Differential Effects of the SR Proteins 9G8, SC35, ASF/SF2, and SRp40 on the Utilization of the A1 to A5 Splicing Sites of HIV-1 RNA*

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Splicing is a crucial step for human immunodeficiency virus, type 1 (HIV-1) multiplication; eight acceptor sites are used in competition to produce the vif, vpu, vpr, nef, env, tat, and rev mRNAs. The effects of SR proteins have only been investigated on a limited number of HIV-1 splicing sites by using small HIV-1 RNA pieces. To understand how SR proteins influence the use of HIV-1 splicing sites, we tested the effects of overproduction of individual SR proteins in HeLa cells on the splicing pattern of an HIV-1 RNA that contained all the splicing sites. The steady state levels of the HIV-1 mRNAs produced were quantified by reverse transcriptase-PCR. For interpretation of the data, transcripts containing one or several of the HIV-1 acceptor sites were spliced in vitro in the presence or the absence of one of the tested SR proteins. Both in vivo and in vitro, acceptor sites A2 and A3 were found to be strongly and specifically regulated by SR proteins. ASF/SF2 strongly activates site A2 and to a lesser extent site A1. As a result, upon ASF/SF2 overexpression, the vpr mRNA steady state level is specifically increased. SC35 and SRp40, but not 9G8, strongly activate site A3, and their overexpression in vivo induces a dramatic accumulation of the tat mRNA, to the detriment of most of the other viral mRNAs. Here we showed by Western blot analysis that the Nef protein synthesis is strongly decreased by overexpression of SC35, SRp40, and ASF/SF2. Finally, activation by ASF/SF2 and 9G8 was found to be independent of the RS domain. This is the first investigation of the effects of variations of individual SR protein concentrations that is performed in vivo on an RNA containing a complex set of splicing sites.

Splicing plays a key role for production of the HIV-1 retroviral proteins. By using the integrated proviral genome as the template, RNA polymerase II of the infected cell produces long primary transcripts that are all identical. Some of these transcripts are transported to the cytoplasm in an intact form to serve as genomes for new virions or as messenger RNAs for the production of the Gag-Pol protein precursor. The other transcripts undergo alternative splicing to produce mRNAs for the auxiliary and regulatory proteins and the Env precursor protein. Production of mRNAs encoding HIV-1 proteins depends on the alternative utilization of four 5'-splice sites D1 to D4 (1) and eight 3'-splice sites (A1, A2, A3, A4a, -b, -c, A5, and A7) (1). An additional 3'-splice site (A6) was only found in one HIV-1 strain (2). Donor sites D1, D2, and D3 can be coupled to any of the A1 to A5 acceptor sites, whereas donor site D4 is exclusively coupled to site A7 and to site A6 when this site is present (1, 2). The combination of these various sites gives rise to at least 35 different mRNAs (1). Although the relative efficiencies of the HIV-1 donor sites seem to depend mainly upon their complementarity to the U1 snRNA 5'-terminal sequence (3, 4), efficiencies of HIV-1 acceptor sites depend upon the presence of cis-regulatory elements (5–21). Several studies have shown that HIV-1 acceptor sites are suboptimal as follows. (i) Their polypyrimidine tracts are short and interrupted by purines (3, 5, 9). (ii) Their branch point sequences are not canonical, and in some cases, a residue other than an adenosine is used as the branch site (22, 23). (iii) Their accessibility is limited by their sequestering in stable secondary structures (24). (iv) Several cis-inhibitory elements have been identified that down-regulate the A2, A3, and A7 sites by binding of hnRNP A1 or hnRNP H proteins (11, 12, 14, 16, 18, 19, 21). Despite these numerous handicaps, the utilization of several of the HIV-1 acceptor sites is essential for virus multiplication. Sites A3 and A7 are used for production of mRNAs for the Tat transcriptional activator (see Ref. 1 and for review see Ref. 25). One of the three A4a, -b, or -c sites and site A7 are used to produce mRNAs for the Rev protein (1). This regulatory protein plays an essential role in the transport of intact primary transcripts to the cytoplasm (for review see Ref. 26). Site A5 is required for production of mRNAs for the Nef and Vpu proteins. Nef down-regulates the cell-surface expression of the HIV-1 receptor glycoprotein and has many additional roles in the infected cell that favor virus multiplication. It is also responsible for AIDS pathology, because of its role in bystander lymphocyte apoptosis (for reviews

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see Refs. 27 and 28). Finally, site A2 is needed for production of mRNAs for the Vpr protein. Vpr is implicated in nuclear import of the HIV-1 pre-integration complex, and it induces cell cycle arrest in proliferating cells (for review see Ref. 29).

The efficiency of the regulated acceptor sites of cellular mRNAs generally depends upon the binding of members of the SR protein family (for reviews see Refs. 30 and 31). To date, 10 distinct members of this family have been identified (for reviews see Refs. 30 and 32). These proteins contain one or two RNA recognition motif(s), together with a carboxy-terminal domain containing numerous arginine-serine amino acid pairs. SR proteins are involved in constitutive splicing where they seem to have redundant functions in assistance to spliceosomal component assembly. Because of their interactions with the 70K protein of U1 snRNP, the 35-kDa subunit of factor U2AF, and proteins of the U4/U6.U5 tri-snRNP, the SR proteins are essential actors in the complex mechanism of spliceosome assembly (for reviews see Refs. 30 and 33). In addition, SR proteins can regulate alternative splicing by binding to cis-regulatory elements. These regulatory elements generally bind specifically one given SR protein and, depending on the identity of this SR protein, different effects may be generated. Binding of SR proteins to specific sequences located downstream from the acceptor site generally reinforces the efficiency of U2AF binding to the polypyrimidine tract, either by direct interaction of the RS domain of the SR protein with the RS domain of U2AF35 or by displacement of the hnRNP A1 protein that blocks the access of spliceosomal components to the 3' splice site (for reviews see Refs. 30, 31, and 33).

The role of SR proteins on viral multiplication has been demonstrated by previous studies on adenovirus. SR proteins activate the production of the early mRNAs, but a limitation of their activity is required for the late phase of infection (34). This limitation results from titration of the SR proteins by the major late transcripts (35) and/or a virus-induced dephosphorylation (36). Although variations of the cellular concentrations of some of the SR proteins, namely SC35 and 9G8, have been observed after cell infection by virus HIV-1 (37, 38), little is known about the action of SR proteins on the competition between the eight splicing acceptor sites and four splicing donor sites of HIV-1 RNA. The present knowledge in this field can be summarized as follows: (i) deep studies, which were performed both in vitro and in vivo, demonstrated the action of proteins ASF/SF2 and SC35 at acceptor site A7 (17, 19, 20, 39, 40); (ii) an in vitro study performed with S100 extract showed that, among the SR proteins SC35, SRp40, SRp55, and SRp70, only protein SC35 is able to activate site A3 in S100 extract (41). For a complete study of the effects of individual SR proteins on the entire set of HIV-1 splicing sites in cellular conditions, we cotransfected HeLa cells with a construct that produces a truncated HIV-1 RNA containing all the splicing sites (13, 24), and a plasmid that overexpresses one of the SC35, SRp40, ASF/SF2, and 9G8 SR proteins. In these ex vivo experiments, very different HIV-1 RNA splicing patterns were observed with different overexpressed SR protein. For interpretation of the data, in vitro splicing assays were performed with transcripts that contained the D1 donor site and several of the A1 to A5 acceptor sites.

EXPERIMENTAL PROCEDURES

Plasmids Used in This Study—Pld-C2, pLD-C3, and pLD-L3-U1 constructs, used for production of the C2, C3, and L3-U1 transcripts, were described previously (11, 23, 24). To build the plasmid pLD-C1, two DNA fragments were generated by PCR amplifications of plasmid pBRU3 (42) containing the HIV-1 BRU/LAI complete DNA (GenBank™ accession number K02013). Amplifications were performed as described previously (23). The DNA fragment encoding the RNA region from position 4313 to 4545 (43) was amplified with the following: (i) a sense primer O-756 (5′-TTATTGATAGTCTTCAAACGCTGAAAC-3′) containing the HIV-1 BRU RNA sequence from positions 4313 to 4325 (underlined) and a BglII restriction site (italic); and (ii) an antisense primer O-764 (5′-CGGAATTCCTTCCCCAGAGGAGCT-3′) complementary to the HIV-1 BRU RNA from positions 4530 to 4545 (underlined) and containing an EcoRI restriction site (italic). The BglII/EcoRI fragment of plasmid pLD-C2 (23) was substituted for the BglIII-EcoRI fragment of the amplified DNA. The pSP ex2 inv 9G8–102 plasmid (44) was used for production of an SP6 transcript allowing us to test for protein 9G8 splicing activation properties in vitro. Plasmids ΔPSpU used for transfection experiments were described previously (11). Plasmids pXJ41-ASF, pXJ41-SRp40, pXJ42–9G8, pXJ42–SC35, or pXJ42–SRp20 were used for overexpression of the corresponding SR proteins. They were used as templates for the production of capped, uniformly labeled RNAs by the T7 or SP6 RNA polymerase in the presence of [α-32P]UTP. In vitro splicing assays were performed in HeLa cell nuclear extract (from the Computer Cell Culture Center S.A., Belgium), pure cytoplasmic S100 extract prepared as described previously (35), or mixtures of S100 and nuclear extracts (4:4 or 7:1), in 22-μl assays using classical splicing reaction conditions for in vitro splicing reaction (44). For splicing reaction, incubation at 30 °C was for 2 h and 30 min. For SR protein assays, 150 or 300 ng of purified 9G8, ASF/SF2, SC35, or SRp40 recombinant protein were used, prepared as described previously (44). The degree of purity of these proteins was verified by electrophoresis followed by argent staining and Western blot analysis. The C1, C2, C3, L3-U1, and Sp1 ex2 inv 9G8–102 splicing products were analyzed by electrophoresis on 5 or 6% polyacrylamide denaturing gels and were visualized by autoradiography. Quantifications were performed with a PhosphorImager (Amersham Biosciences) using the ImageQuant software (version 5.2). Splicing efficiency was expressed as the ratio of spliced products to unspliced RNA (P). For each gel electrophoresis of splicing products, MP values were measured three times using the ImageQuant software. The mean value is given with an estimation of the MP measurement error, shown as bar errors in the histograms representing the MP values (Figs. 1A, E1–E4, 2, C1–C4, and 3, C1–C3). The factor of splicing activation by SR proteins was estimated by dividing the MP ratio obtained in the presence of the recombinant SR protein by that obtained in its absence (fold of activation). The estimated errors on the factors of activation due to the experimental errors on MP measurements mentioned above was deduced and represented as bar errors in the histograms showing activation factors.

Ex Vivo Splicing Assays—HeLa cells in 60-mm plastic Petri dishes were cotransfected with the calcium phosphate precipitation technique as described previously (48). RNA was extracted using the RNA-solv Reagent (Omega Bio-Tek), and it was treated by DNase. Three μg of RNA were reverse-transcribed by the Moloney murine leukemia virus-reverse transcriptase according to the supplier’s specifications (Invitrogen), using oligonucleotide ACC15 (5′-TTACTA-ATCGAATTGCTAC-3′), complementary to the HIV-1 pNL4-3 RNA from positions 8445 to 8463) as the primer. About 10% of each of the RT reactions was PCR-amplified with the forward oligonucleotide 2143 (5′-GGCTGCTGAAACGCGCAGGAGG-3′), containing the HIV-1 BRU RNA sequence from positions 700–727 of the plasmid pSP ex2 inv 9G8–102, which spans the D4 and A7 splice sites (5′-TTGAGGTTGGTT-GCTTTGATAGAG-3′), complementary to pNL4–3 RNA from positions 6029–6041 and 8389–8381). This selectively amplified the 1.8-kb HIV size class mRNAs. Amplification was limited to 30 cycles, in order to detect both major and minor bands. DNA products were analyzed by electrophoresis on a 6% polyacrylamide nondenaturing gel. A typhoon 8600 imager (Amersham Biosciences) was used to scan the gel after ethidium bromide staining. PCR products were quantified using ImageQuant (Amersham Biosciences). The fractioned cDNAs were identified by reference to previous data on HeLa cells transfected with plasmid ΔPSpU (11, 24).

The level of expression of individual SR proteins in the different transfection experiments was estimated by Western blot analysis of total cell extracts using the monoclonal antibody 10H3 directed against the RS domain of the SR protein family and specific antibodies directed against ASF/SF2, SC35, or 9G8 (44). A monoclonal antibody directed...
against the HIV-1 Nef protein (MaT0020), a generous gift of A. M. Auberin, was used to test by Western blot assays the yield of transitory expression of the Nef protein (47), in HeLa cell transfected with the ΔPSP plasmid, with or without overexpressing one of the SR proteins.

RESULTS

We will first describe the in vitro experiments that were used to interpret the data obtained by HeLa cell transfection.

Strategy Used for in Vitro Splicing Assays — As regulation of alternative splicing of HIV-1 RNA was proposed to depend mainly on the suboptimal properties of acceptor sites, the in vitro studies were focused on the effect of SR proteins on acceptor sites. In addition, as we already studied the SR protein effect at site A7 in vitro (21), we concentrated the in vitro study on the effects of SR proteins on the acceptor sites A1–A3, A4a–c, and A5. To this end, we produced in vitro transcripts that contained the same donor site (D1) and one of the A1–A3 sites or the block of five A3, A4a–c, and A5 sites (Figs. 1A and 2A), and we tested the effects of the addition of purified recombinant SR proteins on their in vitro splicing efficiencies. All the transcripts contained the 5′ exon of HIV-1 RNA, which is delimited by site D1, and its 95-nt-long downstream sequence. The 5′ part of the intron was joined to segments of the HIV-1 RNA, containing the studied acceptor site(s). In transcripts C1–C3, the 178, 303, and 187-nt-long segments, located upstream from sites A1–A5, were used, respectively, as the 3′ part of the intron. Transcript L3-U1 contained the same 3′-intronic sequence as transcript C3. As no information was available on cis-regulatory elements at site A1, a 48-nt-long segment (positions 4492–4544) was arbitrarily selected as the second exon of transcript C1 (Fig. 1A). In transcript C2, the second exon (positions 4972–5053) contained ESSV, the cis regulatory element that was described for site A2 (positions 4995–5013) (12). In transcript C3, the second exon (positions 5559–5408) contained the identified cis inhibitory element ESS2p that binds hnRNP H (11). However, the downstream ESS2 inhibitory element (7–9), which binds hnRNP A1 (14, 16), and the recently described ESE2 activator element (41) were not present in this transcript (Fig. 1A). These elements were present in the longer L3-U1 transcript, which ends at position 5637 and also contains sites A4a–c and A5 (Fig. 2A).

Compared with nuclear extracts, S100 extracts contain very low levels of SR proteins and thus are more suitable than nuclear extracts to test for the splicing activation effects of individual SR proteins. However, when recombinant SR proteins were added to the S100 extract, splicing efficiencies of the four tested transcripts were low (Figs. 1, B–D, and 2B). Hence, for a quantitative estimation of splicing site activation by SR proteins, the C1–C3 and L3-U1 RNAs were spliced in mixtures of nuclear and S100 extracts. Either equal volumes (4 μl) of HeLa nuclear and S100 extracts (4:4 NE/S100 mixture) or 1 μl of nuclear extract and 7 μl of S100 extract (1:7 NE/S100 mixture) were used. For simplification, only the results obtained in the 4:4 NE/S100 mixture are shown in detail. For the 1:7 NE/S100 mixture, only the calculated activation factors of each SR protein on each splicing site are given (Figs. 1E4 and 2C4). The splicing activation properties of recombinant 9G8, ASF/SF2, SC35, and SRp40 proteins were tested in these two mixtures and in the S100 extract, and the observed splicing efficiencies were compared with those in the nuclear extract (Figs. 1, B–D, and 3). In all these experiments, the splicing efficiencies and the factors of activation were estimated as explained under “Experimental Procedures.” A comparative analysis of the activation values obtained in four separate experiments performed with different batches of nuclear extracts, different preparations of transcripts, and SR proteins revealed a high degree of reproducibility of the experiments.

Comparative Analysis of Sites A1–A3 Efficiencies in Vitro — As expected, in the absence of SR protein addition, none of the C1–C3 transcripts were spliced in the S100 extract (Fig. 1, B–E1). However, addition of individual SR proteins are sufficient to promote the splicing of each of the acceptor sites. (i) Splicing at site A1 is the most efficient upon addition of proteins ASF/SF2 or SC35. (ii) Splicing at site A2 is the most efficient upon addition of protein ASF/SF2. (iii) In agreement with the data of Zahler et al. (41), limited splicing occurs at site A3 upon addition of protein SC35. Most interesting, in the S100 extract, protein SRp40 only has a low activation property on the A3 site contained in the C3 RNA, but the activation factor increases strongly upon addition of nuclear extract to the S100 extract.

In the 4:4 NE/S100 mixture without SR protein addition, splicing efficiencies were in the order C1 > C2 > C3 (Fig. 1, B–D, lanes 2, and E2). The same relative order of splicing efficiencies was found for assays performed in pure nuclear extract (Fig. 1, B–D, lanes 2 in insets, and E2). Hence, in the context of the transcripts used in these in vitro experiments, site A1 had a higher efficiency than sites A2 and A3. These data confirmed the observation that, even in the absence of the ESS2 inhibitory element, site A3 has a low splicing efficiency (11). In the 4:4 NE/S100 mixture, splicing at site A1 (Fig. 1B) was markedly activated by both ASF/SF2 (lane 6) and SC35 (lane 8) proteins, whereas activations by proteins 9G8 (lane 4) and SRp40 (lane 10) were weak and undetectable, respectively (see Fig. 1, E2 and E3 for quantifications). Splicing at site A2 (Fig. 1C) was also strongly activated by addition of protein ASF/SF2 (lane 6) and to a lower extent by addition of the SC35 (lane 8) and SRp40 (lane 10) proteins. Protein 9G8 (Fig. 1C, lane 4) had a very weak effect (Fig. 1, E2 and E3). Finally, splicing at site A3 (Fig. 1D) was activated by addition of each of the four SR proteins, but again protein 9G8 had the weakest effect. On a quantitative point of view (Fig. 1E3), protein ASF/SF2 had similar activation efficiencies on sites A2 and A3, as well in the 4:4 and the 1:7 NE/S100 mixtures (factors of about 10 and 12, respectively), and the highest activation efficiencies were found for SRp40 and SC35 proteins at site A3 in the 4:4 NE/S100 (factors of ~15 and 19, respectively).

We concluded that in transcripts containing individual acceptor sites in vitro: (i) proteins ASF/SF2 and SC35 activate site A1 utilization to a similar extent; (ii) protein ASF/SF2 has a stronger activation effect than protein SC35 on site A2; (iii) proteins SRp40 and SC35 have a stronger activation capacity than protein ASF/SF2 at site A3; however, the effect of SRp40 depends upon the presence of other components of the nuclear extract; and (iv) protein 9G8 has a modest activity on the three sites.

Comparison of Site A3, A4a–c, and A5 Efficiencies in the L3-U1 Transcript — Transcript L3-U1 (Fig. 2) contained the 5′-splice site D4, complementary to the U1 snRNA, that is required for an efficient utilization of site A3 in the presence of ESS2 and also for utilization of sites A4a–c and A5 (7). As expected in pure S100 extract, no splicing occurred at any of the five sites of the L3-U1 RNA, and addition of each of the purified SR proteins in S100 extract allowed a low yield of utilization of site A3, whereas the four other sites were not used (Fig. 2, B and C1). In the 4:4 NE/S100 mixture, site A5 was preferentially used (factor of 2 compared with site A3; Fig. 2C2), indicating that some component(s) in nuclear extract favors the utilization of site A5 at the expense of site A3. Addition of protein 9G8 had a slight inhibitory effect on the utilization of all the five 3′-splice site (Fig. 2C3). Protein ASF/SF2 slightly increased utilization of sites A4a and -b. However, in contrast to the results obtained for the C3 RNA, it only had...
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Fig. 1. Differential effects of individual SR proteins on in vitro utilization of HIV-1 sites A1–A3 in the C1–C3 RNAs. A, schematic representation of the C1–C3 pre-mRNAs containing the donor site D1 and the acceptor sites A1–A3, respectively. Boxes represent exons, and lines represent introns. The positions in the HIV-1 BRU RNA (43) of the extremities of the HIV-1 sequences, which are joined together in the transcripts (BglII site in the cDNA), are indicated above the constructs. The various splicing sites and splicing regulatory elements that were previously identified (11, 12) are also indicated.

B–D, in vitro splicing assays. The uniformly labeled C1 (B), C2 (C), or C3 (D) transcripts were incubated for 150 min in 8 μl of buffer D (lanes 1 in B–D and lanes 1 of the insets), a mixture of nuclear (NE) and cytoplasmic (S100) extracts (NE/S100 4:4 μl) or pure S100 extract (8 μl), under splicing conditions, in the absence (lanes 2 and 3) or the presence of 300 ng of the individual SR protein 9G8 (lanes 4 and 5), ASF/SF2 (lanes 6 and 7), SC35 (lanes 8 and 9), or SRp40 (lanes 10 and 11). The transcripts were also incubated for 150 min in the presence of 8 μl of HeLa cell nuclear extract (+) (insets in B–D). The products of the reaction were separated by electrophoresis in a 5% polyacrylamide sequencing gel. Positions of pre-mRNAs (P), exon 1 (E), lariat splicing intermediates (L), and splicing products (M) are indicated on the left side.
a modest activation effect on site A3. Protein SRp40 and to a higher extent protein SC35 had strong activation properties on site A3 (factors of about 2 and 5, respectively) but did not modify sites A4a and -b utilization and decreased site A5 utilization (Fig. 2, B and C3). When the L3-U1 transcript was spliced in the 1:7 NE/S100 mixture, the activation by the four SR proteins was qualitatively similar to that observed in the 4:4 EN/S100 (Fig. 2, compare C3 and C4). However, with a quantitative point of view, two significant differences are observed; activation of sites A4a and -b by ASF/SF2 was more efficient in the 1:7 compared with the 4:4 mixture, whereas site A3 activation by SC35 was lower in the 1:7 compared with the 4:4 mixture.

Finally, when the L3-U1 transcript was spliced in nuclear
extract (Fig. 3, A, C1, and C2), site A5 became predominantly used. Addition of protein 9G8 again had a very limited effect on the relative efficiencies of the five sites, and protein ASF/SF2 had a moderate activation effect on site A3 (factor of 2.6) and it slightly decreased site A5 utilization (30% decrease). Protein SRp40 and especially SC35 had a strong activation effect on site A3 (factors of about 5.2 and 15, respectively), and the two proteins had a negative effect on site A5 utilization (60% decrease in both cases) (Fig. 3C2). Utilization of sites A4a and -b is moderately sensitive to activation by SR proteins, because only protein ASF/SF2 slightly increased their utilization. Site A5 is not subjected to an activation by the tested SR proteins and seemed to be regulated by default under the conditions used. To verify that the observed SR activation effects were dependent upon the SR protein concentrations, we compared the activation effects of proteins SC35 and SRp40 on site A3 utilization in the L3-U1 transcript, using pure nuclear extract and two different amounts of recombinant proteins, namely 150 and 300 ng (Fig. 3B) (this latter amount was the same as that used in the previous assays; Fig. 3A). For both proteins, estimation of \( M\) showed that activation increased with increasing protein concentration. Whereas increasing the SC35 protein amount by a factor of 2 augmented splicing efficiency at site A3 by a factor of 2.5, the same increase of the amount of protein SRp40 only augmented site A3 utilization by a factor of 1.6 (Fig. 3C3), which is again in accord with the idea that the action of SRp40 is dependent on that of another nuclear factor.

The Strategy Used for ex Vivo Assays—The above in vitro assays showed how activation of HIV-1 splicing sites by SR proteins, especially site A3, strongly depends upon the RNA used for the in vitro assay. It was thus important to develop a strategy to test the effects of individual SR proteins on an RNA that contained all the HIV-1 splicing sites. Due to limitation in the length of RNAs that can be spliced in vitro, such experiments had to be performed by transfection of HeLa cells. To this end, we used the \( \Delta P\) transcript and in the authentic HIV-1 RNA (13, 24). HeLa cells were cotransfected with \( \Delta P\) and two amounts (50 and 250 ng) of a recombinant vector that expressed one SR protein (either the full-length SR protein 9G8, ASF/SF2, SC35, SRp40, or SRp20 or a shortened form of protein 9G8 or ASF/SF2 that was missing the RS domain). The full-length SR proteins contained an additional histidine tag that decreased their mobility in SDS-PAGE and allowed us to distinguish them from their endogenous counterparts by means of a general SR-detecting antibody 10H3, comparable with monoclonal antibody 104 (44) (see Fig. 4). Our previous experiments\(^2\) showed that among the SRp30 proteins, proteins 9G8 and ASF/SF2, but not protein SC35, are efficiently recognized by the 10H3 antibody. Hence, by using this antibody, two bands corresponding to 32–35-kDa proteins are detected, which mainly represent the endogenous 9G8 and ASF/SF2 proteins (Fig. 4A). In addition, a specific SC35 antibody was also used for the analysis of protein SC35 expression (Fig. 4B). The other endogenous SR proteins (SRp75, SRp55, SRp40, and SRp20) are well detected, which mainly represent the endogenous 9G8 and ASF/SF2 proteins (Fig. 4A).

\(^2\) R. Gattoni and J. Stevénin, unpublished data.
using the monoclonal antibody 104 (41, 48–50), the yield of protein SRp40 detected in HeLa cells may sometimes be lower than that of proteins Srp30 and Srp20. Western blot analysis of the SR protein expression was performed three times on different batches of cells cotransfected with plasmid ΔPSP and the highest amount of SR protein expressing vectors. Reproducible results were obtained, and one example is given in Fig. 4, showing that all recombinant SR proteins were well expressed. By quantification of the Western blot signals, we estimated the relative levels of each recombinant SR protein versus the corresponding endogenous protein to 0.75 for Srp20, 1.70 for ASF/SF2, 1.55 for 9G8, 4.4 for Srp40 (Fig. 4, A and C), as well as 1.75 for SC35 (Fig. 4, B and C). As a whole, considering that about 25% of HeLa cells were transfected with an SR expression vector, the recombinant SR proteins, except Srp40, were estimated to be expressed between 3 and 7 times more than the endogenous SR molecules, for the highest amount of SR expression vectors, and at least twice less with the lowest amount. The values were highest for Srp40, but they are biased due to the lower level of endogenous Srp40, and they should not be considered as abnormal. Taken together, the results of Fig. 4 suggested that the overexpression of all SR proteins remained limited enough to mimic possible natural variations of SR protein concentrations.

The effects of the overexpression of SR proteins on HIV-1 RNA splicing were analyzed by RT-PCR. For the analysis of the levels of the various HIV-1 mRNAs in the absence of SR protein overexpression, HeLa cells were cotransfected with plasmid ΔPSP and an empty SR protein expression plasmid. Several transfection experiments using various sets of SR protein expression vectors have been performed. They gave similar results, and one representative example is given (Fig. 5A). Note that protein Srp20 was found to have no splicing activation capacity in our conditions (data not shown). The RT-PCR analyses have been performed with a reverse primer that encompasses the D4-A7 junction, so that all the mRNAs identified were submitted to the D4-A7 splicing reaction. They were identified by comparison with previous data obtained by the same RT-PCR approach (11, 24). In addition, the identity of the vpr1-2 mRNAs was verified using oligonucleotides specific for vpr mRNAs (data not shown). Quantifications of the results illustrated in Fig. 4A are shown in Fig. 4C.

**Overexpression of Individual SR Proteins Strongly Modifies the Pattern of HIV-1 RNA Splicing Products**—As observed previously by Purcell and Martin (1) and Bilodeau et al. (13), splicing of HIV-1 RNA without overexpression of SR proteins led to a higher production of the nef mRNA family than the tat and rev mRNA families (Fig. 5A, lanes 1). This is in agreement with the stronger utilization of site A5 found in nuclear extract *in vitro* (Fig. 3A). In addition, as observed previously (1), the vpr1 and vpr2 mRNAs were present in low yields. As illustrated in Fig. 5, A and C, the strongest effects of protein ASF/SF2 overexpression are as follows: (i) a strong increase of the vpr mRNA levels, especially vpr1 mRNA, and (ii) a decrease of both the tat1 and the nef2 mRNA levels (Fig. 5, A, lanes 6 and 7, and C). We noted also a slight increase of the nef3, nef5, and tat4 mRNA levels when higher amounts of plasmid pXJ41-ASF/SF2 were used for the transfection. On the basis of the *in vitro* data (Fig. 1), the important increase found for vpr1 mRNA and the slight effects on nef4, nef5, and tat4 mRNAs can result from the strong activation of site A2 by protein ASF/SF2. Indeed, production of these four mRNAs implies a splicing event at site A2 (Fig. 5B). Although site A3 was found to be activated by protein ASF/SF2 *in vitro*, it was probably not activated by protein ASF/SF2 in HeLa cells, because the global amount of the tat mRNAs did not increase significantly upon overexpression of this protein. The global decrease of nef mRNAs upon ASF/SF2 overexpression confirmed the absence of activation of site A5 by this protein, which we also noticed in *in vitro* assays.

Overexpression of proteins SRp40 and SC35 led to a huge increase of the tat1 mRNA, which likely reflects a very strong activation of the D1-A3 intron elimination (Fig. 5, A, lanes 10–13, and C). The tat2 mRNA level was also increased by protein SC35 but to a lower extent, suggesting that elimination of the D1-A3 intron was more favored than the combined elimi-
When protein SRp40 was overexpressed, tat2 mRNA completely disappeared at the benefit of tat3 mRNAs (Fig. 5A, lanes 12 and 13), indicating that protein SRp40 activated markedly site A3 and moderately site A2, which fits with our in vitro data (Fig. 1). The strong activation of site A3 by proteins SC35 and SRp40 resulted in a marked reduction of all other mRNA species, including the nef mRNAs (Fig. 5C). Only nef3 mRNA was moderately affected by the SC35 overexpression. In agreement with the incapacity of protein SRp40 to activate site A1, nef3 mRNA was strongly reduced by SRp40 overexpression. Taken together in HeLa cells, as found in vitro with the L3-U1 transcript (Figs. 2 and 3), site A3 was strongly activated by proteins SRp40 and SC35 at the expense of site A5. Protein 9G8 had the more limited effect on the HIV-1 RNA splicing pattern (Fig. 5A, lanes 2 and 3, and B). Like proteins SRp40 and SC35, it increased tat1 mRNA synthesis, but the factor of activation was lower. It also slightly increased the synthesis of the tat2 and nef3 mRNAs, which might result from the activation of site A1. In contrast, nef4 mRNA, which reflects the use of site A2, was slightly decreased upon 9G8 addition.

The Activation Effects of Proteins ASF/SF2 and 9G8 Are Preserved upon Deletion of Their Activation RS Domain—Since it has been shown that protein ASF/SF2, deleted of its RS domain, remains active in vitro in stimulating splicing of an HIV-1 tat pre-mRNA containing a shortened D4-A7 intron (17), we tested whether this ASF/SF2 deleted form expressed in transfected cells preserves its activation property on the HIV-1 pre-mRNA produced from the AP5P plasmid. We also tested the activation properties of a ΔRS 9G8 protein. Most interest-
ing, the deleted forms of ASF/SF2 and 9G8 had the same global effect as their wild type counterparts, with a slight decrease in the capacity to increase the spr1-2 mRNA synthesis in the case of protein ASF/SF2 (Fig. 5A, lanes 8 and 9). We concluded that the presence of the RS domains of 9G8 and ASF/SF2 is not strictly required for their specific activation properties on site A3 for 9G8 and site A2 for ASF/SF2.

Overexpression of Proteins ASF/SF2, SC35, and SRp40 Decreases Nef Protein Expression—Finally, we determined whether the effects of SR proteins, which we observed on the alternative splicing of HIV-1 RNA, could also be detected at the protein level. To this end, after cotransfection assays using the ∆PSP plasmid and the highest amount of SR-expressing plasmid, total proteins from transfected cells were extracted and analyzed by Western blot with antibodies directed against several viral proteins. Signals too weak to get quantitative results were obtained with specific antibodies directed against the Tat and Rev proteins. However, a monoclonal antibody directed against Nef, which is the more expressed protein during the early period of infection, gave a strong signal for a protein migrating as Nef with proteins extracted from HIV-1-transfected HeLa cells (Fig. 5D). Only a limited reduction in the Nef level was found upon protein 9G8 and SRp20 overexpression. In contrast, in agreement with results observed at the mRNA levels, ASF/SF2, SRp40, and SC35 overexpression resulted in a strong reduction of the Nef level. The effects on Nef protein even appeared to be stronger than that observed on its mRNAs. This result can be due, at least in part, to the different technical approaches used for their respective detection.

Based on these ex vivo experiments, we concluded that the strong activation of site A2 by ASF/SF2 and the strong activation of site A3 utilization by SRp40 and SC35 found in vitro occur in cell cultures and that the relative levels of SR proteins strongly modify the pattern of splicing of HIV-1 RNA and its protein expression as illustrated by the Nef protein analysis.

DISCUSSION

Splicing of RNAs from lentiviruses is a complex process because of the alternative utilization of several suboptimal 3′-splice sites (1). Here we tested the effects of variations of individual SR protein concentrations in HeLa cells on the HIV-1 RNA splicing pattern. This is the first investigation of SR protein effects that is made ex vivo on such a huge number of splicing sites that compete one with another.

When Taken Individually, Three of the HIV-1 Acceptor Sites, A1–A3, Are Activated by Proteins ASF/SF2 and SC35—Two of the SR proteins tested, ASF/SF2 and SC35, have been shown to activate splicing of numerous viral and cellular genes, and they are general splicing activators. The other two, 9G8 and SRp40, could be more specific regulators. For instance, 9G8 was found to enhance the splicing of its own messenger (51) and that of the 12 S adenovirus E1A transcript (45), and SRp40 was found to be involved in alternative splicing of the cardiac tropinin T pre-mRNA (52). Accordingly, in S100 extract and in S100/NE mixtures, proteins ASF/SF2 and SC35 were found to activate the three A1–A3 sites, when these sites were tested individually (RNA C1–C3) (Fig. 1). In contrast, protein 9G8 had only a limited activation effect, and protein SRp40 had activation properties that were dependent upon the NE/S100 ratio. For instance, protein SRp40 only had a very low activity on site A3 in the C3 construct when splicing was done in the S100 extract, whereas it had a strong activation property on this site in the 4:4 NE/S100 mixture. This suggests the requirement of one or more nuclear factor(s) for activation of site A3 by protein SRp40 in this RNA context. In addition, the length of exon 2 strongly modified the activation properties of SR proteins. Although in the L3-U1 RNA, which contains all the A3 regulatory elements and the downstream splicing sites, the strong activation of site A3 by SC35 was found, no significant activation of this site by ASF/SF2 was detected. In addition, protein SRp40 was able to activate site A3 utilization in S100 extract in this RNA context. In agreement with our data, Zahler et al. (41) found that protein SC35 activates site A3 utilization in S100 extract. However, with the RNA that they used, which is shorter than RNA L3-U1, Zahler et al. (41) found no activation of site A3 by protein SRp40 in the S100 extract and gave no information on the activation property of protein ASF/SF2 in this RNA context. Taken together, our data and those of Zahler et al. (41) show how complex is the action of SR proteins on clusters of splicing sites and regulatory elements as found in the HIV-1 RNA region containing the five A3 to A5 splicing sites.

Ex Vivo ASF/SF2 Essentially Activates Site A2, Whereas SC35 and SRp40 Activate Nearly Exclusively Site A3—The differences found between RNA C3 and L3-U1 in vitro are reinforced when comparing results for the C3 RNA and ex vivo data. For instance, whereas site A3 was activated efficiently by ASF/SF2 in the context of the C3 construct and its activation by this SR protein was modest in the L3-U1 RNA, the level of tat1 mRNA spliced between sites D1 and A3 was impaired ex vivo upon ASF/SF2 overexpression. Obviously, we cannot rule out the fact that SR protein overexpression might alter the RNA steady state levels by modification of their stability. However, the more likely explanation of the ex vivo results is a direct effect of SR protein on splicing efficiency. The observed ex vivo effect of ASF/SF2 on the complete set of HIV-1 splicing sites may be explained by a preferential targeting of ASF/SF2 near site A2, at the expense of site A3, in HIV-1 RNAs containing all the sites. In line with this possible explanation, the competition between sites A2 and A1 for ASF/SF2 binding should be less strong than that for sites A2 and A3, because the level of the mRNAs, which were spliced at site A1 but not at the A2 site (tat2 and nef3), was slightly increased in HeLa cells upon overexpression of protein ASF/SF2. In contrast to what occurs with ASF/SF2, the activation effect of site A3 by protein SRp40 was reinforced ex vivo compared with in vitro. Indeed, in HeLa cells, proteins SC35 and SRp40 activate nearly exclusively site A3, revealing that the other acceptor sites are subjected to a strong competition with site A3 in these conditions. Although more modest, protein 9G8 also has a significant activity on site A3 ex vivo. Hence, three of the tested SR proteins activate preferentially site A3 ex vivo. Considering the other acceptor sites, it was most likely that the less efficient control by SR proteins at site A1 may be explained by the higher intrinsic efficiency of this site, as evidenced in vitro (Fig. 1). In addition, according to our ex vivo data, the three A4a–c sites are poorly used, and only site A4b is subjected to a moderate activation by SR proteins. We noticed that the strong increase of site A2 or A3 utilization due to SR protein overexpression in HeLa cells is accompanied by a marked decreased of site A5 utilization. This suggests that site A5 is at least in part regulated by default.

The HIV-1 Splicing Sites, Which Are the More Sensitive to SR Activation, Were Previously Found to Be Controlled by ESS Elements—Remarkably, sites A2 and A3, whose activities are strongly dependent upon SR proteins, were previously found to be subjected to negative controls by splicing silencer elements. Three distinct hnRNP A/B-binding sites were identified downstream from site A2 and have been found to be responsible for the ESSV inhibitory property (12). As found for site A7 (19, 21), ASF/SF2 may activate site A2 by competing with the binding of hnRNP A/B to ESSV. Two distinct inhibitory elements binding hnRNP H and hnRNP A1, respectively, were found at site A3 (7–9, 13, 14, 16, 41). When these two inhibitory elements ESS2 and ESS2p are present in pre-mRNA substrates, as is the case...
for the L3-U1 RNA (Fig. 3) and the full-length HIV RNA (Fig. 5), the use of site A3 remains weakly efficient in the presence of normal levels of SC35 or SRp40 proteins. This was also observed previously (24, 41). However, when their amount was increased, proteins SC35 and SRp40 are both able to counteract the inhibitory effects of these elements. The dramatic splicing shift observed with SC35 is in full agreement with the recent identification of an SC35-specific ESE, located just upstream of the ESS2 element (41). SC35 recognizes a large variety of sequences (44, 53, 54), but most interesting, the sequence element encompassing this ESE, CCAGUAGAUC, exhibits six consecutive matches with one of the high affinity motifs identified by SELEX (44, 54). However, because we show that SC35 also improves splicing of the C3 transcript, which does not contain this element, it is most likely that additional SC35-responsible motifs could be located in the first 50 residues downstream from the A3 site, or in the 3’ part of the intron. The role of SRp40 in activation of site A3, which we demonstrate here, has to be clarified. Protein SRp40 has a definite specificity for a long RNA sequence UGGGAGCGRUYGUGGYY (55); however, protein SRp40 may also activate splicing by interaction with shorter and more degenerate RNA motifs (53). No sequence homologous to the specific SRp40 recognition motif was detected in the HIV-1 RNA segment containing sites A1 to A5, in line with the observation that no strong binding of SRp40 occurs on the 100-residue region encompassing the SC35-specific ESE (41). As mentioned above, our data suggest that the action of SRp40 on site A3 in a short RNA context (C3 RNA) is dependent upon the presence of a nuclear component, perhaps another SR protein. In contrast, a direct activation of site A3 occurs in S100, in a longer RNA context (L3-U1 RNA), which suggests the occurrence of one binding site downstream from position 5408, referred to as the BRU RNA nomenclature.

We detected several putative binding sites for protein ASF/SF2, which are close to the A1–A3 binding sites, possibly explaining why these three sites were activated by ASF/SF2 in the C1–C3 transcripts. In contrast, no RNA sequences specific for protein 9G8, which are mainly based on repetitions of GAC triplets, as found in ESE element that respond to this protein (44, 45, 51, 56, 57), were detected in the HIV-1 RNA segment containing sites A1 to A5, in line with the observation that no strong binding of SRp40 occurs on the 100-residue region encompassing the SC35-specific ESE (41). As mentioned above, our data suggest that the action of SRp40 on site A3 in a short RNA context (C3 RNA) is dependent upon the presence of a nuclear component, perhaps another SR protein. In contrast, a direct activation of site A3 occurs in S100, in a longer RNA context (L3-U1 RNA), which suggests the occurrence of one binding site downstream from position 5408, referred to as the BRU RNA nomenclature.

Most interesting, as previously found for site A7 (17, 19), the activation property of ASF/SF2 on site A2 is not strictly dependent upon the presence of the R5 domain. The same is true for 9G8. This reinforced the idea that, at least in part, the action of ASF/SF2 consists in competing with the binding of inhibitory proteins on the ESS elements, rather than facilitating directly splicing assembly. At site A2, ASF/SF2 may compete with hnRNP A1 binding at ESSV. Taken together, the present data and previous data on site A7 (17, 19, 21, 39, 40, 58) show the strong sensitivity of two HIV acceptor sites A2 and A7 to ASF/SF2 and of a third one (A3) to a series of SR proteins (SC35, SRp40, and 9G8).

Variations of SR Protein Composition Is Expected to Alter HIV Protein Expression in Infected Cells—Based on the very different effects of ASF/SF2 on the one hand and SC35 and SRp40 on the other hand regarding the pattern of mRNAs produced from HIV-1 RNA, we can anticipate that variations of the relative ratios of SR proteins in infected cells will significantly modify the pattern of viral protein expression. Indeed, we demonstrate the strong reduction of the Nef protein production upon overexpression of proteins ASF/SF2, SRp40, and especially SC35. Thus, if ASF/SF2 expression is increased in the infected cell, the Vpr protein is expected to be produced in larger amounts, whereas the Nef protein level is expected to decrease. Protein Vpr is required for virus multiplication in nondividing cells because of its action at an early step of nuclear import of the preintegration complex (59). Protein Vpr also induces cell cycle arrest in proliferating cells, stimulates virus transcription, and regulates activation and apoptosis of infected cells (for review see Ref. 29). The Nef protein whose level might be decreased is for a large part responsible for the AIDS pathology (for review see Ref. 60). Indeed, by activating the production of Fas-L at the surface of the infected cells, Nef participates to the apoptoposis of neighboring cytotoxic T lymphocytes (61, 62). On the contrary, Nef protects the infected cell from apoptosis by interacting with the ASK1 kinase that is needed for transduction of the apoptosis signal (63). Hence, a high amount of ASF/SF2 compared with other SR proteins in infected cells may modify the fate of the infected cell and surrounding lymphocytes. If SC35 or SRp40 is present in excess compared with other SR proteins in the infected cell, the Tat protein is expected to be produced in large amounts. By taking into account the central role played by the Tat protein in transcription of full-length HIV-1 RNA (for review see Ref. 25), an increase of the SC35 and SRp40 concentration or activity in the infected cell may induce the entry in a viral multiplication cycle. Most interesting, Maldarelli et al. (37) observed a strong increase in the SC35 concentration after infection of T cells by HIV-1 virions. This increase, which is of the same order as the one we observed with the lower amount of SC35 construct in transfected cells, might favor tat mRNA production and facilitate the initiation of a cycle of virus multiplication. As the HIV-1 virus may remain silent for a long time in some infected cells (64), production of the Tat protein is probably not the unique parameter involved in the initiation of a viral multiplication cycle. It should also be considered that the Tat protein activity depends upon several post-translational modification events (65, 66). Another recent report (39) showed that the 9G8 protein concentration was selectively decreased in HIV-1-infected MT4 cells. According to the moderate effect of protein 9G8 on the HIV-1ΔPSP splicing pattern, this decrease is not expected to have marked consequences on HIV-1 protein production.

Altogether, the good agreement between our ex vivo and in vitro data with recent in vitro data on site A3 (41) brings support to the new approach, which we used in this study to investigate the activation properties of SR proteins on a very complex array of splicing sites. We think that this approach can be used to study other complex sets of splicing sites.

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Differential Effects of the SR Proteins 9G8, SC35, ASF/SF2, and SRp40 on the Utilization of the A1 to A5 Splicing Sites of HIV-1 RNA
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