A Second Exon Splicing Silencer within Human Immunodeficiency Virus Type 1 tat Exon 2 Represses Splicing of Tat mRNA and Binds Protein hnRNP H*

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An equilibrium between spliced and unspliced primary transcripts is essential for retrovirus multiplication. This equilibrium is maintained by the presence of inefficient splice sites. The A3 3′-splice site of human immunodeficiency virus type I (HIV-1) is required for Tat mRNA production. The infrequent utilization of this splice site has been attributed to the presence of a sub-optimal polypurinucleotide tract and an exonic splicing silencer (ESS2) in tat exon 2 – 60 nucleotides downstream of 3′-splice site A3. Here, using site-directed mutagenesis followed by analysis of splicing in vitro and in HeLa cells, we show that the 5′ extremity of tat exon 2 contains a second exonic splicing silencer (ESS2p), which acts to repress splice site A3. The inhibitory property of this exonic silencer was shown when inserted down-stream of another HIV-1 3′-splice site (A2). Protein hnRNP H binds to this inhibitory element, and two U-to-C substitutions within the ESS2p element cause a decreased hnRNP H affinity with a concomitant increase in splicing efficiency at 3′-splice site A3. This suggests that hnRNP H is directly involved in splicing inhibition. We propose that hnRNP H binds to the HIV-1 ESS2p element and competes with U2AF35 for binding to the exon sequence flanking 3′-splice site A3. This binding results in the inhibition of splicing at 3′-splice site A3.

Because the unique transcript produced from the integrated proviral cDNA of retroviruses serves as the genome for newly synthesized virions and also for the production of mRNAs by alternative splicing, retrovirus multiplication depends upon an equilibrium between spliced and unspliced primary transcripts. To ensure this equilibrium, retroviral RNAs generally have splice sites that are used with low efficiencies. In human immunodeficiency virus type I (HIV-1), 3′-splice sites (3′ss)1

and several central 3′ss (A3, A4a, A4b, A4c, and A5) compete with each other (Fig. 1A). Site A3 is required for production of tat mRNAs, sites A4a, b, and c for production of rev and env mRNAs and site A5 for production of nef and env mRNAs (Fig. 1A) (1). Metazoan 3′ss consist of three critical elements: the branchpoint sequence (2, 3), a polypurinucleotide tract (PPT) sequence (4, 5) and an AG dinucleotide at the 3′-end of the intron (for reviews, see Refs. 6–9). HIV-1 branchpoint sequences are highly divergent in comparison to the metazoan consensus sequence (10–12), and HIV-1 PPTs are suboptimal (short and interspersed by purines) (13–15). The affinity of factor U2AF for the PPT depends upon the presence of a long stretch of U residues (9, 16). Factor U2AF consists of two proteins, U2AF65 and U2AF35, with molecular weight of 65 and 35, respectively (17). Introns with suboptimal PPTs, like those in HIV-1 RNA, require binding of U2AF35 at the intron-exon junction for stable interaction of U2AF65 with the PPT (9, 18, 19). Furthermore, suboptimal 3′ss are often the subject of positive or negative regulation by cis-regulatory elements, which are frequently located in the 3′ exon. Exonic splicing enhancers (ESEs) increase the utilization of upstream 3′ss by binding of nuclear components that favor the association of spliceosomal components. Several identified ESEs were found to bind serine arginine-rich proteins (20–24). In contrast, exonic splicing silencers (ESSs) decrease the utilization of the upstream 3′ss. To date, the nuclear components that bind to the ESSs have been identified for only a limited number of ESSs: serine arginine-rich proteins for the bovine papillomavirus type-1 (25), heterogeneous nuclear ribonucleoprotein H (hnRNP H) for the rat β-tropomyosin exon 7 ESS (26) and the Rous sarcoma virus NRS (27), hnRNP A1 for the fibroblast growth factor receptor 2 K-SAM ESS (28) and for an ESS in exon v5 of CD44 (29). To date, one ESE (30, 31) and three ESSs (15, 30–33) have been identified in HIV-1 RNA. One of the ESSs (ESS2) is located within tat exon 2 and regulates the utilization of the A3 3′ss (Fig. 1A) (14, 30, 32). The second ESS (ESS3) is located within tat-rev exon 3 and regulates the utilization of the A7 3′ss (15, 30, 31). The third ESS (ESSV) was recently discovered downstream of 3′-splice site A2 (33). HnRNP A/B proteins were found to selectively bind both the HIV-1 ESS2 and ESSV ele-
ments, and this binding is necessary for the inhibitory properties of these ESS elements (33, 34). The hnRNP proteins are a family of nuclear proteins that package nascent pre-messenger RNAs early after their transcription by RNA polymerase II (for review, see Ref. 35). HnRNP reactions are involved in several steps of mRNA production, including transcription regulation, modulation of alternative splicing, and mRNA stabilization and localization (for review, Ref. 35). HnRNP A1 was previously found to modulate the choice of alternative 5′ss (36–40). Its involvement in inhibition of 3′ss utilization by binding to ESSs was found more recently, and, in this case, the mechanism of inhibition is still unknown. HnRNP H was found to bind both ESE (c-src ESE (41) and ESS (26)) elements.

We recently proposed a model for the secondary structure of the HIV-1 RNA region containing the five central 3′ss (A3, A4a, A4c, A5, and A5) (42). Splice site A3 is contained in the terminal loop of a conserved stem-loop structure (SLS2), and its suboptimal PPT is in the helix. To complete this previous study, we looked for the interdependence between sequence and secondary structure at site A3 and generated mutations in both the PPT and the opposite strand of SLS2. Analysis of these mutants revealed the presence of a second ESS element acting to repress splicing at 3′-splice site A3 (ESS2p). Demonstration of the presence of this ESS2p element and the possible involvement of protein hnRNP H as a mediator of its inhibitory property are presented in this report.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Plasmid pHU3 (43) was used as the source of cDNA sequences from the HIV-1 BRU/LAI strain (GenBank™ accession number K02013). PCR amplifications were done according to Nour et al. (44). Three DNA fragments, H1, H2, and H3, corresponding to the HIV-1BRU cDNA regions 1–365, 5172–5408, and 5172–5637, respectively (numbering according to ref. 45), were PCR-amplified with the primer pairs O-605/O-606, O-567/O-608, and O-567/O-1114, respectively (Table I). All the constructs were made in plasmid pBluescriptKSI cleaved with the BamHI and PstI nuclease. PCR-amplified fragments were digested at restriction sites generated by the primers (see Table I). In plasmid pHD-C5, fragments H1 and H2 have been ligated together using their own restriction sites. Similarly, in plasmid pHD-L3-U1, fragments H1 and H3 have been ligated together. Plasmid pHD-L2-C2 contains the HIV-1BRU RNA regions 1–365 and 4696–5053 (10). Plasmid pHPS, used for the transfection experiments, was constructed in the following way. Infectious HIV-1 plasmid pNL4-3 (GenBank™ accession number M19921) was cleaved with SpeI and BsuIII nucleases to generate fragments E3-C3-WT and E3-C3-A. This fragment was ligated together with oligonucleotides 5′-CTAGACCCTGTTTG-3′ and 5′-GAAAGCTTGTACTCC-3′, which had been previously annealed, to form a double-stranded linker. This created an HIV-1 plasmid deleted between nt 1511 and 4551.

Site-directed Mutagenesis—To generate plasmid pSJ-C3-1227, the inserted BamHI-PstI fragment of plasmid pHD-C3 was cloned into plasmid pHU3 containing phage M13mp9 and site-directed mutagenesis was performed according to Kramer et al. (46), using oligonucleotide O-1227 (see Table I), the mutated BamHI-PstI DNA fragment was reinserted into plasmid pBluescriptKSI™. All other site-directed mutagenesis analyses were performed by the PCR method, using the Stratagene QuikChange™ site-directed mutagenesis kit. The plasmids shown in Table I pPSF variants were constructed by replacement of the HIV-1pNL4-3 EcoRI-BsuIII fragment (positions 5743–5985) by the corresponding HIV-1BRU EcoRI-BsuIII fragment (positions 5325–5577) from plasmids pHD-L3-U1, pSJ-L3-U1-1227, or pSJ-L3-U1-1228. To create the pPSF-1695 variant plasmid, pPSF was cleaved with EcoRI and SalI nucleases and ligated together with oligonucleotides L3A-sense and L3A-antisense (see Table I), which had been previously annealed, to form a double-stranded mutated linker with EcoRI and SalI sticky ends. To create variants pPSF-1691, pPSF-1693, and pPSF-1696, the EcoRULuxhol fragment from pPSF WT was cloned in plasmid pBluescriptKSI™ cleaved by the same enzymes and the mutations were generated by the PCR method, using the appropriate primers (Table I). The EcoRULuxhol fragments from these mutant constructs in pBluescriptKSI™ (nt 5743–8465) were ligated into pPSF, which was cleaved with EcoRI and BamHI. All plasmids were sequenced to confirm the expected base changes or deletions.

The HIV-1BRU cDNA regions encoding the WT or variant second exon of the C3 transcript, produced from plasmid pHD-C3, were PCR-amplified from plasmid pHD-C3 with the antisense primer O-608 (see Table I) and a sense primer generating a T7 RNA polymerase promoter (for gel shift experiments) or a SP6 RNA polymerase promoter (for in vitro splicing assays) of the appropriate precursor (positions 6690–6715 (SP6), 1734 (T7)) or 10–1992 (SP6), or 1738 (T7) or O-1990 (SP6) (Table I) were used to amplify fragments E2-C3-WT, E2-C3-A, and E2-C3-C, respectively. Similarly, sense primer O-1989 and antisense primer O-608 were used to amplify fragment E2-C3LESS2p. Finally, fragments E2-C2-WT and E2-C2ESS2p were PCR-amplified from plasmid pHD-C2 with the sense primer O-755 and the antisense primer O-1988 and O-1987, respectively, which generated an SP6 RNA polymerase promoter (Table I).

Enzymatic Probing of RNA Secondary Structure—Synthesis of nonradioactive transcripts for RNA secondary structure analysis and enzymatic digestions with V1 RNase and S1 nuclease were performed as previously described (42). Positions of enzymatic cleavages were identified by primer extension analysis with the avian myeloblastosis virus reverse transcriptase (Life Science) using primer O-608 (Table I). Oligonucleotide primers were 5′-end-labeled with γ-32P[ATP 3000 Ci/ mmol (Amersham Pharmacia Biotech). Annealing of primers and primer extension was made as previously described (42).

In Vitro Splicing Assays—Prior to transcription with T7 RNA polymerase, constructs O-5671C3 and O-5671C2 constructs and its derivatives were linearized with the PstI nuclease, whereas plasmid pHD-C2 construct and all its derivatives were linearized with the EcoRI nuclease. For splicing assays, uniformly labeled capped transcripts were synthesized and in vitro splicing assays were performed with HeLa cell nuclear extracts from the Computer Cell Culture Center S.A. (Belgium), using 100,000 Cerenkov cpm (~40 fmol) of RNA transcript per assay (10). The reaction mixture was prepared on ice and then incubated at 30°C for 120 min. Spliced products were deproteinized with proteinase K, phenol-extracted, and analyzed on a 5% polyacrylamide sequencing gel. Splicing efficiency was estimated by scanning the gel with a Molecular Dynamics PhosphorImager using ImageQuant software, version 3.3. The M/P ratio (amount of mature mRNA versus the amount of residual precursor) was determined for each transcript, taking into account the estimated radioactivity and the number of uracil residues per molecule.

In Vivo Splicing Assays—HeLa cells were transfected by the modified calcium phosphate coprecipitation technique with 12 μg of plasmid DNA as described above (47). Total cellular RNA was isolated from transfected HeLa cells 48 h post-transfection, and 3 μg of RNA was reversed-transcribed and PCR-amplified with forward oligonucleotide 5′-GTCGAGCCGGCGGCGGCT-3′ and reverse oligonucleotide 3′-GGCTTGCTGAAGCGCGCACGGCAAGAGG-5′, nt 700–727 and reverse primer SJ47A, which spans sites D4 and A7 (5'-TTGGGAGTTGGGTTCCTGTATGAG-3', nt 8369–8381 and 6032–6044) (47). After verification of the presence of amplified spliced products by PAGE, amplification products (100 ng) were radiolabeled by performing a single round of PCR with the addition of 10 μCi of [α-32P]dCTP, and the products were analyzed by electrophoresis on a 6% polyacrylamide 7× urea gel.

Electrophoretic Mobility Shift Assays—DNA matrices were produced by PCR amplification using adapted primers (Table I), one of them generating a T7 or an SP6 promoter. Transcriptions were carried out on 0.9 pmol of PCR product in a 10-μl reaction mixture containing 20 μM MgCl2, 10 mM NaCl, 40 mM Tris-HCl, pH 7.9, 10 mM DTT, 0.1 mg/ml bovine serum albumin, 10 units of RNAsin, 4 mM each dATP, dCTP, rGTP, 0.4 mM UTP, 8 μM of [α-32P]UTP (800 Ci/mmol) (ICN), and 70 units of T7 RNA polymerase (USB Pharmacia Biotech). Transcripts were then treated as previously described (10). For RNP complex formation, the following RNA binding mixture was used: 20 μM HEPES (pH 7.9), 3 mM MgCl2, 0.1 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.25 mM phenylmethylsulfonyl fluoride, 20% glycerol, 1 μg/ml yeast tRNA, and 0.5–4 μl of HeLa cell nuclear extract (Computer Cell Culture Center S.A., Belgium) in a total volume of 10.5 μl. For competition experiments, the RNA binding mixture was preincubated for 15 min at 30°C with 20–200 ng of unradiolabeled competitor RNA, synthesized from 1.8 pmol PCR product with the T7-MEGASHortscript kit (Ambion). All EMSAs (with or without competitor RNA) were performed with 50,000 cpm (6 pmol) of 32P-labeled transcript added to the RNA binding mixture. Incubation was performed for 15 min at 30°C, heparin (5 μg/ml) was then added as a nonspecific competitor, and the incubation was continued for 10 min at room temperature. To test for the effect of antibodies directed against individual hnRNP proteins on the stability and the electrophoretic mobility of the shifted RNA-protein complexes, before the heparin treatment, 1 μl of a polyclonal anti-hnRNP H antibody directed against an hnRNP H peptide located at the C terminus
A Second ESS Element Acting on HIV-1 3' Splice Site A3

The number, the sequence, and the utilization of each oligonucleotide are given. Sequences identical (i) or complementary (c) to HIV-1 regions are underlined. Nucleotide positions of these HIV-1/BRU regions are numbered according to the GenBank™ accession number K00213. Restriction sites introduced by the oligonucleotides are indicated and the sequence corresponding to this site is in italics. T7 and SP6 promoter sequences are in boldface. Lowercased letters indicate the mutations introduced by site-directed mutagenesis in HIV-1/BRU RNA. The name of the resulting plasmids is given.

| Primer number | Sequence (5’ → 3’) | Restriction site | HIV-1/BRU sequence | Utilization | Generated variant |
|---------------|---------------------|------------------|--------------------|-------------|-------------------|
| O-605         | TATTCTGGATCCGTCCTGC TGTG | BamHI | 1–12 (i) | PCR | |
| O-606         | TATTCTAAGTCTCCATGCATCTA | BgII | 373–385 (c) | PCR | |
| O-607         | AGAAGACCAAGACCTAGGAGGACCA | BgII | 5155–5165 and PCR | | |
| O-608         | TATTCTGCGAGTTGCTCTCTT | PsI | 5397–5408 (c) | PCR | Primer extension |

molecular of the gene (a generous gift of D. Black, University of California) (41) or 1 μl of the anti-hnRNP A1 monoclonal antibody 4B10 (a generous gift of G. Dreyfuss, University of Pennsylvania) were added and incubation was continued for 15 min at 30 °C. RNP complexes were then fractionated onto a 6% polyacrylamide (38:2) gel with 5% (w/v) glycerol in 1× TBE buffer, 45 mM Tris borate (pH 8.3), and 0.1% (v/v) Tween 20 (48). To separate the free from the bound RNA or shifted protein complexes were soaked in 10 mM DTT, 10% glycerol, 0.1% (v/v) Tween 20, and heated for 10 min at 95 °C. The digested cross-linked products were then incubated with the T7 and SP6 promoter sequences are in boldface. Lowercased letters indicate the mutations introduced by site-directed mutagenesis in HIV-1/BRU RNA. The name of the resulting plasmids is given.

UV Cross-linking Reactions, Immunoprecipitation, and Immunoblotting—Radiolabeled RNAs were produced by SP6 transcription using the suitable PCR products as matrices (Table I). SP6 RNA transcripts were synthesized in a 15-μl reaction mixture containing 20 mM MgCl₂, 10 mM NaCl, 40 mM Tris-HCl, pH 7.9, 10 mM DTT, 0.1 mg/ml bovine serum albumin, 10 units of RNAsin, 0.3 mM each rATP, rCTP, rUTP, 3 μM GTP, 30 μCi of [α-32P]GTP (3000 Ci/mmol, ICN), and 40 units of SP6 RNA polymerase (USB Pharmacia Biotech). Incubation in nuclear extract was for 15 min at 30 °C as for EMSA, except that 500,000 cpm (50 fmol) of radiolabeled transcripts was used. Formation of RNP complexes was verified by EMSA. Reaction mixtures containing RNP complexes were transferred to 96-well plates for irradiation at 4 °C with 254-nm UV light 1 cm from the source for 10 min. RNA components were then directly digested in the 96-well plates by addition of 50 units of T1 RNase in each well, and incubation was for 30 min at 50 °C. Protein G-Sepharose beads (Amersham Pharmacia Biotech) were precoted with 1 μl of anti-hnRNP H antisera or 1 μl of anti-hnRNP A1 antibody 4B10 for 2 h at 4 °C.

The digested cross-linked products were then incubated with the precoted beads for 2 h at 4 °C in 400 μl of immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Nonidet P-40) containing 0.1 mg/ml bovine serum albumin. Subsequently, beads were washed three times with the immunoprecipitation buffer containing 0.25% Nonidet P-40. After centrifugation, the beads were resuspended in 20 μl of SDS-PAGE loading buffer (80 mM Tris-HCl, pH 6.8, 3% SDS, 10% glycerol, 0.1% bromophenol blue) and boiled for 5 min for elution of the immunoprecipitated proteins. The proteins were resolved by 10% SDS-PAGE. Each sample of the eluted proteins was then directly digested in the 96-well plates by addition of 50 units of T1 RNase in each well, and incubation was for 30 min at 50 °C. Protein G-Sepharose beads (Amersham Pharmacia Biotech) were precoted with 1 μl of anti-hnRNP H antisera or 1 μl of anti-hnRNP A1 antibody 4B10 for 2 h at 4 °C.

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To test for the presence of protein hnRNP H or hnRNP A1 in the fractionated RNA-protein complexes, the gel slices containing the RNA-protein complexes were soaked in 10 μl of SDS-PAGE loading buffer for 1.5 h at 37 °C and boiled for 5 min. The piece of gel and the eluate were then divided into two parts: One part was fractionated on a gel used for elution of the immunoprecipitated proteins. The proteins were resolved by 10% SDS-PAGE. Each sample of the eluted proteins was then directly digested in the 96-well plates by addition of 50 units of T1 RNase in each well, and incubation was for 30 min at 50 °C. Protein G-Sepharose beads (Amersham Pharmacia Biotech) were precoted with 1 μl of anti-hnRNP H antisera or 1 μl of anti-hnRNP A1 antibody 4B10 for 2 h at 4 °C.

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RESULTS

Modulation of the in vitro Utilization of Site A3 by Its Downstream Sequence—To test for the effects of the sequence and the RNA secondary structure of the splice site A3 PPT on usage
of this splice site, RNA transcripts of the WT or variant pLD-C3 constructs (Fig. 1AII, C3 transcripts) were produced and used for in vitro splicing assays. WT and variant C3 transcripts contained the HIV-1/BRU RNA portion from positions 1 to 385 fused to the HIV-1/BRU RNA region from positions 5172 to 5408, plus 52 nt arising from the p Bluescript vector at the 5′ terminus (Fig. 1AII). Hence, D1 5′s and the A3 3′s are present in these transcripts, but the previously identified ESS2 element is absent (Fig. 1AII). Splicing efficiency of transcript C3 WT in a HeLa cell nuclear extract was relatively low as determined by the M/P ratio (0.03) between spliced (M) and unspliced RNA (P) (Fig. 1C). In agreement with previous results (14), after two A-to-U substitutions in the PPT of transcript C3 WT (variant C3-1227, Fig. 1AIII), the in vitro utilization of site A3 was increased by a factor of about 7.5 (M/P = 0.22) (Fig. 1C). According to the secondary structure established for the HIV-1 RNA region containing site A3 (42), the two A-to-U substitutions in variant C3-1227 destabilized the SLS2 helix 2. This was verified experimentally by V1 and S1 nucleases probing, and the results are schematically presented in Fig. 1B.

To discriminate between sequence and secondary structure effects on splicing efficiency, two other mutant C3 RNAs (C3-1228 and C3-1695) were produced. In variant C3-1228, two U-to-A substitutions were introduced in the 3′-strand of helix 2, such that a helix with similar stability to that of helix 2 in the WT RNA was formed. As shown in Fig. 1B, formation of the expected helix 2 was demonstrated experimentally by use of V1 and S1 nucleases. Fig. 1C shows that no spliced RNA was detected after a 120-min incubation of RNA C3-1228 in a HeLa cell nuclear extract. Interestingly, this absence of splicing was also observed for variant C3-1695, where only the two U-to-A substitutions in the downstream sequence were introduced (Fig. 1, AIII and C). This suggested that the absence of splicing observed for variant C3-1228 was not due to the restoration of the WT RNA secondary structure but to the alteration of the sequence located downstream from site A3. Accordingly, the U-to-A substitution at position 5362 (variant 1691) was sufficient to abolish splicing (Fig. 1, AIII and C). In contrast, when the U residue was substituted for an A residue only at position 5366 (variant 1693), splicing efficiency was slightly higher as compared with WT RNA (M/P of 0.05 instead of 0.03). Finally, when the two U residues at positions 5362 and 5366 were substituted by C residues (variant 1606), splicing efficiency was markedly increased (M/P of 0.10 versus 0.03). The in vitro splicing efficiencies of the WT and variant C3 RNAs were tested several times, and reproducible results were obtained. These results suggested that splicing efficiency at site A3 is modulated by the sequence within tat exon 2 immediately downstream of splice site A3.

Because utilization of site A3 was previously shown to be negatively regulated by the ESS2 element located about 60 nt downstream from site A3 (14, 32), it was important to test whether the effects of the A3 downstream sequence on site A3 efficiency were also observed in the presence of ESS2. For this purpose, the above mutations were also tested in plasmid pLD-L3.U1, which contains the entire tat exon 2 (Fig. 1AII). As found for the C3 series, mutations 1606 and 1693 increased in vitro splicing efficiency at site A3 in the L3 series, whereas mutations 1691 and 1695 drastically decreased splicing efficiency at this site (Fig. 1D). No significant differences in splicing at 3′-splice sites A4a, A4b, A4c, and A5 were detected for the various transcripts studied (Fig. 1D). Therefore, our results indicate that, both in the presence or the absence of ESS2, the utilization of site A3 in the in vitro splicing assays was modulated by the identity of its downstream sequence.

Site A3 Utilization Is Also Modulated by Its Downstream Sequence in an HIV-1 RNA Context—To verify that the results obtained for the WT and variant C3 and L3.U1 RNAs were not artifacts of in vitro splicing assays, we tested the effects of the mutations (1227, 1228, 1606, 1691, 1693, and 1695) on splicing of HIV-1 RNA in HeLa cells (42, 47). For this purpose, we used a plasmid (p∆PSP) (see “Experimental Procedures”) that contains the HIV-1/pNL4-3 proviral genome deleted between nt 1511 and 4550 in the D1-A1 intron. This construct contains all the HIV-1 splicing sites, and the relative usage of these splice sites in cells transfected with this construct is similar to virus-infected T cells (1).

To test for the effect of substitutions in the A3 PPT and/or the downstream sequence, a fragment of ~200 nt containing splice site A3 was replaced by the corresponding HIV-1/BRU sequence. Either the WT (plasmid p∆PSP-WT) or the variant HIV-1/BRU sequences (plasmids p∆PSP-1227, p∆PSP-1228, p∆PSP-1606, p∆PSP-1691, p∆PSP-1693, or p∆PSP-1695) were used. HeLa cells were transfected with the various p∆PSP plasmids and mRNAs produced by splicing of the mini-HIV-1 primary transcript were analyzed by RT-PCR (47). As a consequence of the two A-to-U substitutions in the A3 PPT (plasmid p∆PSP-1227), the relative yields of both Tat 1 and Tat 2 mRNAs, both of which are spliced at site A3, were strongly increased. On the other hand, the relative yields of nef mRNAs (e.g. Nef 3 and Nef 4) were decreased (Fig. 2A). However, the increase of Tat 1 and Tat 2 was reduced for plasmid p∆PSP-1228, which contained, in addition to the two A-to-U substitutions in the PPT, two U-to-A substitutions in the downstream sequence. Little or no product spliced at site A3 was detected for plasmid p∆PSP-1695, which carried the two downstream U-to-A substitutions without optimization of the PPT. A single U-to-A substitution at position 5362 in the downstream sequence (plasmid p∆PSP-1691) also increased the yields of Tat 1 and Tat 2 mRNAs but to a lesser extent. As found by in vitro splicing assays, the U-to-A substitution at position 5366 (plasmid p∆PSP-1693) slightly increased the level of Tat 1 and Tat 2 mRNAs, whereas the two U-to-C substitutions (plasmid p∆PSP-1606) strongly increased the yields of Tat 1 and Tat 2 mRNAs with a corresponding decrease of nef and rev mRNAs (Fig. 2B). Thus, the effect of the downstream mutation in the presence of an optimized PPT was less deleterious in HeLa cells than in vitro. However, in the presence of the WT PPT, variations of site A3 efficiency as a consequence of downstream mutations were similar in vitro and in HeLa cells, demonstrating the importance of the downstream sequence on site A3 utilization in the context of HIV-1 RNA.

A 9-nt Sequence Located Downstream from Site A3 Has an ESS Activity—Because the U-to-A mutations downstream from site A3 strongly decreased splicing efficiency at this site, whereas U-to-C substitutions increased utilization of splice site A3, we determined whether the WT A3 downstream sequence contained an ESE or an ESS element. To answer this question, we generated a template (pSJ-C3AESS2p) used to synthesize pre-mRNA deleted between nt 5360 and 5368 (Fig. 3A). As shown in Fig. 3B, this deletion resulted in an approximate 6-fold increase in in vitro splicing at site A3 (M/P = 0.56 versus 0.09). This increase suggested that an exonic splicing silencer (ESS) was present downstream from site A3. This potential silencer was designated ESS2p (proximal ESS2) to distinguish it from the previously identified ESS2 element acting on site A3.

To demonstrate ESS activity of the ESS2p silencer element in a heterologous context, we inserted this element downstream from another HIV-1 3′s, site A2 (pLD-C2 construct).
FIG. 1. Base substitutions downstream from site A3 modulate its in vitro splicing efficiency. A, schematic representation of the HIV-1 genome is given in panel AI. The 5′ss (D) and 3′ss (A) are shown. Boxes indicate open reading frames. The C3 WT and the L3.U1 WT transcripts are shown in panel AII. Numbering of the HIV-1/BRU RNA sequences is according to Ratner et al. (45). Exon sequences are represented by rectangles, introns by thin lines, the thick horizontal line at the beginning of the transcripts correspond to sequences generated by plasmid pBluescriptKSII. The junction between the two HIV-1 RNA regions within the mini-intron is indicated by a vertical broken line. The C3 and L3.U1 variant sequences generated by site-directed mutagenesis are aligned with the WT sequence in part AIII. The base substitutions are underlined.

B, schematic representation of data from enzymatic probing of the WT and three variant RNAs on the SLS2 structures. Cleavages by S1 nuclease (arrows surmounted with circles) and V1 RNase (arrows surmounted with squares) are shown. Three black circles or squares indicate a strong cleavage, two gray circles or squares a medium cleavage, one white circle or square a low cleavage. Base substitutions in variant SLS2 s are indicated by circled nucleotides. The free energy of the proposed stem-loop structures at 37 °C, in 1 M NaCl was calculated with MFold software. Positions of nucleotides in the HIV-1/BRU RNA region are given. The A3 3′ss is indicated by an arrow.

C, polyacrylamide gel electrophoresis of the in vitro splicing products of the WT and variant C3 transcripts. Uniformly labeled RNAs were prepared as described under "Experimental Procedures" and were incubated for 120 min in a HeLa cell nuclear extract under splicing conditions. The untreated C3 transcript was fractionated.
The C2 RNA, transcribed from construct pLD-C2, contained the HIV-1/BRU RNA region from positions 1 to 385 fused to the HIV-1/BRU RNA sequence between nt 4669 and 5053 (10) (Fig. 3A). In the variant C2ESS2p RNA, the distance between site A2 and the inserted ESS2p element was identical to that between site A3 and the ESS2p element in the WT A3 site context (Fig. 3A). As shown in Fig. 3B, insertion of the 9-nt ESS2p element decreased splicing at site A2 by a factor of ~5 (M/P = 0.27 versus 0.05). Splicing was blocked before the first step of the reaction, because production of the first exon and the lariat intermediate were also strongly decreased (Fig. 3B). From these data, we concluded that the 9-nt sequence downstream from site A3 (nt 5360–5368) was sufficient to inhibit splicing at site A2 and, therefore, that this sequence had the properties of a splicing silencer.

**U-to-A Substitutions in the ESS2p Element Modify Interaction with Nuclear Components**—Because effects of ESSs have been shown to be mediated by the binding of nuclear proteins (25, 28, 29, 49), the interaction of nuclear components with ESS2p was investigated by electrophoretic mobility shift assays (EMSA). To this end, radiolabeled E2-C2 transcripts containing the WT or the mutated HIV-1/BRU RNA region from positions 5359 to 5408 (3’-exon of the C3 RNA) were produced by in vitro transcription with T7 or SP6 RNA polymerase (Fig. 4A). To limit nonspecific RNA-protein interactions, mobility shift experiments were performed in the presence of heparin and competitor tRNAs. After incubation in a nuclear extract, complexes were formed with the three T7 RNA polymerase transcripts tested (Fig. 4B). Similar results were obtained with the SP6 RNA polymerase transcripts (data not shown). One major complex (I) was formed with the E2-C3-WT and E2-C3-C (containing the two U-to-C substitutions) transcripts at all the tested concentrations of nuclear extract. This complex had a lower electrophoretic mobility compared with the major complex II that was formed with the E2-C3-A transcript. At higher nuclear extract concentrations, a diffuse band corresponding to complex II was also detected for transcript E2-C3-A. These data suggested that distinct RNA-protein complexes were formed, depending on the nucleotide sequence of the ESS2p element.

As shown in Fig. 4B, E2-C3-WT and E2-C3-C transcripts on
FIG. 4. EMSA performed with the WT or variant C3 exon 2 and nuclear extract. A, schematic representation of the E2-C3-WT, E2-C3-A, and E2-C3-C transcripts used for electrophoretic mobility shift assays. The ESS2p base substitutions in transcripts E2-C3-A and E2-C3-C are indicated by underlined letters. B, detection of an electrophoretic mobility shift after incubation of transcripts E2-C3-WT, E2-C3-A, and E2-C3-C with increasing amounts (0.5, 1, 2, 3, and 4 μl) of nuclear extract, under conditions described under “Experimental Procedures.” Gel electrophoresis was in 6% polyacrylamide (38:2)-5% glycerol gel in TBE buffer. Positions of the complex I formed with transcripts E2-C3-WT and E2-C3-C and the complex II formed with the E2-C3-A transcript and of free RNA are indicated on the right of the panel. The C 1F (bound RNA in complex I/free RNA) and C 1F (bound RNA in complex II/free RNA) ratios were calculated by estimation of radioactivity in the bands of gel with a PhosphorImager as described under “Experimental Procedures” and are given below the lanes. C, detection of an electrophoretic mobility shift after incubation of about 100,000 cpm of radiolabeled E2-C3-WT or E2-C3-A RNA (indicated above the lanes) in 4 μl of nuclear extract, in the presence of increasing amounts (0, 50, 100, and 200 ng) of unlabeled competitor RNA (E2-C3-A or E2-C3-WT as indicated above the lanes). Positions of complexes I and II and free RNA are indicated. D, displacement curves of complex I formed with the radiolabeled E2-C3-WT RNA by unlabeled competitor RNAs: E2-C3-WT (black squares) or E2-C3-C (white squares). About 100,000 cpm of radiolabeled E2-C3-WT RNA were incubated in 4 μl of nuclear extract in the presence of increasing amounts (30, 50, 100, and 200 ng) of competitor RNA under conditions described under “Experimental Procedures.” The percentage of bound RNA to total RNA calculated from PhosphorImager measurements was plotted versus the amount of competitor RNA together with standard deviation estimated from three separate experiments.

Together, the EMSA and competition data suggested that two different proteins or sets of proteins of the nuclear extract were binding to ESS2p depending on whether pyrimidines or A residues were present at positions 5362 and 5366. This may explain the higher inhibitory activity of ESS2p after the U-to-A substitution. The data also suggested that the same protein or set of proteins was binding to the WT ESS2p sequence and the ESS2p sequence with C substitutions at positions 5362 and 5366, with, however, substantial differences in affinity for this protein or set of proteins, which may explain the increase in splicing efficiency observed after U-to-C substitutions.

hnRNP HBinds to the C3 Exon 2—Interestingly, the sequence generated by the two U-to-A substitutions in ESS2p contained the winner binding sequence for hnRNP H, selected from a pool of randomized RNA sequences (50) (Fig. 4A). On the other hand, the WT ESS2p sequence shows some homology with the ESS element found in the rat β-tropomyosin pre-mRNA, which binds hnRNP H (26) (Fig. 4B). Proteins hnRNP A1 and hnRNP H have molecular masses of 35 and 55 kDa, respectively. Hence, binding of hnRNP A1 to the E2-C3-A RNA might be expected to generate an RNP complex of higher electrophoretic mobility as compared with binding of protein hnRNP H to the E2-C3-WT or E2-C3-C RNAs. This could explain the difference of electrophoretic mobility of RNP complexes depending on the ESS2p sequence. To test for the involvement of hnRNP H and hnRNP A1 in complex I and II formation, we performed immunoprecipitation assays of the UV cross-linked proteins using specific antibodies directed against hnRNP H or hnRNP A1 proteins, respectively.

We had found that G residues added at the extremities of T7 RNA polymerase transcripts can generate additional hnRNP A1 binding sites of low affinity. Therefore, because T7 and SP6 transcripts gave similar results in EMSA, SP6 transcripts were used for immunoprecipitation assays. Because the ESS2p elements of the three RNAs studied (E2-C3-WT, E2-C3-C, and E2-C3-A) contain the same number of G residues, but variable numbers of A, U, and C residues, the E2-C3-WT, E2-C3-C, E2-C3-A, and E2-C3-ΔESS2p RNAs were uniformly labeled by

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Fig. 5. Cross-linking of proteins hnRNP A1 and H to the WT and mutated C3 and C2 exon 2. A and B, the E2-C3-WT or variant RNAs and E2-C2-WT and variant RNA were incubated in 4 μl of nuclear extract. After RNA-protein cross-linking at 254 nm as described under “Experimental Procedures” and digestion with T1 RNase, proteins hnRNP A1 and H were each immunoselected with a specific antibody bound to G-Sepharose beads (anti-hnRNP H antiserum, panel A upper gel, and anti-hnRNP A1 monoclonal antibody 4B10, panel B upper gel). The eluted material was fractionated by 10% SDS-PAGE, and radiolabeled proteins hnRNP H and A1 were detected by autoradiography. As a control of hnRNP H and A1 immunoselections, the proteins in the polyacrylamide gel were transferred to a nitrocellulose membrane, probed with anti-hnRNP H (panel A, lower gel) or anti-hnRNP A1 (panel B, lower gel) antibodies and followed by ECL detection. The ratios (a/b) of radiolabeled hnRNP protein (a) to total immunoselected hnRNP protein (b), indicated below the lanes, were calculated as described under “Experimental Procedures.”

incorporation of [α-32P]GTP. Labeled RNAs were incubated in a HeLa cell nuclear extract followed by UV-cross-linking. After T1 RNase digestion, hnRNP A1 and hnRNP H were each immunoselected with specific antibodies bound to protein G-Sepharose beads (anti-hnRNP A1 antibody, generous gift of G. Dreyfuss and anti-hnRNP H antiserum, generous gift of D. Black). The presence of radiolabeled nucleotides cross-linked to hnRNP H or hnRNP A1 was detected by SDSPAGE, followed by autoradiography (Fig. 5, A and B).

As shown in Fig. 5A, a high amount of cross-linked hnRNP H was detected for RNA E2-C3-WT. This amount was decreased by a factor of 3 for RNA E2-C3-C. Only trace amounts of labeled hnRNP H were detected for RNA E2-C3-WT. This amount was decreased by a factor of 3 for RNA E2-C3-A and RNA E2-C2-WT and variant RNA were each immunoselected with a specific antibody bound to G-Sepharose beads (anti-hnRNP H antiserum, panel A upper gel, and anti-hnRNP A1 monoclonal antibody 4B10, panel B upper gel). The eluted material was fractionated by 10% SDS-PAGE, and radiolabeled proteins hnRNP H and A1 were detected by autoradiography. As a control of hnRNP H and A1 immunoselections, the proteins in the polyacrylamide gel were transferred to a nitrocellulose membrane, probed with anti-hnRNP H (panel A, lower gel) or anti-hnRNP A1 (panel B, lower gel) antibodies and followed by ECL detection. The ratios (a/b) of radiolabeled hnRNP protein (a) to total immunoselected hnRNP protein (b), indicated below the lanes, were calculated as described under “Experimental Procedures.”

We then used two approaches to confirm the presence of protein hnRNP H in complex I and protein hnRNP A1 in complex II. In these experiments a two-step gel electrophoresis was used followed by immunoblotting with anti-hnRNP H (Fig. 6, A and B) or anti-hnRNP A1 (Fig. 7C) antibodies. As illustrated in Fig. 7, C and D, protein hnRNP A1 but not protein hnRNP H was detected in complex II formed with RNA E2-C3-A. The trace amounts of protein hnRNP A1 seen in the control lane may be due to the presence of endogenous RNA in the extract bound to this protein. Protein hnRNP H was detected in complexes I formed with the E2-C3-WT and E2-C3-C RNAs (Fig. 7B). As a control, the band of gel at the same level as complex I in the lane corresponding to the E2-C3ΔESS2p RNA was analyzed. It contained no protein hnRNP H (Fig. 7B), which reinforced the idea that ESS2p is required to bind protein hnRNP H. The above immunoprecipitation data suggested a lower affinity of protein hnRNP H for E2-C3-C RNA as compared with E2-C3-WT RNA. Accordingly, a lower amount of protein(s) hnRNP H was detected for the E2-C3-C RNA compared with the E2-C3-WT RNA in the Western blot analysis illustrated in Fig. 7B. Two closely spaced bands were present when the total proteins of the nuclear extract (NE) or proteins in complex I were analyzed by the anti-hnRNP H antibodies. This is in contrast to the immunoprecipitation experiments, in which only one band was detected with anti-hnRNP H antibodies (Fig. 5A).

However, protein heterogeneity may be masked in this case by the presence of cross-linked ribonucleotides. One possible explanation for the double band in Fig. 7B is the presence of isoforms
of hnRNP H, which bind to E2-C3-WT and E2-C3-C RNAs as monomers or heterodimers. Based on the peptide used to produce the anti-hnRNP H antibodies, the homologous hnRNP F protein (51) should not be detected by these antibodies (41). The two detected proteins may simply differ by the degree of post-translational modification.

All the data obtained from EMSA, immunoprecipitation assays, supershift assays, and two-step gel electrophoresis were in good agreement and were consistent with the hypothesis that protein hnRNP H binds to the WT ESS2p and to the ESS2p mutant with 2C residues, whereas protein hnRNP A1 binds to the ESS2p mutant with 2A, explaining the difference of splicing efficiency.

The ESS2p Sequence Is Sufficient to Generate an hnRNP H Binding Site—As shown above, insertion of ESS2p downstream from site A2 limited site A2 utilization. It was important to verify that the insertion of ESS2p downstream from this site generated an hnRNP H binding site, which could explain the observed inhibition. To this end, UV-cross-linking and immunoselection experiments were performed as described above with the E2-C2-WT transcript corresponding to the 5′ portion of the 3′-exon of the C2 RNA and with the same RNA portion

where the 5′-AUUGGGUGU-3′ ESS2p sequence had been inserted (transcript E2-C2-ESS2p, Fig. 5, A and B). In agreement with recent data of Bilodeau et al. (33), both the E2-C2-WT and E2-C2-ESS2p transcripts cross-linked at high levels to hnRNPs A1 (Fig. 5B). Only trace amounts of hnRNPs H were cross-linked with transcript E2-C2-WT, whereas a significant amount of cross-linked hnRNPs H was observed for transcript E2-C2-ESS2p (Fig. 5A). Because the immunoblotting experiment confirmed that similar amounts of hnRNPs H were immunoselected (Fig. 5A, lower gel), we concluded that insertion of the ESS2p sequence was sufficient to generate a binding site for hnRNPs H. These data are consistent with the hypothesis that ESS2p inhibits splicing by binding to hnRNPs H.

**DISCUSSION**

**Highly Controlled Utilization of Site A3 in HIV-1 RNA—** Utilization of the A3 3′s of HIV-1 RNA is required for tat mRNA production. It has previously been shown that splicing at 3′-splice site A3 is repressed by the ESS2 cis-acting element
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(14, 30, 31). Here, we show that a second inhibitory element, ESS2p, also acts to repress splicing at site A3. Because ESS2p inhibits splicing in the absence of ESS2, it indicates that these two inhibitory elements act independently on site A3. To our knowledge, this is the first example of a 3′ss that is negatively regulated by two independent ESS elements. Why is there such stringent control over site A3 utilization? The A3 PPT is suboptimal compared with efficient 3′ss of cellular pre-mRNAs. However, splice site A3 contains a larger number of U residues and is less interrupted by purines compared with the PPTs of sites A4, b, and c and A5. Although no ESS element has been shown to act on sites A4a, b, and c and A5, splice site A4b acts as a repressor of splice site A5, and removal of splice site A4b results in a large increase in splicing at the A5 site. It was proposed that the inhibition results from competition between factors binding to branch-site sequences and factors binding to the splice site A4b AG dinucleotide (12). The equilibrium for utilization of the competing five A3 to A5 sites may therefore depend upon both this competition for splicing factors and negative regulation of the most optimized splice site (A5) by ESS elements. In addition, the Tat protein encoded by mRNAs spliced at site A3 has been found to be cytotoxic or apoptotic to infected and uninfected cells (52–60). Thus to maintain efficient replication, it is possible that HIV-1 controls production of this toxic viral protein by maintaining tight control of tat mRNA production.

Implication of hnRNP H in the Regulation of Site A3 Utilization—Whereas ESS2 binds hnRNP A/B proteins (34), our data show that ESS2p binds hnRNP H. To date, it has not been shown which RNA sequences have the highest affinity for proteins hnRNP H. However, hnRNP H is known to associate with tracts of G residues (61). Furthermore, the ESS element of the rat β-tropomyosin pre-mRNA (UGUGGGGAC), which binds hnRNP H (26), contains, as does ESS2p, a UGGG sequence. Also, a 31-nt segment of the Rous sarcoma virus NRS, that binds hnRNP H (27), has two G-rich motifs, one of which contains the UGGG sequence (UGGGA). Hence, our identification of hnRNP H binding to the UUGGGU sequence of ESS2p agrees with the limited sequence data on hnRNP H binding sites. Conversion of the UUGGGU sequence of ESS2p into a UCGGGG sequence decreased hnRNP H affinity for ESS2p. Consistent with the hypothesis that binding of hnRNP H to ESS2p is responsible for the inhibitory property, splicing at site A3 in HIV-1 RNA was increased by a factor of about 2 in HeLa cells as the result of the two U-to-C substitutions. HnRNP A/B have been implicated in the regulation of HIV-1 splice sites A2 (33), A3 (through ESS2 (34) and A7 (13)). Our data are the first to implicate hnRNP H in the regulation of HIV-1 RNA splicing.

How does protein hnRNP H regulate site A3 utilization by binding to ESS2p? As mentioned above, introns with suboptimal 3′ss require the binding of the U2AF35 subunit to the intron-exon boundary for a stable association of factor U2AF with the PPT (18, 19). For such introns, U2AF35 binding was found to encompass the terminal AG dinucleotide of the intron and about 10 nt at the exon flanking the 3′-splice site (18) (Fig. 8A). At site A3, the 10-nt sequence at the exon 5′ extremity contains the ESS2p element. Thus, it is likely that factor U2AF35 competes with hnRNP H for binding to the intron-exon junction (Fig. 8B). Such a mechanism could also explain the increased splicing efficiency observed after the two U-to-C substitutions in ESS2p. Indeed, these two mutations, which decrease affinity of hnRNP H for ESS2p, may favor U2AF35 binding and therefore increase splicing efficiency (Fig. 8C).

Interestingly, the ESS element of the rat β-tropomyosin pre-mRNA, which also binds hnRNP H, is also located only 5 nt downstream of the regulated 3′ss (26). Thus, the proposed mechanism for repression of splicing at site A3 may also explain the inhibitory property of the β-tropomyosin ESS.

According to the data presented in this paper, binding of hnRNP H to ESS2p may be complex: (i) two different proteins recognized by the anti-hnRNP H antibodies were detected in complex I (Fig. 7B) and in the present stage of the study we do not know whether they bind individually or as an heterodimer, binding of a dimer of proteins to E2-C3-WT and E2-C3-C RNA would be in agreement with the very low electrophoretic mobility of complex I, (ii) also we cannot exclude the possibility that two types of complex I, which differ by internal structural rearrangements of the partners without marked changes in the electrophoretic mobility, are formed with the WT ESS2p, because ESS2p with two U-to-C only displaced part of the complex I formed with WT ESS2p (Fig. 4D). Further experiments are underway to answer these questions.

Some Point Mutations in ESS2p Increase Silencer Activity—When the UUGGGU sequence of ESS2p was converted into UAGGGA, the silencer activity of the variant ESS2p element with the UAGGGA sequence was consistent with the high affinity of hnRNP A1 for the UAGGGA sequence (Kp of 1 nm) (50) and with the higher abundance of protein hnRNP A1 in nuclei and nuclear extract compared with hnRNP H (for review, see Ref. 62). According to the model of inhibition proposed above, hnRNP A1 should compete strongly with factor U2AF35 for binding to the ESS2p sequence with two U-to-A substitutions.
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Fig. 9. The sequence required for hnRNP H binding to ESS2p is conserved in HIV-1 strains from the groups M, N, and O and in the SIVcpzGAB strain. The sequences of the RNA segments from the BRU (GenBank™ accession number K02013), pNL4-3 (M19921), HXB2 (K03455), ELI (K03454), MAL (X04415), YBF30 (AJ006022), ANT70C (L20587) HIV-1 strains, and the SIVcpzGAB strain (X52154) corresponding for generous gifts of specific antibodies for hnRNP A1 and hnRNP H

Because the U-to-A substitution at position 5362 was also sufficient for a stronger inhibitory effect in HeLa cells, this suggests that this substitution is sufficient to convert the ESS2p binding site for hnRNP H into a binding site for hnRNP A1. In connection with our observation, a UAGG motif is present in the ESS element of the K-SAM exon of FGFR-2 pre-mRNA (63). This ESS element was found to bind protein hnRNP A1, and this protein is thought to be involved in the splicing inhibition mechanism (28). In contrast to the strong effect of the U-to-A substitution at position 5362, the U-to-A substitution at position 5366 alone did not increase inhibition by ESS2p. It appeared to have a slightly negative effect on splicing inhibition. This suggests that hnRNP H and/or hnRNP A1 binds to the UUGGGA sequence but with a slightly lower affinity compared with binding of hnRNP H to the WT UUGGGG sequence and hnRNP A1 to the UAGGGG sequence.

The ESS2p Sequence Required to Bind hnRNP H Is Strongly Conserved in HIV-1 Strains—Sequence comparison of 61 different strains of HIV-1 virus belonging to the M, N, or O groups of HIV-1 strains and of strains of the related group of SIV virus, revealed a strong conservation of the UUGGGG sequence of ESS2p in these various strains (64). The sequence found in the most studied members of each group of HIV-1 or SIVcpz strains are aligned in Fig. 9. As shown in the alignment, the UUGGGG sequence at the 5′-end of ESS2p is strongly conserved even though U5362 (marked with an asterisk) corresponds to a wobble position in the Vpr coding sequence. Substitution of U5362 by a C or an A would not alter the coding capacity of the RNA. However, only a very limited number of strains have a U-to-C substitution at this position (5 among 61 examined). Furthermore, no U-to-A substitution occurred at this position, which would convert the hnRNP H binding site into an hnRNP A1 binding site. Hence, according to this sequence comparison, the capacity of ESS2p to bind hnRNP H is strictly conserved in all the strains compared, including the SIVcpzGAB virus. This is a strong indication that ESS2p is important for the propagation of HIV-1 and SIVcpz related viruses.

Interestingly, recent data of Bourara et al. (65) suggest that HIV-1 RNA may be edited in chronically infected human H9 cells. Among five reported editing sites, one is within ESS2p. The first G residue in the ESS2p sequence UUGGGU appears to be converted to an A residue. This conversion may abrogate binding of hnRNP H and therefore interfere with splicing regulation in HIV-1-infected cells.

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REFERENCES

1. Purcell, D. F., and Martin, M. A. (1993) J. Virol. 67, 6365–6378
2. Parker, R., Siliciano, P. G., and Guthrie, C. (1987) Cell 49, 229–239
3. Wu, J., and Manley, J. L. (1989) Genes Dev. 3, 1553–1561
4. Zamore, P. D., and Green, M. R. (1991) EMBO J. 10, 207–214
5. Ruskin, B., Zamore, P. D., and Green, M. R. (1998) Cell 92, 207–219
6. Mount, S. M. (1982) Nucleic Acids Res. 10, 459–472
7. Green, M. R. (1986) Ann. Rev. Genet. 20, 671–708
8. Keller, B. R., and Noon, W. A. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 7417–7420
9. Reed, R. (1989) Genes Dev. 3, 2113–2123
10. Damier, L., Domenjoud, L., and Branlant, C. (1997) Biochem. Biophys. Res. Commun. 237, 182–187
11. Dreyfuss, G., and Khachi, S. (1995) J. Biol. Chem. 270, 2460–2466
12. Swanson, A. K., and Stoltzfus, C. M. (1998) J. Biol. Chem. 273, 34551–34557
13. Si, Z., Rauch, D., and Stoltzfus, C. M. (1998) Mol. Cell. Biol. 18, 5404–5413
14. Si, Z., Amendt, B. A., and Stoltzfus, C. M. (1999) Nucleic Acids Res. 27, 861–867
15. Staffa, A., and Cochrane, A. (1994) J. Virol. 68, 3071–3079
16. Bosquejo, R., Weiner, M., and Garcia-Blanco, M. A. (1999) J. Biol. Chem. 268, 11222–11229
17. Zamore, P. D., Patton, J. G., and Green, M. R. (1992) Nature 355, 609–614
18. Wu, S., Romo, C. M., Nisen, T. W., and Green, M. R. (1999) Nature 402, 832–835
19. Guth, S., Martinez, C., Gaur, R. K., and Valcarcel, J. (1999) Mol. Cell. Biol. 19, 8263–8271
20. Stark, J. M., Bazett-Jones, D. P., Herfort, M., and Roth, M. B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2163–2168
21. Bourgeois, C. F., Pupier-Mazur, J., Willems, K., and Stevenin, J. (1999) Mol. Cell. Biol. 17, 7374–7387
22. Schaal, T. D., and Maniatis, T. (1999) Mol. Cell. Biol. 19, 261–273
23. Selvakumar, M., and Helfman, D. M. (1999) RNA (N. Y.) 5, 378–394
24. Sun, Q., Mayeda, A., Hampson, R. K., Krainer, A. R., and Rottman, F. M. (1993) Genes Dev. 7, 2389–2402
25. Zheng, Z. M., Huyben, M., and Baker, C. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14088–14093
26. Chen, C. D., Kobayashi, R., and Helfman, D. M. (1999) Genes Dev. 13, 593–606
27. Fogel, B. L., and McNally, M. T. (2000) J. Biol. Chem. 275, 32371–32378
28. Del Gatto-Konczak, F., Olive, M., Gesnel, M. C., and Breathnach, R. (1999) Mol. Cell. Biol. 19, 251–260
29. Mattier, N., Marx, M., Weg-Remers, S., Ponta, H., Herrlich, P., and König, H. (2000) J. Biol. Chem. 275, 35335–35340
30. Amendt, B. A., Si, Z. H., and Stoltzfus, C. M. (1995) Mol. Cell. Biol. 15, 4606–4615
31. Staffa, A., and Cochrane, A. (1995) Mol. Cell. Biol. 15, 4597–4605
32. Amendt, B. A., Hessele, D., Chang, L. J., and Stoltzfus, C. M. (1994) Mol. Cell. Biol. 14, 2980–2990
33. Bilodeau, P. S., Domsic, J. K., Mayeda, A., Krainer, A. R., and Stoltzfus, C. M. (2001) J. Virol. 75, 8487–8497
34. Caputi, M., Mayeda, A., Krainer, A. R., and Zalmer, A. (1999) EMBO J. 18, 4060–4067
35. Dreyfuss, G., Matanis, M. J., Pinol-Roma, S., and Burd, C. G. (1993) Annu. Rev. Biochem. 62, 289–321
36. Mayeda, A., and Krainer, A. R. (1992) Cell 68, 365–375
37. Wang, X., Bani, M., Lu, S. J., Rowan, S., Ben-David, Y., and Chabot, B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6924–6928
38. Caceres, J. F., Stamm, S., Helfman, D. M., and Krainer, A. R. (1994) Science 263, 1706–1709
39. Chabot, B., Blanchette, M., Lapierre, I., and La Branche, H. (1997) Mol. Cell. Biol. 17, 1776–1786
40. Blanchette, M., and Chabot, B. (1999) EMBO J. 18, 1939–1952
41. Chou, M. Y., Rooke, N., Turck, C. W., and Black, D. L. (1999) Mol. Cell. Biol. 19, 69–77
42. Jacquenet, S., Ropers, D., Damier L., Mougin, A., Bilodeau, P. S., Stoltzfus, C. M., and Branlant, C. (2001) Nucleic Acids Res. 29, 464–478
43. Charneau, P., Alizon, M., and Clavel, F. (1992) J. Virol. 66, 2814–2820
44. Nour, M., Naimi, A., Beck, G., and Branlant, C. (1995) Curr. Microbiol. 31, 270–276
45. Ratner, L., Haseltine, W., Patarca, R., Livak, K. J., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K., Ivanoff, L., Lautenberger, J. A., Popas, T. S., Ghrayeb, J., Chang, N. T., Gallo, R. C., and Wong-Staal, F. (1985) Nature 313, 277–283
46. Kramer, W., Drutsa, V., Jansen, H. W., Kramer, B., Pflugfelder, M., and Fritz, H. J. (1984) Nucleic Acids Res. 12, 9441–9456
47. Bilodeau, P. S., Domsic, J. K., and Stoltzfus, C. M. (1999) J. Virol. 73, 9764–9772
48. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
49. Kan, J. L., and Green, M. R. (1999) Genes Dev. 13, 462–471
50. Burd, C. G., and Dreyfuss, G. (1994) EMBO J. 13, 1197–1204
51. Min, H., Chan, R. C., and Black, D. L. (1995) Genes Dev. 9, 2659–2671
52. Vogel, J., Hinrichs, S. H., Reynolds, R. K., Luciw, P. A., and Jay, G. (1988) Nature 335, 606–611
53. Vogel, J., Hinrichs, S. H., Napolitano, L. A., Ngo, L., and Jay, G. (1991) Cancer Res. 51, 6686–6690
54. Sabatier, J. M., Vives, E., Mabrouk, K., Benjouad, A., Rochat, H., Duval, A., Hue, B., and Bahraoui, E. (1999) J. Virol. 63, 961–967
55. Magnusson, D. S., Knudsen, B. E., Geiger, J. D., Brownstone, R. M., and Nath, A. (1995) Ann. Neurol. 37, 373–380
56. Lotz, M., Clark-Lewis, I., and Ganu, V. (1994) J. Cell Biol. 124, 35–371
57. Li, C. J., Friedman, D. J., Wang, C., Metelev, V., and Pardee, A. B. (1995) Science 268, 429–431
58. Ensoli, B., Gendelman, R., Markham, P., Fiorelli, V., Colombini, S., Raffeld, M., Cafaro, A., Chang, H. K., Brady, J. N., and Gallo, R. C. (1994) Nature 371, 674–680
59. Ensoli, B., Buonaguro, L., Barillari, G., Fiorelli, V., Gendelman, R., Morgan, R. A., Wingfield, P., and Gallo, R. C. (1993) J. Virol. 67, 277–287
60. Barillari, G., Gendelman, R., Gallo, R. C., and Ensoli, B. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7941–7945
61. Swanson, M. S., and Dreyfuss, G. (1988) Mol. Cell. Biol. 8, 2237–2241
62. Krecic, A. M., and Swanson, M. S. (1999) Curr. Opin. Cell Biol. 11, 363–371
63. Del Gatto, F., Gesnel, M. C., and Breathnach, R. (1996) Nucleic Acids Res. 24, 2017–2021
64. Knikken, C., Foley, B., Hahn, B., Marx, P., McCutchan, F., Mellors, J., Mullins, J., Wolinsky, S., and Korber, B. (1999) Human Retroviruses and AIDS: A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences, Los Alamos National Laboratory, Los Alamos, NM
65. Bourara, K., Litvak, S., and Araya, A. (2000) Science 289, 1564–1566
66. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680
