is a truncated version of hnRNP A1 which lacks the glycine-rich C-terminal domain (Mayeda *et al.*, 1994). M(RRM1,2) has both phenylalanines of the RNP-1 submotif in each RNA recognition motif (RRM) replaced with aspartic acid; these mutations

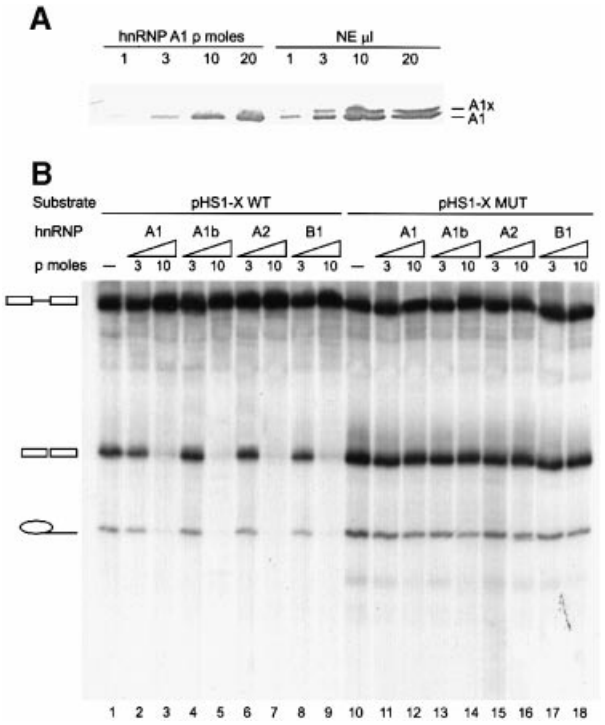

Fig. 3. Reconstitution of wild-type HIV-1 splicing inhibition by addition of hnRNP A/B proteins. (**A**) Determination of the concentration of hnRNP A1 protein in HeLa cell nuclear extract. Varying amounts of recombinant hnRNP A1 protein (1, 3, 10 and 20 pmol) and HeLa cell nuclear extract (1, 3, 10 and 20 µl) were separated by SDS–PAGE, transferred to nitrocellulose and immunoblotted with mAb 4B10. By comparing the signals between the known amounts of purified hnRNP A1 protein and the nuclear extract, we estimate that hnRNP A1 is present in this preparation of HeLa cell nuclear extract at ~3 pmol/µl. (**B**) Reconstitution of wild-type HIV-1 splicing inhibition by addition of hnRNP A/B proteins. *In vitro* splicing reactions were performed with a HeLa cell nuclear extract reaction mixture depleted twice with beads containing covalently linked ESS wild-type RNA. The reaction mixture was then complemented by addition of total HeLa cell SR proteins. This extract was tested for splicing of the pHS1-X WT substrate RNA (lanes 1–9) and the pHS1-X MUT substrate RNA (lanes 10–18) in the absence or presence of hnRNP A/B proteins. A 3 or 10 pmol aliquot of purified recombinant hnRNP A1 (lanes 2, 3, 11 and 12), hnRNP $A1^B$ (lanes 4, 5, 13 and 14), hnRNP A2 (lanes 6, 7, 15 and 16) or hnRNP B1 (lanes 8, 9, 17 and 18) was added to the splicing reaction mixtures prior to the addition of the splicing substrate.

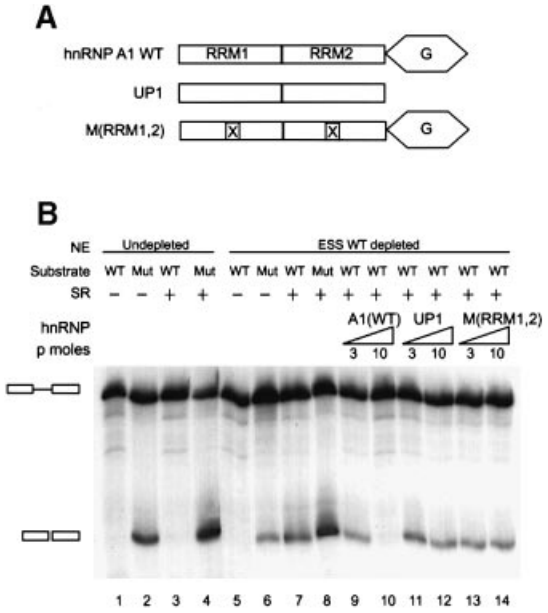

Fig. 4. The RNA-binding and glycine-rich domains of hnRNP A1 are required for splicing inhibition. (**A**) Schematic representation of mutant hnRNP A1 proteins. Wild-type hnRNP A1 showing the two RRMs and glycine-rich C-terminal domain. Mutant UP1 lacks the C-terminal glycine-rich domain. Mutant M(RRM1,2) contains two phenylalanine to aspartic acid replacements in the RNP-1 submotif of each RRM (Mayeda *et al*., 1994). (**B**) Mutant hnRNP A1 proteins cannot reconstitute splicing inhibition. *In vitro* splicing reactions were performed with an undepleted HeLa cell nuclear extract (lanes 1–4) or a HeLa cell nuclear extract reaction mixture depleted twice with beads containing covalently linked ESS wild-type RNA (lanes 5–14). The pHS1-X WT substrate RNA was tested in lanes 1, 3, 5, 7 and 9–14. The pHS1-X MUT substrate was tested in lanes 2, 4, 6 and 8. A HeLa cell SR protein mixture was used to complement the reaction mixtures in lanes 3, 4 and 7–14. Recombinant hnRNP A1 protein was added to the reaction mixtures in lanes 9 and 10 (3 and 10 pmol respectively). A 3 or 10 pmol aliquot of recombinant wild-type hnRNP A1 (lanes 9 and 10), UP1 (lanes 11 and 12) or mutant M(RRM1,2) (lanes 13 and 14) was added to the reaction mixtures. hnRNP A1 could reconstitute splicing inhibition (lanes 9 and 10) but the mutant proteins could not (lanes 11–14).

disrupt RNA-binding activity of both RRMs (Mayeda *et al*., 1994). The X-ray crystal structure of UP1 has been determined (Xu *et al*., 1997) and it shows proper folding of UP1 into two RRMs. The phenyalanines mutated in M(RRM1,2) are solvent exposed, therefore their substitution with acidic residues should not influence the folding of the protein. Neither of these hnRNP A1 mutant proteins could reconstitute the splicing inhibition of the wild-type HIV-1 substrate when added to the depleted extract (Figure 4B, lanes 11–14). These results indicate that both the N-terminal RRMs and the C-terminal glycine-rich domain are required for the splicing inhibitory activity of hnRNP A1.

A high-affinity hnRNP A1-binding site can substitute efficiently for the HIV ESS

High-affinity hnRNP A1-binding RNA sequences have been identified through iterative selection (SELEX) experiments (Burd and Dreyfuss, 1994). To elucidate further the relationship between sequences bound by the hnRNP A/B proteins and splicing inhibition, we substituted a 20 nucleotide fragment containing the hnRNP A1 high-affinity binding sequence for the ESS in both the pHS1-X WT *in vitro* splicing substrate and in the 60 nucleotide ESS wild-type RNA bead-linked substrate (pHS1-X WA1 and HIV-WA1, Figure 5A). In addition, we substituted a 20 nucleotide scrambled version of the high-affinity binding sequence as a control (pHS1-X SCA1 and HIV-SCA1; Figure 5A). HIV-WA1 RNA and HIV-SCA1 RNA substrates were covalently linked to agarose beads and incubated with nuclear extracts as described above. The HIV-WA1 substrate recruited hnRNP A/B proteins with higher affinity than the wild-type HIV-1 sequence (ESS WT) (Figure 5B, lanes 1, 3, 7 and 9). Nuclear extracts depleted of hnRNP A/B proteins with the HIV-WA1 substrate immobilized to beads were activated to splice the pHS1-X WT pre-mRNA substrate (data not shown). In addition to the hnRNP A/B proteins, a 50 kDa protein (p50K) was recruited specifically to the HIV-WA1 RNA substrate. Previous work similarly showed specific UV cross-linking of a 50 kDa protein from HeLa cells to the hnRNP A1 high-affinity binding sequence (Burd and

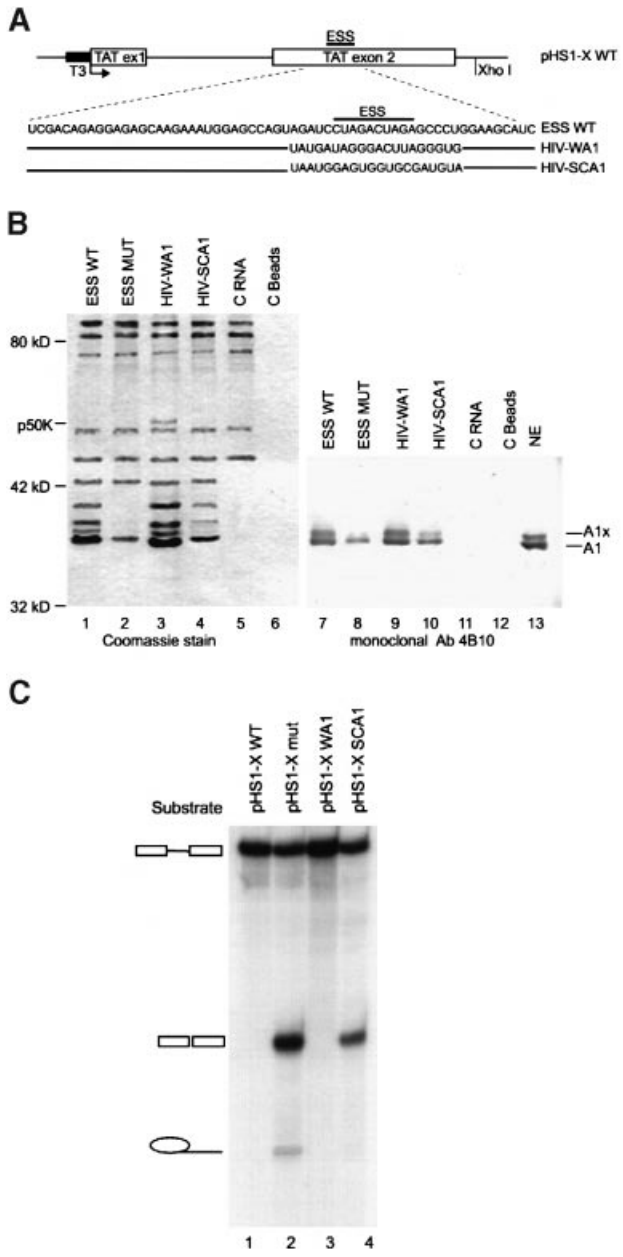

Fig. 5. A high-affinity hnRNP A1-binding site placed in *tat* exon 2 functions as an ESS. (**A**) Schematic representation of splicing substrate derived from pHS1-X. A 20 nucleotide segment containing the HIV-1 ESS was substituted with a high-affinity hnRNP A1-binding sequence determined by SELEX (Burd and Dreyfuss, 1994) (HIV-WA1) or a scrambled sequence version of this A1-binding site (HIV-SCA1). The sequences of both HIV-WA1 and HIV-SCA1 RNAs utilized in the binding assays are shown. These same sequences were substituted in the pHS1-X WT *in vitro* splicing substrate to give the pHS1-X WA1 and pHS1-X SCA1 substrates. (**B**) Analysis of proteins that interact with the hybrid HIV-1–hnRNP A1 high-affinity binding sequence. The indicated RNAs were covalently linked to agarose beads and incubated in HeLa cell nuclear extract. The proteins remaining on the beads after washing were separated by SDS–PAGE and stained with Coomassie Blue (lanes 1–6) or immunostained with anti-hnRNP A1 mAb 4B10 (lanes 7–13). C Beads are control beads lacking bound RNA. Lane 13 contained 10 µl of HeLa cell nuclear extract. (**C**) Effect on splicing of hnRNP A1 high-affinity binding sequence substitution for the ESS of *tat* exon 2. Wild-type (pHS1-X WT), ESS mutated (pHS1-X MUT) and substituted (pHS1-X WA1, pHS1X-SCA1) pre-mRNAs were incubated under splicing conditions in HeLa cell nuclear extract.

Dreyfuss, 1994). The HIV-SCA1 RNA substrate had reduced affinity for hnRNP A/B proteins relative to the HIV-WA1 and ESS wild-type RNAs, but had higher affinity for these proteins than the ESS mutant RNA (lanes 4 and 10).

We next determined whether the relative affinity for hnRNP A/B proteins detected in the binding assay for the HIV wild-type and mutant ESS sequences tested in Figure 5B could be correlated with splicing efficiency. *In vitro* splicing substrates containing the wild-type and three mutant *tat* exon 2 ESS sequences were incubated in a splicing reaction mixture with HeLa cell nuclear extract and the splicing efficiency of the different substrates was analyzed (Figure 5C). Substitution of the ESS with the high-affinity hnRNP A1-binding sequence in the pHS1-X WA1 substrate (lane 3) strongly inhibited splicing, while the scrambled version in pHS1-X SCA1 (lane 4) increased the efficiency of splicing to a level intermediate between those of the wild-type (pHS1-X WT) and the original mutation of the ESS sequence (pHS1-X MUT) (lane 2). This result shows that there is an inverse correlation between the recruitment of hnRNP A/B proteins to *tat* exon 2 (Figure 5B) and splicing efficiency.

Discussion

Splicing of the HIV-1 pre-mRNA is highly regulated and is dependent on the host splicing machinery. Exonic sequences, distinct from the splice sites, have been identified as the binding sites for cellular factors that repress the usage of upstream splice sites (Amendt *et al*., 1994; Staffa and Cochrane, 1995; Si *et al*., 1998). Using a combination of RNA affinity chromatography and *in vitro* splicing, we have demonstrated that hnRNP A/B proteins are required for inhibition of splicing of HIV-1 *tat* exon 2. The hnRNP A/B proteins which assemble on the *tat* exon 2 ESS consist of two RRMs and a glycine-rich auxiliary domain at the C-terminus. hnRNP A2 and hnRNP B1 share 80% amino acid identity with hnRNP A1 in the RRMs (Dreyfuss *et al*., 1993). hnRNP A1, A2 and B1 previously were shown to influence alternative splice site selection *in vitro* and *in vivo* (Mayeda and Krainer, 1992; Mayeda *et al*., 1993, 1994; Yang *et al*., 1993; Chabot *et al*., 1997; Bai *et al*., 1999). Inclusion of excess hnRNP A/B proteins in an *in vitro* splicing reaction mixture can lead to a slight stimulation of the overall level of splicing of certain pre-mRNA substrates (Mayeda and Krainer, 1992; Burd and Dreyfuss, 1994). However, addition of excess hnRNP A1 inhibits splicing of a bovine growth hormone pre-mRNA, apparently by counteracting the recognition of an exonic splicing enhancer by an SR protein (Sun *et al*., 1993). Unfortunately, not much is known about the sequence, location or number of hnRNP-binding sites on those substrates. The alternatively spliced K-SAM exon of FGF receptor 2 contains an ESS element that mediates skipping of that exon. This ESS can be UV cross-linked to hnRNP A1 in HeLa cell nuclear extract (del Gatto-Konczak *et al*., 1999). Consistent with our finding of stable association of hnRNP A1 with the *tat* exon 2 ESS, this recent work showed that substitution of the K-SAM ESS with 20 nucleotides encompassing the HIV *tat* exon 2 ESS also promoted UV cross-linking of

hnRNP A1 to the RNA substrate (del Gatto-Konczak *et al*., 1999).

HIV-1 *tat* exon 3 also carries an ESS. Previous work has suggested that these two ESSs use a similar mechanism to inhibit splicing, since the two sequences can be exchanged maintaining the same inhibitory activity (Si *et al*., 1998). Preliminary data suggest that the *tat* exon 3 ESS can also bind hnRNP A/B proteins with high affinity (data not shown). However, splicing regulation of *tat* exon 3 seems to be far more complex than in exon 2, since at least two other sequences distinct from the ESS have been shown to interact with cellular factors to modulate splicing properly at the upstream 3′ splice site (Amendt *et al*., 1995; Staffa and Cochrane, 1995; Mayeda *et al*., 1999). These sequences have the ability to act as exonic splicing enhancers and can interact with SR proteins. The SR proteins SF2/ASF and SC35 have been identified as factors that productively recognize the regulatory sequences (Mayeda *et al*., 1999). Depletion of the SR protein SF2/ASF from cells *in vivo* can activate splicing of substrates containing this splicing regulatory region (Wang *et al*., 1998). In *tat* exon 2, there is no evidence for regulatory sequences other than the ESS, and no specific SR protein binding to the regulatory region has been observed (data not shown).

The sequence CUAGACUAGA of the *tat* exon 2 ESS is similar to other known binding sequences for hnRNP A1. The UACCUUUAGAGUAGG intron element regulating 5′ splice site selection in the human hnRNP A1 pre-mRNA (Chabot *et al*., 1997) and the mouse hepatitis virus RNA transcription regulatory region UUAGAUUAGA (Li *et al*., 1997) are both bound by hnRNP A1. Utilizing the SELEX technique, Burd and Dreyfuss (1994) have identified a 'winner' high-affinity binder RNA for hnRNP A1, UAUGAUAGGGACUUAGGGUG with an apparent $K_d = 1\times10^{-9}$. All of these hnRNP A1-binding sequences contain tandem repeats of the sequence UAG at their core. In Figure 5, we demonstrated that the hnRNP A1 SELEX winner sequence can substitute efficiently for the *tat* exon 2 ESS. In the same experiment, we noticed that the control RNA constituted by the scrambled winner sequence, SCA1, had some affinity for hnRNP A1 (Figure 5B, lanes 3 and 10) though at a much lower level than the winner A1 sequence. This suggests that hnRNP A1 can achieve a broad range of binding affinities on suboptimal sequences and that our knowledge of what constitutes an hnRNP A1-binding sequence is incomplete. The *tat* exon 3 ESS also contains a UAG motif, AGAUCCAUUCGAUUAGUGAA, although not in a tandem repeat. Recently, an ESS in the K-SAM exon of human FGF receptor 2 was shown to have interactions with hnRNP A1 protein that are important for exon skipping (del Gatto-Konczak *et al*., 1999). The K-SAM ESS sequence UAGGGCAGGC also bears homology to these hnRNP A1-binding sequences. In their study, chimeric proteins containing parts of hnRNP A1 linked to the bacteriophage MS2 coat protein RNA-binding domain were expressed in cells along with a splicing substrate containing the MS2-binding sequence substituted for the ESS in the K-SAM exon of FGF receptor 2. This *in vivo* study demonstrated the importance of the association of the C-terminal domain of hnRNP A1 with the pre-mRNA to generate exon skipping of the K-SAM exon (del Gatto-Konczak *et al*., 1999). This is consistent with previous findings showing that the C-terminal domain of hnRNP A1 is essential for alternative splicing and stable RNA binding activities of hnRNP A1 (Mayeda *et al*., 1994) and with our demonstration that both the C-terminal glycine-rich domain and the two RRMs are essential for the hnRNP A1 splicing inhibition activity. Together, these studies demonstrate the importance of high-affinity hnRNP A1-binding sites in negative splicing regulatory elements. The core of the hnRNP A1 winner SELEX sequence, UAGGG, has varying degrees of homology to the functionally active hnRNP A1-binding sequences described above. Reliable prediction of ESS elements based solely on SELEX-determined sequences is not yet possible, so functional studies like these are critical for identifying the range of hnRNP A1 interactive elements.

Recently, Chen *et al*. (1999) have uncovered an unrelated ESS in the rat β-tropomyosin gene exon 7. This ESS sequence at the 5′ end of the exon is involved in a secondary structure important for splicing inhibition.They have demonstrated that hnRNP H, a three RRM-containing member of the hnRNP protein family, can be UV cross-linked to this ESS. Partial immunodepletion of hnRNP H from nuclear extracts can activate splicing of an ESS-containing substrate RNA *in vitro*. The sequence of the hnRNP H-binding ESS is different from that of the hnRNP A/B-binding ESSs discussed above in that it lacks the UAG motif. It appears that there may be multiple mechanisms available in the nucleus for regulated splicing repression.

hnRNP A/B proteins are required for the negative regulation of HIV-1 *tat* exon 2 pre-mRNA splicing. A possible mechanism of action for this specific splicing inhibition could involve the recruitment of small nuclear ribonucleoprotein particles (snRNPs) toward non-functional splicing signals present on the viral pre-mRNA. This model is similar to the mechanism described for somatic inhibition of splicing of the *Drosophila* P-element third intron involving an hnRNP A-like protein, hrp48 (Hammond *et al*., 1997). Consistent with this model, hnRNP A1 can interact with U2 and possibly U1 snRNP (Mayrand and Pederson, 1990; Buvoli *et al*., 1992). An *in vitro* splicing substrate containing the *tat* exon 2 ESS region and the downstream intron can assemble a non-functional complex containing U2 and U1 snRNPs (Dyhr-Mikkelsen and Kjems, 1995). A similar mechanism of splicing inhibition has been characterized recently by Kan and Green (1999) in the IgM M2 exon. An ESS in the M2 exon inhibits assembly of the splicing machinery at the upstream intron while promoting assembly of a splicing-inactive ATP-dependent complex containing U2 snRNP.

The proteins required for inhibition of splicing of *tat* exon 2 identified here, hnRNP A/B proteins, are widely expressed in mammalian tissues (Kamma *et al*., 1995; Hanamura *et al*., 1998) and are probably involved in essential cellular mechanisms. Therefore, hnRNP A/B proteins may not be an ideal target for therapy aimed at slowing down or arresting viral replication by decreasing the pool of unspliced viral messages. Nevertheless, these proteins are involved in an important conserved mechanism for regulation of splicing efficiency.

Materials and methods

Immobilization of RNA on agarose beads and binding assays

Substrate RNAs for bead immobilization were synthesized by *in vitro* transcription using T7 RNA polymerase and DNA oligonucleotide templates. These RNAs contained the sequence GGC at their 5′ ends in order to improve transcription efficiency. RNAs were covalently linked to adipic acid dihydrazide agarose beads by modification of a published procedure (Langland *et al*., 1995). RNA (500 pmol) was placed in a 400 µl reaction mixture containing 100 mM sodium acetate pH 5.0 and 5 mM sodium *m*-periodate (Sigma). Reaction mixtures were incubated for 1 h in the dark at room temperature. The RNA was then ethanol precipitated and resuspended in 500 µl of 0.1 M sodium acetate pH 5.0. A 400 µl aliquot of adipic acid dihydrazide agarose bead 50% slurry (Sigma) was washed four times in 10 ml of 0.1 M sodium acetate pH 5.0 and pelleted after each wash at 300 r.p.m. for 3 min in a clinical centrifuge. After the final wash, 300 µl of 0.1 M sodium acetate pH 5.0 was added to the beads and the slurry was then mixed with the periodate-treated RNA and rotated for 12 h at 4°C. The beads with the bound RNA were then pelleted and washed three times in 1 ml of 2 M NaCl and three times in 1 ml of buffer D [20 mM HEPES-KOH, pH 7.6, 5% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT)]. The efficiency of binding of RNA to the beads was between 70 and 80% as determined using 5′-end-labeled RNA.

The beads containing immobilized RNA were incubated in a 650 µl *in vitro* splicing reaction mixture containing 250 µl of HeLa cell nuclear extract for 20 min at 30°C. Beads were then pelleted by centrifugation at 1000 r.p.m. for 3 min and washed four times with 1 ml of buffer D containing 4 mM $MgCl_2$. After the final centrifugation, the proteins bound to the immobilized RNA were eluted by addition of 60 µl of protein sample buffer and heated for 5 min at 90°C.

Protein analysis

Proteins were separated on 13.5% SDS–polyacrylamide gels and visualized by Coomassie Blue staining or electroblotted onto a nitrocellulose membrane and probed with antibodies. mAb 4B10 against hnRNP A1 (Piñol-Roma *et al*., 1988) and mAb 4F4 against hnRNP C1/C2 (Choi and Dreyfuss, 1984) were kindly provided by Dr G.Dreyfuss (University of Pennsylvania). Rabbit polyclonal anti-A1 antiserum was kindly provided by Dr S.Riva (Istituto di Biochimica Genetica and Evoluzionistica of Pavia, Italy). Immunoblots were stained using the Vectastain horseradish peroxidase staining kit (Vector labs).

Nuclear extract depletion/reconstitution assay

Two consecutive rounds of depletion were performed as follows: a 650 µl splicing reaction mixture containing 250 µl of nuclear extract, 4 mM $MgCl_2$, 4 mM ATP, 5 mM creatine phosphate, 2.5% (w/v) polyvinyl alcohol in buffer D was incubated for 20 min at 30°C with beads containing 500 pmol of bound RNA. The beads were pelleted by centrifugation and the splicing reaction mixture supernatant saved. A 25 µl aliquot of the splicing mixture containing the twice-depleted nuclear extract was assayed for splicing activity in the presence or absence of 200 ng of total SR proteins prepared from HeLa cells as previously described (Zahler *et al*., 1992). Recombinant hnRNP A1, $A1^B$, A2, B1 and mutants M(RRM1,2) and UP1 were expressed in *Escherichia coli* and purified as described (Mayeda and Krainer, 1992; Mayeda *et al*., 1994).

Plasmid construction for splicing substrates

Plasmids pHS1-X WT and pHS1-X MUT used for generating *in vitro* splicing substrates were a generous gift from Dr C.M.Stoltzfus (University of Iowa) and have been described previously as pHS1-X and pESS10, respectively (Amendt *et al*., 1994). Plasmids pHS1-X WA1 and pHS1-X SCA1 were constructed using PCR-based mutagenesis to substitute a 20 nucleotide region containing the ESS sequence in pHS1-X for the 20 nucleotide hnRNP A1 high-affinity binding sequence previously described (TATGATAGGGTCTTAGGGTG) (Burd and Dreyfuss, 1994) or for a scrambled version of this sequence (TAATGG-AGTGGTGCGATGTA), respectively.

In vitro pre-mRNA splicing assays

Capped, ^{32}P-labeled run-off transcripts were synthesized by *in vitro* transcription using T3 RNA polymerase (Promega) from *Xho*I-linearized plasmids. HeLa cell nuclear extracts were prepared and splicing reactions were performed in a total volume of 25 µl as described (Zahler *et al*., 1992). The reaction mixtures were incubated at 30°C for 2–3 h. RNAs recovered from the splicing reaction mixtures were separated on an 8 M urea–6% polyacrylamide gel and visualized with a PhosphorImager (Molecular Dynamics).

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