A Novel Splice Donor Site in the \textit{gag-pol} Gene Is Required for HIV-1 RNA Stability*

Received for publication, December 23, 2005, and in revised form, March 23, 2006. Published, JBC Papers in Press, May 4, 2006, DOI 10.1074/jbc.M513698200

Martin Lützelberger†, Line S. Reinert‡, Atze T. Das§, Ben Berkhouët†, and Jørgen Kjems*†

From the †Department of Molecular Biology, University of Aarhus, C. F. Møller Allé 130, 8000 Århus C, Denmark and the §Department of Human Retrovirology, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, P. O. Box 22660, 1100 DD Amsterdam, The Netherlands

Productive infection and successful replication of human immunodeficiency virus 1 (HIV-1) requires the balanced expression of all viral genes. This is achieved by a combination of alternative splicing events and regulated nuclear export of viral RNA. Because viral splicing is incomplete and intron-containing RNAs must be exported from the nucleus where they are normally retained, it must be ensured that the unspliced HIV-1 RNA is actively exported from the nucleus and protected from degradation by processes such as nonsense-mediated decay. Here we report the identification of a novel 178-nt-long exon located in the \textit{gag-pol} gene of HIV-1 and its inclusion in at least two different mRNA species. Although efficiently spliced \textit{in vitro}, this exon appears to be tightly repressed and infrequently used \textit{in vivo}. The splicing is activated or repressed \textit{in vitro} by the splicing factors ASF/SF2 and heterogeneous nuclear ribonucleoprotein A1, respectively, suggesting that splicing is controlled by these factors. Interestingly, mutations in the 5′-splice site resulted in a dramatic reduction in the steady-state level of HIV-1 RNA, and this effect was partially reversed by expression of U1 small nuclear RNA harboring the compensatory mutation. This implies that U1 small nuclear RNA binding to optimal but non-functional splice sites might have a role in protecting unspliced HIV-1 mRNA from degradation.

The basic genome organization of the human immunodeficiency virus 1 (HIV-1) provirus is similar to all other retroviruses with respect to the three major open reading frames (ORFs) encoding the structural proteins (Gag), the protease, reverse transcriptase, and integrase enzymes (Pol), and the envelope glycoproteins (Env) (Fig. 1A). Gag and Pol are produced from the unspliced transcript, whereas Env is produced from a mRNA in which an intron, defined by a major 5′-splice site (SD1) in the 5′-untranslated region and one of several 3′-splice sites (SA3-SA5) at the end of the pol ORF, is spliced out. HIV-1 contains 6 additional genes termed tat, rev, vif, vpr, vpx, and nef that are produced from alternative splicing. In total, five 5′-splice sites and 11 3′-splice sites have been identified, which give rise to more than 40 different mRNAs grouped into three different classes: the unspliced primary transcript (~9 kb), a class of singly spliced RNAs (~4 kb), and a class of two or multiple spliced RNAs (~2 kb) (1–6). In the early phase of HIV-1 infection, only completely spliced mRNAs are exported to the cytoplasm, encoding the Tat, Rev, and Nef proteins. Subsequently Rev binds to its target sequence on incompletely spliced HIV-1 RNAs, termed Rev response element (RRE), and mediates their nuclear export (7, 8).

The accumulation of unspliced and partially spliced RNAs in the cytoplasm requires that the removal of introns from the primary HIV-1 transcript is inefficient and delayed. Thus, a hallmark of the HIV-1 genome is the presence of optimal 5′-splice sites that match the consensus sequence and non-consensus 3′-splice sites with short polypyrimidine tracts interrupted by purines and non-canonical branch point sequences. The recognition and modulation of these splice signals is controlled by intronic and exon splice enhancers and silencers situated in the vicinity of the splice sites. The splice enhancers are recognized by members of the SR protein family (9–12), whereas the splice silencers recruit members of the heterogeneous ribonucleoprotein (hnRNP) family to suppress splice site recognition (13–18). Moreover, the removal of HIV-1 introns has been suggested to be sequential, proceeding from the 5′-end, because splicing of introns in the 3′-untranslated region can be detrimental due to the induction of nonsense-mediated decay. Bohne et al. (19) have shown that splicing of a 3′-intron in HIV-1 is tightly inhibited unless the 5′-introns are removed. Accordingly, mutating the major splice donor site (SD1) blocks all downstream splice events.

Another intriguing feature is the connection between splice sites, RNA stability, and nuclear export. The SD4 5′-splice site in HIV-1 has been shown to exert a stabilizing effect on the steady-state level of HIV-1 RNA and modulates Rev-mediated nuclear export and stability (20, 21). In these studies it was shown that insufficient hydrogen bonding between the splice donor SD4 and the 5′-end of U1 snRNA leads to (nuclear) degradation of HIV-1 RNA. Thus, the 5′-splice sites provide an RNA protective function in addition to their role in pre-mRNA splicing.

In this study we have identified two novel HIV-1 mRNA species by cloning cDNAs amplified with the polymerase chain reaction (PCR). Both mRNAs contain a new 178-nt-long exon, positioned in the gag-pol gene, and the flanking 5′- and 3′-splice sites are well conserved among different HIV-1 subtypes. Splicing of this exon is tightly repressed during the course of an HIV-1 infection, suggesting another role for this exon. In this report we provide evidence that the highly conserved and intrinsically strong 5′-splice site may serve an important function in protecting and stabilizing the unspliced HIV-1 mRNA.

EXPERIMENTAL PROCEDURES

Plasmids and Protein Expression—Plasmids pUC18 HIV-E1a-E2 and pUC18 HIV-E1-E2 were generated by PCR, using pHIV-1 LAI derived from HIV-1 isolate LAI as a template and primers 476U45 (5′-GCC GAT CCT AAT ACG ACT CAC TAT AGG GTC TCT CGT GTT AGA CCA-3′), 4602U45 (5′-GCC GAT CCT AAT ACG ACT CAC
TAT AGG GAA GAT GGC CAG TAA AAA-3'), and 5027L28 (5'-TGC GTC GAC CTT GCC AGA GGT TTG C-3'). The resulting EcoRI-Sall-cleaved PCR products were inserted between the EcoRI and Sall sites of pUC18 vector (22). The plasmid pUC18 HIV-E1-E2(ΔClal/BsrGI) was constructed by deleting 3627 bp of the first HIV-1 intron between the Clal and BsrGI sites in pUC18 HIV-E1-E2, followed by a fill-in using Klenow polymerase and blunt end ligasion. Plasmid pCMVΔR8.2 3U was generated by PCR from plasmid pCMVΔR8.2 (23) using the QuickChange site-directed mutagenesis kit (Stratagene) and the primers 4789L35 (5'-GCG GAT CCT CCC ATT TGC TGT CCC TGG C-3') and 476U45 (5'-GCG GAT CCC TGG C-3') using the QuikChange site-directed mutagenesis kit (Stratagene) and 4789L35 (5'-GCG GAT CCT CCC ATT TGC TGT CCC TGG C-3') and 476U45 (5'-GCG GAT CCC TGG C-3') using the QuikChange site-directed mutagenesis kit (Stratagene) and 4789L35 (5'-GCG GAT CCT CCC ATT TGC TGT CCC TGG C-3'). Recombinant His6-ASF/SF2 and GST-hnRNP A1 were expressed in E. coli as described previously (24-26).

**RT-PCR**—Reverse transcription (RT) PCR was performed on total RNA purified from SupT1 cell cultures with moderate to complete syncytia formation after 48-72 h of infection with HIV-1 (subtype LAI).

For first strand synthesis up to 2 μg of total RNA were mixed with 2 pmol primer, denatured at 70 °C for 5 min, and chilled on ice. The mixture was then incubated for 60 min at 42 °C in the presence of 1× AMV reverse transcriptase reaction buffer, 4 mM sodium pyrophosphate, 1 mM each dNTP, 40 units of RNasin RNase inhibitor (Promega), and 30 units of AMV reverse transcriptase (Promega). Seven amplification primers were used in this work. Primer 589S1L32 binds to exon 4 (antisense strand) and was used for first strand synthesis. Primer 768U28 (sense strand) spans the major 5'-splice site and splice acceptor SA1A. Primer 499I1L25 (antisense strand) spans SA1 and SD1A. Primers 733U26 and 760U31 (sense strand) bind to the first HIV-1 exon upstream of SD1 and were used as nested primers. Primer 5039L25 is located in exon 2 (antisense strand) and used in combination with primer 4812L28 (antisense strand) binding to exon 1A for nested PCR. The sequences of the primers are as follows: 768U28, 5'-GCG GAT GCC AGG GGA GGC GAC TGG AGG A-3'; 499I1L25, 5'-GCG GAT CCT CTC TTC TCC CCG CTA TTA TCC C-3'; 5039L25, 5'-GCG GAT CCT TTG GTC CTT TCC A-3'; 4812L28, 5'-GCG GAT GCC CTT CCG CCT ATA ATT TTC TTT A-3'; 733U26, 5'-GAG GAT AAT TCG GAC TCG GTG TCT TCC AG3-3'; 589S1L32, 5'-GCG GAT CCC CCA A-3'; 4974L60C, 5'-GCG GAT CCC CCA A-3'; 760U31, 5'-GAG GAT AAT TCG GAC TCG GTG TCT TCC AG3-3'.

**Cell Culture and Transfection**—293T cells were propagated and transfected for Northern blot analysis using Lipofectamine reagent (Invitrogen) as described by the manufacturer’s protocol with minor modifications. For each transfection, 35 μl of Lipofectamine reagent and 20 μg of DNA were mixed with 500 μl of RPMI medium without serum (Invitrogen). The amount of DNA in all cotransfection experiments was kept constant by adding plasmid pGL3 (Promega). Transfection efficiency was monitored by including pDS-Red1-N1 (Clontech). The diluted DNA and diluted Lipofectamine reagent were combined and incubated at room temperature for 20 min to allow DNA-liposome complexes to form. The mixture was then added to 293T cells grown in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal calf serum on a 10-cm plate to 50–60% confluency. Total RNA was isolated 24 h after transfection using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions.

**Northern Blot Analysis**—Total RNA isolated 24 h after transfection was subjected to electrophoresis on a denaturing 1% agarose gel containing 5% formaldehyde. The RNA (15 μg in each lane) was transferred onto positively charged nylon membrane (Hybond N+; Amersham Biosciences). After immobilizing the RNA by UV cross-linking (0.5 J/cm2; Stratalinker), the membrane was hybridized at 42 °C in ULTRA-hyb solution (Ambion). HIV-1-derived RNA was detected with a 32P-labeled probe synthesized from the 463-bp EcoRI/Sall fragment of pUC18 HIV-E1a-E2. To monitor transfection efficiency, the membrane was hybridized with a probe specific for green fluorescent protein (GFP), which was synthesized from the 246-bp Stul/NotI fragment of pDS-Red1-N1. The membranes were autoradiographed and quantified using Bio-Rad phosphorimaging and ImageQuant software.

**RT-PCR**—Reverse transcription (RT) PCR was performed on total RNA purified from SupT1 cell cultures with moderate to complete syncytia formation after 48–72 h of infection with HIV-1 (subtype LAI). For first strand synthesis up to 2 μg of total RNA were mixed with 2 pmol primer, denatured at 70 °C for 5 min, and chilled on ice. The mixture was then incubated for 60 min at 42 °C in the presence of 1× AMV reverse transcriptase reaction buffer, 4 mM sodium pyrophosphate, 1 mM each dNTP, 40 units of RNasin RNase inhibitor (Promega), and 30 units of AMV reverse transcriptase (Promega). Seven amplification primers were used in this work. Primer 589S1L32 binds to exon 4 (antisense strand) and was used for first strand synthesis. Primer 768U28 (sense strand) spans the major 5'-splice site and splice acceptor SA1A. Primer 499I1L25 (antisense strand) spans SA1 and SD1A. Primers 733U26 and 760U31 (sense strand) bind to the first HIV-1 exon upstream of SD1 and were used as nested primers. Primer 5039L25 is located in exon 2 (antisense strand) and used in combination with primer 4812L28 (antisense strand) binding to exon 1A for nested PCR. The sequences of the primers are as follows: 768U28, 5'-GCG GAT GCC AGG GGA GGC GAC TGG AGG A-3'; 499I1L25, 5'-GCG GAT CCT CTC TTC TCC CCG CTA TTA TCC C-3'; 5039L25, 5'-GCG GAT CCT TTG GTC CTT TCC A-3'; 4812L28, 5'-GCG GAT GCC CTT CCG CCT ATA ATT TTC TTT A-3'; 733U26, 5'-GAG GAT AAT TCG GAC TCG GTG TCT TCC AG3-3'; 589S1L32, 5'-GCG GAT CCC CCA A-3'; 4974L60C, 5'-GCG GAT CCC CCA A-3'; 760U31, 5'-GAG GAT AAT TCG GAC TCG GTG TCT TCC AG3-3'.

**Cell Culture and Transfection**—293T cells were propagated and transfected for Northern blot analysis using Lipofectamine reagent (Invitrogen) as described by the manufacturer’s protocol with minor modifications. For each transfection, 35 μl of Lipofectamine reagent and 20 μg of DNA were mixed with 500 μl of RPMI medium without serum (Invitrogen). The amount of DNA in all cotransfection experiments was kept constant by adding plasmid pGL3 (Promega). Transfection efficiency was monitored by including pDS-Red1-N1 (Clontech). The diluted DNA and diluted Lipofectamine reagent were combined and incubated at room temperature for 20 min to allow DNA-liposome complexes to form. The mixture was then added to 293T cells grown in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal calf serum on a 10-cm plate to 50–60% confluency. Total RNA was isolated 24 h after transfection using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions.

**Northern Blot Analysis**—Total RNA isolated 24 h after transfection was subjected to electrophoresis on a denaturing 1% agarose gel containing 5% formaldehyde. The RNA (15 μg in each lane) was transferred onto positively charged nylon membrane (Hybond N+; Amersham Biosciences). After immobilizing the RNA by UV cross-linking (0.5 J/cm2; Stratalinker), the membrane was hybridized at 42 °C in ULTRA-hyb solution (Ambion). HIV-1-derived RNA was detected with a 32P-labeled probe synthesized from the 463-bp EcoRI/Sall fragment of pUC18 HIV-E1a-E2. To monitor transfection efficiency, the membrane was hybridized with a probe specific for green fluorescent protein (GFP), which was synthesized from the 246-bp Stul/NotI fragment of pDS-Red1-N1. The membranes were autoradiographed and quantified using Bio-Rad phosphorimaging and ImageQuant software.

**RESULTS**

In a search for new splice signals within the HIV-1 genome, we identified a putative splice donor site with the sequence AG/GUAAGA at nucleotide position 4721 (numbering according to HXB2). It closely matches the optimal 5'-splice site consensus sequence AG/GURAGY (where R is A or G, and Y is C or U) except for the last nucleotide and is

---

**JOURNAL OF BIOLOGICAL CHEMISTRY**

**Pre-mRNA Processing in HIV-1**

---

**RESULTS**

In a search for new splice signals within the HIV-1 genome, we identified a putative splice donor site with the sequence AG/GUAAGA at nucleotide position 4721 (numbering according to HXB2). It closely matches the optimal 5'-splice site consensus sequence AG/GURAGY (where R is A or G, and Y is C or U) except for the last nucleotide and is
situated in the \textit{pol} gene, 190 nucleotides upstream of the previously characterized splice acceptor site SA1 (Fig. 1).

To test whether this 5'-splice site is used \textit{in vivo}, RT-PCR analysis with a primer positioned in the first HIV-1 exon and a primer spanning the putative splice donor site and splice acceptor site SA1 was performed on total RNA isolated from HIV-1-infected cells. We obtained a PCR product of 230 bp, which was subsequently cloned and sequenced. It revealed that the major 5'-splice site (SD1) was joined to a novel splice acceptor site with the sequence AAUUAG/CA at nucleotide position 4543. This implies that the PCR product was derived from an mRNA containing HIV-1 exon 1, a novel exon of 178 nucleotides in length, and exon 2. The new splice acceptor site contains a short polypyrimidine tract of only 9 consecutive pyrimidines characteristic for all splice acceptor sites in HIV-1 (Fig. 1B). The novel splice donor and acceptor sites were denoted SD1A and SA1A, respectively, and the new exon named exon 1A (Fig. 1B).

To address the question whether exon 1A is joined to exons other than exon 2, we performed RT-PCR using a primer in exon 1A and primers positioned in exon 3 or 4. A PCR product of 200 bp was obtained using the exon 4 primer, which was subsequently cloned and sequenced. The fragment derived from an mRNA containing exon 1A and exon 4. PCR products containing exon 1A and exon 4 in combination with exon 2 and/or exon 3 were not detected (data not shown).

To investigate whether the newly identified splice donor and acceptor sites are conserved in different HIV-1 isolates, we compared a representative set of 367 HIV-1 sequences retrieved from the HIV database (hiv-web.lanl.gov) both on the protein and nucleotide levels (Fig. 2, A and B). Because splice acceptor SA1A and splice donor SD1A are located within the \textit{pol} open reading frame, we analyzed the frequency of codons overlapping with both splice sites. As shown in Fig. 2C 82% of the leucine residues at position 886 are encoded by a UUA codon. In contrast, the average fraction of UUA codons in HIV-1 and human ORFs is only 26 and 8%, respectively. Similar values can be found for the other codons overlapping with the exon 1A 5'– and 3'–splice sites (Fig. 2C). Thus, the strong bias for codons that are otherwise infrequently used in HIV-1 and human indicates that these splice sites are phylogenetically conserved at the RNA level and that their integrity might be important for the viability of the virus.

To quantify the inclusion of exon 1A into HIV-1 mRNA, we performed an S1 nuclease-mapping analysis using RNA isolated from SupT1 cells infected with wild-type HIV-1 for 0–3 days (Fig. 3). To
detect splicing of exon 1A, we designed four 60-nucleotide-long single-stranded DNA probes spanning either the splice acceptor SA1A (Fig. 3A, Oligo 4974L60A), splice donor SD1A joint to splice acceptor SA1A (Fig. 3A, Oligo 4974L60B), splice donor SD1 joint to splice acceptor SA1A (Fig. 3A, Oligo 4974L60C), or splice donor SD1A joint to splice acceptor SA3 (Fig. 3A, Oligo 5838L60). S1 mapping using oligonucleotide 4974L60A (Fig. 3B, lanes 1–4) resulted in protected bands of 50 (unspliced) and 38 nucleotides in length (SD1 spliced either to SD1A or SA1). Oligonucleotide 4974L60B results only in 41- and 39-nucleotide bands (corresponding to unspliced and exon 1 to exon 2 spliced RNA, respectively, suggesting that no or very little product is formed between exon 1A and exon 2 (Fig. 3B, lanes 5–8). In contrast to the RT-PCR experiment above, splicing of exon 1A to exon 4 could not be detected by S1 mapping using oligonucleotide 5838L60 (Fig. 3C, lanes 14–17). This is based on the observation that only a 41-nucleotide band, corresponding to unspliced RNA, was observed. As shown in Fig. 3C, lanes 10–13, increasing amounts of both unspliced (39-nucleotide band) and RNA that has been spliced from SD1 to SA1 (50-nucleotide band) are detectable in SupT1 cells from the first day of infection and increase at about the same rate. Collectively our data suggest that splice donor SD1A is only infrequently used in vivo.

Previous investigations have shown that processing of HIV-1 RNA is highly regulated by splicing factors such as members of the SR (serine/arginine) protein and hnRNP family that bind to splice enhancer and silencer elements surrounding the splice sites (10, 13, 15, 17). To study whether any of these splicing factors has an effect on exon 1A inclusion in vitro, we created a set of mini-gene constructs shown in Fig. 4A. To test splicing between SD1 and SA1A, we constructed a splicing substrate containing only HIV-1 exons 1 and 1A. To enable in vitro splicing analysis the region from nucleotide 832 to 4422 (numbering according to HXB2) was deleted to reduce the intron size to 208 nt (Fig. 4A). The remaining intron is composed of an 88-nucleotide region downstream of the major 5′-splice site (SD1) and a 120-nucleotide region upstream of SA1A. RNA transcribed from this construct was added as a substrate
to in vitro splicing reactions using HeLa nuclear extract supplemented with either recombinant ASF/SF2 or hnRNP A1 protein. As shown in Fig. 4B, removal of the intron is highly inefficient (Fig. 4B, lane 1) and only detectable when ASF/SF2 was added to the reactions (Fig. 4B, lanes 2 and 3). In contrast, splicing of SD1A to SA1 is readily detectable in HeLa nuclear extract without the addition of ASF/SF2 (Fig. 4D, lane 1) and is stimulated when supplemented with ASF/SF2 (Fig. 4D, lanes 2 and 3). Addition of hnRNP A1 had the opposite effect and repressed splicing (Fig. 4D, lanes 4 and 5). This indicates the presence of splicing enhancer and silencer elements within this construct that act as binding sites for ASF/SF2 and hnRNP A1. The presence of a splicing enhancer within the second HIV-1 exon was further supported by the observation that its replacement with the second exon of PIP7.A pre-mRNA abolished the stimulatory effect of ASF/SF2 on splicing to SD1A (data not shown).

A third construct, containing all three exons, was used to investigate
whether any of the two splicing factors is able to induce exon 1A inclusion. A splicing analysis using this construct yielded only spliced products containing exons 1 and 2, and addition of ASF/SF2 or hnRNP A1 did not affect splicing of this substrate significantly (Fig. 4C). We conclude that the effect observed on SD1A to SA1 splicing is not observed in the 3-exon construct, possibly because splicing between SD1 and SA1 is dominating. Alternatively, the deletion made in the intron between SD1 and SA1 may contain sequences that contribute to the proper recognition of the splice acceptor SA1.

In light of the low splicing activity of the new exon we found it intriguing that its splice sites are so well conserved in different HIV-1 isolates. This prompted us to investigate another potential role of exon 1A in more detail. Recently, it was demonstrated that the integrity of 5′/H11032-splice sites can be an important determinant of mRNA stability (20, 21). Therefore, we tested whether a point mutation in SD1A affects the stability of HIV-1 RNA correspondingly. For this purpose, we changed the sequence of the splice donor site SD1A from AG/GUAAGA to AG/GUUAGA by site-directed mutagenesis. A similar point mutation, denoted 3U, reduced the HIV-1 RNA to non-detectable levels when it was introduced into SD4 (21). Total RNA isolated from 293T cells transfected with a construct expressing the wild-type and mutant splice donor site SD1A was subjected to Northern analysis in order to compare the steady-state levels of HIV-1 RNA. As is shown in Fig. 5A, lane 3, the 3U mutation reduced the level of HIV-1 RNA significantly to only 19% of the wild-type level (Fig. 5B). It is important to note that all transfections were of comparable efficiency (Fig. 5A, lanes 1–7, lower panel, GFP). To confirm this result, we performed S1 nuclease analysis on the RNA using the same oligonucleotides as for the quantification of exon 1A splicing in Fig. 3. As shown in Fig. 5C, both oligonucleotides detected similar levels of unspliced HIV-1 RNA in cells transfected with the wild-type construct (lanes 3 and 6), and the amount of transcript detectable for the 3U mutant was profoundly reduced (lanes 2 and 5). Although the steady-state level of HIV-1 RNA was greatly reduced by the 3U mutation, spliced mRNA containing exon 1 to exon 2 was still detectable as a 39-nucleotide protected band (Fig. 5C, lane 5), indicating that splicing was not affected by the U3 mutation.

To show that U1 snRNA binding is important for RNA expression, a construct expressing the U1 snRNA with a Gly to Ala mutation at position 6, U1-6A snRNA, was cotransfected into the cells. This snRNA contains a compensatory base change (ACUAAC/CUG) that restores the number of hydrogen bonds between the mutated 5′-splice site and

---

**FIGURE 4.** A, mini-gene constructs to study splicing of exon 1A in vitro. Exons are represented by open boxes. Introns are drawn as horizontal lines. The length of the exon and intron sequences (nt) is indicated. Relevant restriction sites used for cloning are indicated (see “Experimental Procedures” for details). All constructs include a T7 promoter to allow run-off transcription. Pre-mRNA substrates transcribed from these constructs were used in in vitro splicing reactions between exons 1 and 1A (B), exons 1, 1A, and 2 (C), and exons 1A and 2 (D). The reactions were supplemented with 100 and 200 ng of ASF/SF2 (lanes 2 and 3, respectively) and 100 and 200 ng of hnRNP A1 (lanes 4 and 5, respectively). The positions of the substrate, intermediates, and spliced products are indicated to the left of each panel. The size of the pUC18 HpaII marker bands in lane 6 is given to the right of each panel.
Pre-mRNA Processing in HIV-1

A

![Image](https://example.com/image1)

**FIGURE 5.** A, Northern blot analysis of total RNA isolated from 293T cells transfected with plasmids as indicated. Mock, pGL3; WT, pCMVΔR8.2; 3U, pCMVΔR8.2 3U; U1-6A, pUC13 U1-6A. To detect HIV-1 RNA, a probe spanning the region from exon 1A to exon 2 was used for hybridization (upper panels, HIV-1). To compare transfection efficiency the membrane was hybridized with a probe specific for GFP (lower panels, GFP). B, quantitation of Northern blots from three independent experiments as shown in panel A. The signal intensity is given in percent (%) relative to the samples from cells transfected with the wild type (lanes 2 and 6). White bars, HIV-1 RNA; shaded bars, GFP RNA. C, S1 nuclease analysis of the RNA shown in panel A, lanes 1–3, using the oligonucleotides 4974L60A and 4947L60B.

the 5′-end of U1 snRNA. Coexpression of U1-6A snRNA with the 3U mutant restored HIV-1 RNA expression almost to the wild-type level (Fig. 5A, lane 5), indicating that it is the interaction between the U1 snRNA and the 5′-splice site that is important for RNA stability. It is important to note that the level of wild-type HIV-1 RNA remained unchanged when the U1-6A snRNA was cotransfected (Fig. 5A, lane 7), showing that the suppression of the 3U mutation was specific. Collectively, these data suggest that the new splice donor site SD1A characterized in this report has a function in stabilizing HIV-1 RNA.

**DISCUSSION**

While evaluating the HIV-1 genome for new splice signals, we discovered a novel exon located in the gag-pol gene. To the best of our knowledge, this exon has never been observed before in any of the mRNA species generated by the provirus despite being intensely studied (1–4,6). The relatively infrequent use of the exon, at least in cultured HIV-1-infected cells, may explain this.

Splicing of exon 1A is affected by both the SR protein ASF/SF2 and hnRNP A1, which are known to act antagonistically on different HIV-1 splice sites (9, 10, 13–15). The most pronounced effect is observed for splicing of SD1A to exon 2. The activation of splicing by ASF/SF2 is probably related to a splicing enhancer element consisting of a GGAAAGG repeat that has been recently identified in HIV-1 exon 2.3 However, ASF/SF2 was not sufficient to induce inclusion of exon 1A in vitro using a 3-exon construct, which could be related to the inefficient use of splice acceptor site SA1A (Fig. 4B). This raises the possibility that SD1A in some transcripts might be used as an alternative 5′-splice site instead of the major splice donor SD1. This would result in an mRNA with exon 1 extended from 288 to 4302 nucleotides, encoding only 891 amino acids of the pol ORF and a C terminus of varying length (depending which downstream acceptor site is used). However, such an mRNA species was not detected in our RT-PCR analysis. The mRNA produced from splicing exon 1A in combination with exon 1 and either exon 2 or 4 contains three potential start codons within exon 1A (Fig. 1B, ATG, underlined), but none of them is positioned in a reading frame that can be translated to a protein of significant length, either alone or in combination with one of the downstream exons. Therefore, exon 1A probably does not give rise to new proteins.

In HIV-1, several cryptic splice sites have been identified that become active when the authentic splice sites are mutated or deleted or parts of the HIV-1 genome are placed into constructs of heterologous context (1). It has been postulated that these cryptic splice sites are part of a viral evolution strategy leading to the synthesis of novel chimeric proteins (27, 28). For instance, mutations in the HIV-1 genome that normally would be considered lethal to the virus can be suppressed by alternative splicing events that replace faulty sequence elements (29). Such a genomic plasticity may be important for viral evolution but can hardly account for conservation of splice sites flanking exon 1A. Our results strongly suggest that the SD1A also fulfills a function different from splicing, namely control of the steady-state level of HIV-1 RNA in the cell.

In a series of genetic experiments we demonstrate that U1 snRNP binding to SD1A is the important factor for the observed increase in RNA level. This resembles the previous observation that the 3U mutant in a different splice donor site, SD4, stabilizes the RNA (21). In that study it was shown that reduction of the number of hydrogen bonds between the splice donor site SD1A and the 5′-end of U1 snRNA correlates closely with the cellular HIV-1 RNA level. Thus, a mechanism might exist that prevents degradation of unspliced RNA by occupying all HIV-1 5′-splice sites with U1 snRNP. Such a mechanism seemingly conflicts with the observation that binding of snRNPs leads to nuclear retention of unspliced RNA and that completion of the splicing reaction removes this obstacle to nucleocytoplasmic export of the RNA (30–32). However, in the case of HIV-1, export of unspliced RNA is mediated by the Rev protein that may circumvent the retention exerted by U1 snRNP.

1. J. W. Fuxreiter, J. A. Kedersha, C. W. Earnshaw, and V. J. Robert, Nature (Lond.) 344, 61 (1990).
2. H. Schaal, personal communication.

3. H. Schaal, personal communication.
(33–37). Our finding that addition of ASF/SF2 to the splicing reaction increases use of the splice donor site SD1A, but does not enhance exon 1A inclusion, supports the idea that this splice site is conserved to function as a U1 snRNP binding site and that this interaction is improved by ASF/SF2. Binding of U1 snRNP may have different effects. It may aid in protecting and stabilizing unspliced HIV-1 mRNA, potentially by recruiting ASF/SF2 that has been shown to stabilize the binding of Rev to the RRE leading to more efficient nuclear export (38). Alternatively, U1 snRNP may stimulate transcription by interacting with part of the transcriptional machinery (39). Both of these mechanisms would lead to increased RNA levels in the cell.

Two 5′-splice sites, SD1A and SD4, have been shown to mediate the RNA-stabilizing effect. The question remains whether more of the HIV-1 5′-splice sites have a dual function for splicing and RNA stability.

Acknowledgments—We thank D. Trono for pCMVΔR8.2 and Susanne Kammer and Christian Damgaard for fruitful discussions.

REFERENCES

1. Purcell, D. F., and Martin, M. A. (1993) J. Virol. 67, 6365–6378
2. Schwartz, S., Felber, B. K., Benko, D. M., Fenyo, E. M., and Pavlakis, G. N. (1990) J. Virol. 64, 2519–2529
3. Furtado, M. R., Balachandran, R., Gupta, P., and Wolinsky, S. M. (1991) Virology 185, 258–270
4. Muesing, M. A., Smith, D. H., Cabradilla, C. D., Benton, C. V., Lasky, L. A., and Capon, D. J. (1985) Nature 313, 450–458
5. Robert-Guroff, M., Popovic, M., Gartner, S., Markham, P., Gallo, R. C., and Reitz, M. S. (1990) J. Virol. 64, 3391–3398
6. Smith, J., Azad, A., and Deacon, N. (1992) J. Gen. Virol. 73, Pt. 7, 1825–1828
7. Malin, M. H., Hauber, J., Le, S. Y., Maizel, J. V., and Cullen, B. R. (1989) Nature 338, 254–257
8. Daly, T. J., Cook, K. S., Gray, G. S., Maione, T. E., and Rusche, J. R. (1989) Nature 342, 816–819
9. Ropers, D., Ayadi, L., Gattoni, R., Jacquenet, S., Damier, L., Branlant, C., and Stevenin, J. (2004) J. Biol. Chem. 279, 29963–29973
10. Zhai, J., Mayeda, A., and Krainer, A. R. (2001) Mol. Cell 8, 1351–1361
11. Jacquenet, S., Decimo, D., Muriaux, D., and Darlix, J. L. (2005) Retrovirology 2, 33
12. Caputi, M., Mayeda, A., Krainer, A. R., and Zahler, A. M. (1999) EMBO J. 18, 4060–4067
13. Domniz, J. K., Wang, Y., Mayeda, A., Krainer, A. R., and Stoltzfus, C. M. (2003) Mol. Cell. Biol. 23, 8762–8772
14. Tange, T. O., Damgaard, C. K., Guth, S., Valcarcel, J., and Kjems, J. (2001) EMBO J. 20, 5748–5758
15. Bilodeau, P. S., Domniz, J. K., Mayeda, A., Krainer, A. R., and Stoltzfus, C. M. (2001) J. Virol. 75, 8487–8497
16. Bilodeau, P. S., Okada, Y., Nishio, Y., and Malim, M. H. (1999) J. Biol. Chem. 274, 1351–1361
17. Caputi, M., Mayeda, A., Krainer, A. R., and Zahler, A. M. (1999) EMBO J. 18, 4060–4067
18. Marchand, V., Mereau, A., Jacquenet, S., Thomas, D., Mougin, A., Gattoni, R., Stevenin, J., and Branlant, C. (2002) J. Mol. Biol. 323, 629–652
19. Bohne, J., Wodrich, H., and Krausslich, H. G. (2005) Nucleic Acids Res. 33, 825–837
20. Lu, X. B., Heimer, J., Rekosh, D., and Hannam, M. L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7598–7602
21. Kwek, K. Y., Murphy, S., Furger, A., Thomas, B., O’Gorman, W., Kimura, H., Proudfoot, N. J., and Akoulitchev, A. (2002) Nat. Struct. Biol. 9, 800–805
22. Naldini, L., Blomer, U., Gage, F. H., Trono, D., and Verma, I. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11382–11388
23. Vier, B. G., Ajuh, P., Aksasjarvi, G., Lamond, A. I., and Krevi, J. P. (2000) Nucleic Acids Res. 28, E14
24. Blanchette, M., and Chabot, B. (1999) EMBO J. 18, 1939–1952
25. Chang, D. D., and Sharp, P. A. (1989) Cell 57, 789–795
26. Ge, H., Zuo, P., and Manley, J. L. (1991) Cell 66, 373–382
27. Benko, D. M., Schwartz, S., Pavlakis, G. N., and Felber, B. K. (1990) J. Virol. 64, 2505–2518
28. Sallfeld, T., Gottlinger, H. G., Sia, R. A., Park, R. E., Sodroski, J. G., and Haseltine, W. A. (1990) EMBO J. 9, 965–970
29. lemon, K., Bileadou, P. S., van Wamel, J. L., Kjems, J., Stoltzfus, C. M., and Berkhout, B. (2003) J. Virol. 77, 3495–3500
30. Dong, D. A., and Sharp, P. A. (1989) Cell 57, 573–583
31. Huang, Y., and Carmichael, G. G. (1996) Mol. Cell. Biol. 16, 6046–6054
32. Legrain, P., and Rosbash, M. (1989) Cell 57, 800–805
33. Fischer, U., Pollard, V. W., Luehrmann, R., Teufel, M., Michael, M. W., Dreyfuss, G., andalm, M. H. (1999) Nucleic Acids Res. 27, 4128–4134
34. Emerman, M., Vazquez, R., and Peden, K. (1989) Cell 57, 1155–1165
35. Felber, B. K., Hadzopoulou-Cladaras, M., Cladaras, C., Copeland, T., and Pavlakis, G. N. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1495–1499
36. Nasioi, G., Zolotukhi, A. S., Tabenero, C., Solomin, L., Cunningham, C. P., Pavlakis, G. N., and Felber, B. K. (1994) J. Virol. 68, 2986–2993
37. Fischer, U., Meyer, S., Teufel, M., Heckel, C., Luehrmann, R., and Rauh, G. (1994) EMBO J. 13, 4105–4112
38. Powell, D. M., Amaral, M. C., Wu, Y. Y., Maniatis, T., and Greene, W. C. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 973–978
39. Kwok, K. Y., Murphy, S., Furger, A., Thomas, B., O’Gorman, W., Kimura, H., Proudfoot, N. J., and Akoulitchev, A. (2002) Nat. Struct. Biol. 9, 800–805
A Novel Splice Donor Site in the *gag-pol* Gene Is Required for HIV-1 RNA Stability

Martin Lützelberger, Line S. Reinert, Atze T. Das, Ben Berkhout and Jørgen Kjems

*J. Biol. Chem.* 2006, 281:18644-18651.
doi: 10.1074/jbc.M513698200 originally published online May 4, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M513698200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 39 references, 20 of which can be accessed free at
http://www.jbc.org/content/281/27/18644.full.html#ref-list-1