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Insights into the selective activation of alternatively used splice acceptors by the human immunodeficiency virus type-1 bidirectional splicing enhancer

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ABSTRACT

The guanosine-adenosine-rich exonic splicing enhancer (GAR ESE) identified in exon 5 of the human immunodeficiency virus type-1 (HIV-1) pre-mRNA activates either an enhancer-dependent 5′ splice site (ss) or 3′ ss in 1-intron reporter constructs in the presence of the SR proteins SF2/ASF2 and SRp40. Characterizing the mode of action of the GAR ESE inside the internal HIV-1 exon 5 we found that this enhancer fulfils a dual splicing regulatory function (i) by synergistically mediating exon recognition through its individual SR protein-binding sites and (ii) by conferring 3′ ss selectivity within the 3′ ss cluster preceding exon 5. Both functions depend upon the GAR ESE, U1 snRNP binding at the downstream 5′ ss D4 and the E42 sequence located between these elements. Therefore, a network of cross-exon interactions appears to regulate splicing of the alternative exons 4a and 5. As the GAR ESE-mediated activation of the upstream 3′ ss cluster also is essential for the processing of intron-containing *vpu/env*-mRNAs during intermediate viral gene expression, the GAR enhancer substantially contributes to the regulation of viral replication.

INTRODUCTION

After integration into the host genome HIV-1 gene expression is driven by a single promoter leading to a single primary transcript. Translational start codons can efficiently inhibit downstream translation, although discontinuous ribosome scanning has been reported for *env* translation (1,2). To allow translation of all eight viral ORFs encoded by the primary transcript, alternative splicing is required to remove inhibitory translational start codons. Indeed, up to now more than 40 viral mRNA isoforms have been described many of which only differ in their 5′ untranslated regions (UTR) (3–8).

HIV-1 alternative splicing shows tight temporal regulation during viral replication (9). Progressive changes in the splicing pattern of the primary transcript shift the mRNA pool towards isoforms with increasing intron content. While in the early phase of viral gene expression only 1.8-kb mRNAs are generated encoding the regulatory proteins Tat and Rev and the accessory protein Nef, additional expression of the 4-kb Env glycoprotein precursor mRNA is caused in the intermediate phase by the interaction of Rev with an RNA secondary structure (Rev Responsive Element, RRE). Finally expression of the Gag and Gag/Pol precursors encoded by unspliced transcripts arises in the late phase of viral gene expression (9). It has been shown that disturbing the temporal regulation of viral precursor mRNA splicing can influence infectivity and pathogenesis of HIV-1 (10–12).

In the early as well as in the intermediate phase of viral RNA processing more than 90% of the mRNAs are spliced at one of the 3′ ss A4c, A4a, A4b or A5, which accumulate in a 3′ ss cluster upstream of exon 5. The progress from early to intermediate RNA splicing pattern is hallmarked by the appearance of intron-containing mRNA due to interference with splicing at 5′ ss D4. Therefore, differential activation of the 3′ ss cluster upstream of exon 5 and of the downstream flanking 5′ ss D4 is crucial for the regulated expression of the viral proteins Rev, Nef, Vpu and Env. The proteins Rev and Nef play important regulatory roles in HIV-1 gene expression. Rev is essential for viral replication, because it fulfils the adaptor function for feeding intron-containing HIV-1 mRNA into the CRM1-mediated export pathway. Nevertheless, for efficient viral replication the accessory

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protein Nef and the structural protein Env also have to be expressed, which are encoded by mRNAs spliced at the most distal 3′ ss of the cluster A5. It has been shown that the viral protein Nef modulates intracellular signalling by binding to a number of proteins involved in signal transduction pathways including Hck, Lyn and Raf1 (13,14).

Although it was originally postulated that alternative splicing of the HIV-1 pre-mRNA depends on combination of strong 5′ ss with consistently weak 3′ ss (15), we found that 3′ ss with a strong intrinsic strength are also present within the HIV-1 pre-mRNA (16). From these studies it can be concluded that the regulation of alternative 3′ ss usage in HIV-1 relies on the combination of intrinsic weak 3′ ss with enhancer elements and of intrinsic strong 3′ ss with silencer elements allowing trans-mediated alterations in splice site activation.

A number of *cis*-regulatory elements neighbouring almost all splice sites of the primary transcript substantially regulate alternative splicing of HIV-1 mRNAs (11,16–24). Most of the *cis*-regulatory elements are located in an exonic position with respect to the multiply spliced class of mRNAs and were shown to regulate 3′ ss usage, although one of these regulatory elements has also been described within an intronic position (Figure 1) (19).

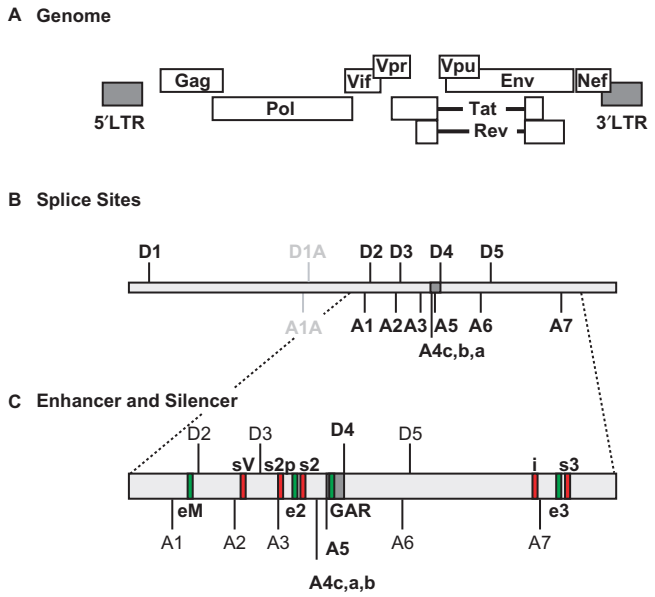

Figure 1. Distribution of splicing regulatory elements in the HIV-1 pre-mRNA. (**A**) Schematic depiction of the HIV-1 genome. Open reading frames are indicated by open boxes. Flanking long terminal repeats (LTRs) are shown as grey boxes. (**B**) Distribution of 5′ ss (D1–D5) and 3′ ss (A1–A7) within the genomic HIV-1 RNA. The position of exon 5 is indicated (dark grey box). A1A and D1A have been published to be preferentially involved in pre-mRNA stabilization (8). The nomenclature of HIV-1 splice sites refers to (50). (**C**) The enlargement of the 3′ half of the pre-mRNA depicts the location of the known enhancer (green) and silencer elements (red) regulating viral pre-mRNA splicing [eM: ESEM1/M2, sV: ESSV, s2p: ESS2p, e2: ESE2, s2: ESS2, I: ISS, e3: ESE3, s3: ESS3; adapted from (24)]. Exon 5 is flanked by 5′ ss D4 and 3′ ss A5 and contains the GAR ESE in the 5′ part. The 3′ ss A4c, A4a and A4b cluster directly upstream of exon 5.

The cellular effectors described so far to mediate the HIV-1 *cis*-regulating enhancer and silencer functions belong to the serine-arginine rich (SR) protein family or to the heterogeneous nuclear ribonucleoprotein particle (hnRNP) family. According to the current model, enhancer-bound SR proteins support splicing by stabilizing spliceosomal components at splice sites through protein–protein interactions mediated by their arginine-serine rich (RS) domains. HnRNP proteins bound to silencers inhibit splicing by interfering with spliceosome assembly in part by counteracting SR protein-mediated stabilizing interactions. In addition to the formation of protein–protein interactions, recent reports have shown that the RS domains of enhancer-bound SR proteins are also able to stabilize the binding of U1 snRNA and U2 snRNA at the 5′ ss and the branch point sequence (BPS), respectively (25,26). Subsequent work has demonstrated that the necessity for the contact between the RS domain and the splicing signals depends on the individual splice site strength (27).

Previously we identified an exonic splicing enhancer termed GAR ESE (guanosine-adenosine-rich exonic splicing enhancer) in the exon 5 of HIV-1 (28), which is targeted by the SR proteins SF2/ASF and SRp40 (22). In an *in vitro* splicing assay the GAR ESE showed comparable activation of an enhancer-dependent heterologous 3′ ss in the presence of either purified SF2/ASF or SRp40. Here we further characterized the role of the GAR enhancer for HIV-1 gene expression starting with the investigation of the differential impact of SF2/ASF- and SRp40-binding sites on exon recognition in a subgenomic context preserving the exon 5 sequence including the flanking viral splice sites. We show that every two of the three individual binding sites for SF2/ASF and SRp40 synergistically mediate the enhancer function. While the exon recognition function of the GAR enhancer showed no clear preference for activation by either SF2/ASF or SRp40, we identified a novel function of the proximal SF2/ASF-binding site of the GAR ESE in specifically activating A5 of the alternative 3′ ss cluster, which is assisted by an additional upstream SF2/ASF-binding site. Although the 3′ ss selection was solely conferred by the GAR enhancer, binding of U1 snRNP at the downstream 5′ ss enforces selective 3′ ss activation. In addition to the GAR ESE, the presence of an additional enhancer activity in the 3′ half of exon 5 is absolutely required for recognition of the internal exons flanked by D4. The role of the GAR enhancer is not restricted to splicing events in the early phase of viral RNA processing, but remains essential for activation of the 3′ ss cluster during the generation of Rev-dependent intron-containing HIV-1 mRNA.

MATERIALS AND METHODS

Oligonucleotides

Unlabelled oligonucleotides were synthesized as previously described (29). Cy5-labelled oligos #1544 and #1225 for RT–PCR were purchased from Metabion GmbH (Martinsried, Germany).

Oligo sequences for cloning were:
#640: 5′-CAATACTACTTCTTGTGGGTTGG
#1660(SRp40⁻anti-sense): 5′-ATCGAGCTCTTCGTC GCTAAATCCGCTTCT
#1737(GTV 15Asense): 5′-ATCTCATGAAATGCAA CCTATAAT
#1831: 5′-AGAGCTAGCTATCTGTTTTAAAG
#1903(SF2(1)⁻ SF2(2)⁻ anti-sense): 5′-ATCGAGCTCTT AGCGCTGTCTCCGCTAATTCCTGCCGTAGGAG
#1987(SF2(1)⁻ anti-sense): 5′-ATCGAGCTCTTCGT CGCTGTCTCCGCTAATTCCTGCCGTAGGAG
#1988(SF2(2)⁻ anti-sense): 5′–ATCGAGCTCTTAAG CGCTGTCTCCGCTTC
#2000(SRp40⁻ SF2(2)⁻ anti-sense): 5′-ATCGAGCTC TTAAGCGCTAAATCCGCTTCTTCCTGC
#2067(SF2(1)⁻ SRp40⁻ anti-sense): 5′-ATCGAGCTC TTCGTCGCTAAATCCGCTAATTCCTGCCGTAGG AG
#2499(SF2(3)⁻ anti-sense): 5′-ATCGAGCTCTTCGT CGCTGTCTCCGCTTCTGCCTGCCGTAGGAGAT
#2501(SF2(3)⁻ GARmut anti-sense): 5′-ATCGAGCTC TTAAGCGAAGTCGCCGCTAATGCCTGCCGTAGG AGAT
#2555(cs-2^{14} sense): 5′-ATCGAGCTCATCAGAACA GTCAGACTCATCAAGCTTCTCTATCAAAGAGGT AACATCTACATGTAATGCAAC
#2556(ΔE42 sense): 5′-ATCGAGCTCGCAGTAAGT AGTACA
#2559(HIV#18 sense): 5′-ATCGAGCTCGTGGTATA TAAAATTATTCATAATGATAGTAGGAGGCGCAG TAAGTAGTACA
#2598(ESE⁻ anti-sense): 5′-ATCGAGCTCTTAAGCG CTAAATCCGCTAATTCCTGCCGTAGGAG
Oligo sequences for RT-PCR were:
#1224(3′ RT–PCR primer hGH): 5′-TCTTCCAGCCT CCCATCAGCGTTTGG
#1225(5′ RT–PCR primer hGH): 5′-CAACAGAAATC CAACCTAGAGCTGCT
#1542(3′ RT–PCR primer): 5′-CACCTTCTTCTTCTA TTCCTT
#1543(3′ RT–PCR primer): 5′-CCCCCATCTCCACAA
#1544(5′ RT–PCR primer): 5′-CTTGAAAGCGAAAG TAAAGC

Plasmids

Generation of the HIV-1 NL4/3 (GenBank Accession No. M19921) derived subgenomic reporter construct pSV-1-env and mutant derivatives pSV-1-env 3U and pSV-1-env SF2⁻ SRp40⁻ has been described (22).

For generation of mutant constructs pSV-1-env ESE⁻, pSV-1-env SRp40⁻ SF2(2)⁻, pSV-1-env SF2(1)⁻ SRp40⁻, pSV-1-env SF2(1)⁻ SF2(2)⁻, pSV-1-env SRp40⁻, pSV-1-env SF2(2)⁻, pSV-1-env SF2(1)⁻, pSV-1-env SF2(3)⁻ and pSV-1-env SF2(3)⁻ GARmut the EcoRI–SacI fragment of pSV-1-env was substituted for PCR products amplified with the 5′ primer #1544 and 3′ primers #2598, #2000, #2067, #1903, #1660, #1987, #1988, #2499 or #2501, respectively, using pSV-1-env as template.

The construct pSV-1-env cs+1[12] was generated by substituting the SacI–NheI fragment of pSV-1-env containing 5′ ss D4 for the respective fragment of SV E/X tat⁻ rev⁻ cs+1[12].

Substitution of D4 for 5′ ss cs-2^{14} in construct pSV-1-env cs-2^{14}, of the E42 sequence for the HIV#18 control in pSV-1-env HIV#18 and deletion of the E42 sequence in the mutant construct pSV-1-env ΔE42 was performed by substitution of the SacI–NdeI fragment of pSV-1-env for the PCR products amplified with 5′ primers #2555, #2559, or #2556, respectively, and 3′ primer #1831 using pSV-1-env as substrate.

Mutant construct pSV-1-env GTV was generated by substituting the SacI–NheI fragment of pSV-1-env with the SacI–NheI fragment from SV E/X tat⁻rev⁻ GT V (28). Mutation of the cryptic 5′ ss 13-nt downstream of D4 from GT to GA was achieved by substituting the BspHI–NdeI fragment with the PCR product amplified with primer pair #1737/#640 using pSV-1-env as template generating pSV-1-env GTV 15A.

The Rev expression plasmid SVcrev (30) and the U1 snRNA expression plasmids pUCBU1 9T10G11C (31), and pUCBU1 6A (28) have already been described.

All constructs were confirmed by DNA sequencing of substituted subgenomic or PCR-amplified fragments. Sequences of all constructs are available on request.

Cell culture and transfection

In DMEM supplemented with 10% FCS 2×10^5 HeLa-T4⁺ cells (32) were plated per 6-well 24 h before transfection. Cells were transfected with 1 μg of pSV-1-env or mutant derivatives and 1 μg of pXGH5 encoding the human growth hormone (33) to monitor transfection efficiency using FuGENE™6 according to the manufacturer's protocol (Roche Molecular Biochemicals). For cotransfection experiments, 1 μg of the respective plasmids were used and DNA amounts were equalized with pSP73. RNA was isolated 30 h after transfection.

RNA preparation and RT–PCR

Total RNA isolation using a modified guanidinium isothiocyanate protocol and RT–PCR were performed as described before (22).

For analysis under denaturating conditions RT-PCR products were fluorochrome-labelled by using Cy5-labelled 5′ primer #1544 for alternatively spliced reporter mRNA or #1225 for hGH-mRNA. Separation and detection were performed by Automated Laser Fluorescence (ALF) using a DNA Sequencer (A.L.F. DNA Sequencer; LKB Pharmacia) operated by ALFwin™ ver. 1.0 software (Pharmacia). Fluorescence curve data were further processed by P2 software. Exon inclusion was quantified as the percentage of doubly spliced mRNA isoforms relative to total mRNA in each sample. For quantification of 3′ ss selectivity the ratio of 1.5.7- and 1.4a.7-mRNA in each sample was calculated. The activation of 5′ ss D4 was quantified as the sum of the percentages of doubly and singly spliced RNA using D4 (ss) relative to total mRNA in each sample.

Viral infection experiments

Cell cultures were maintained in RPMI 1640 medium containing 10% FCS (Pansystems GmbH) and antibiotics (penicillin and streptomycin). Viral stocks were prepared by transfecting 293T cells with the provirus expression vector pNL4-3 followed by ELISA (Innotest HIV p24 Antigen mAB; Innogenetics N.V.) of culture supernatant for p24 content. For HIV-1 infection 5×10^6 PM1 cells were re-suspended in 500 µl culture medium and incubated at 37°C for 3 h with 100 ng of the viral stock. After infection, cells were washed twice with PBS without Ca^{2+} and Mg^{2+} and further cultured in 5 ml medium for another 5 days. Subsequently, total RNA was prepared using Trizol reagent (Invitrogen) according to the protocol provided by the manufacturer.

RESULTS

Individual SR protein-binding sites of HIV-1 GAR ESE synergistically increase recognition of alternatively spliced exons flanked by D4

Previously we identified a purine-rich exonic splicing enhancer (GAR ESE) located in the 5′ part of HIV-1 exon 5 (28), which according to ESEfinder (34) consists of two predicted SF2/ASF-binding sites, an SRp40 and an SRp55-binding site (Figure 1). Both, purified SF2/ASF2 and SRp40 were shown to comparably restore *in vitro* splicing of a GAR ESE-dependent heterologous *dsx*-substrate in splicing-deficient HeLa S100 extracts, while the enhancer did not respond to SRp55 (22). However, mutation of the predicted SRp40-binding site, *in vitro* as well as *in vivo*, only slightly impaired splicing, while mutation of both SF2/ASF-binding sites strongly reduced activation of 5′ ss D4 and the heterologous 3′ ss, respectively (22). Therefore, we set out to determine in more detail the contribution of the SF2/ASF- and SRp40-binding sites to the enhancer function. To investigate whether a discrete modulation of exon 5 recognition could be achieved by different binding sites for SF2/ASF and SRp40 of the GAR ESE, individual and combinatorial binding site mutations were introduced into our previously described 2-intron HIV-1 minigene (pSV-1-env) (Figure 2A and B). None of the binding site mutations introduced into the reporter construct in this study generated additional SR protein-binding site predictions by the ESEfinder. RT–PCR analysis of transiently transfected HeLa-$T4^+$ cells revealed that RNA from the reporter construct is processed into several mRNA isoforms thereby generating doubly spliced mRNA containing an internal exon due to the activation of the alternative 3′ ss A5 (1.5.7) or A4a (1.4a.7), singly spliced mRNA with the distal intron removed (ss), and to a lesser extent unspliced mRNA (us) (Figure 2C, lane1). Introducing mutations for all three predicted SR protein-binding sites completely abolished exon recognition (Figure 2C, lane 2) underlining the essential role of the GAR ESE for exon recognition in accordance with a different triple binding site mutation generated earlier (Figure 2C, lane E). The strongest effect of a single binding site mutation on exon inclusion was exerted by loss of the proximal SF2/ASF-binding site [SF2(1)] resulting in a decrease of exon inclusion from 88 to 49% and concomitant exon skipping (Figure 2C and D, cf. lanes 1 and 8). Mutating either the distal SF2/ASF [SF2(2)] or the SRp40-binding site alone only led to a slight decrease in exon recognition of about 10% (Figure 2C and D, lanes 6 and 7). Combinatorial mutations of two of the SR protein-binding sites, however, dramatically reduced exon recognition to inclusion rates below 14% independent of which site had been mutated. Together, these results show that each SR protein-binding site of the GAR ESE analysed here contributes to exon recognition. Furthermore, we conclude that any two SR protein-binding sites synergistically mediate exon recognition, whereas the presence of the third only leads to an additive effect on exon recognition. These data also support our previous observation that the GAR enhancer is essential for efficient internal exon recognition, which we showed here to depend synergistically on all three binding sites for SR proteins SF2/ASF and SRp40, with the proximal SF2/ASF-binding site having the highest impact on the extent of exon recognition.

The proximal SF2/ASF-binding site specifically contributes to A5 usage

Analysing the ratio of exon 5 to exon 4a inclusion, we detected a dominant influence of the GAR ESE's proximal SF2/ASF-binding site [SF2(1)] on the efficiency of exon 5 recognition. In the parent reporter construct, exon 5 was three times more frequently included than exon 4a, which is comparable to the splicing pattern in HIV-1 NL4/3-infected cells (data not shown). Irrespective of the overall exon inclusion efficiency, mutation of SF2(1) led to a decrease in exon 5 and a concomitant increase in exon 4a recognition (Figure 2C and E, cf. lanes 1 and 8). In contrast, mutation of the distal SF2/ASF- or SRp40-binding site not only maintained this ratio but even slightly increased A5 over A4a usage suggesting that the distal SR protein-binding sites might preferentially activate A4a (Figure 2C and E, cf. lanes 1, 6 and 7). However, combining the mutations of the proximal SF2/ASF and one distal SR protein-binding site pronounced the preferential activation of A4a rather than causing an intermediate ratio of 3′ ss activation (Figure 2C and E, cf. lanes 4, 5 and 8). Besides its general enhancing role in internal exon recognition this indicates that only the proximal SF2/ASF-binding site confers selective activation of 3′ ss A5. From these results we conclude that the HIV-1 exon 5 GAR ESE fulfils a dual role in the regulation of alternative splicing by (i) ensuring recognition of the alternative exons 5 and 4a and (ii) determining the ratio of internal exon recognition in favour of exon 5.

A predicted third SF2/ASF-binding site covering the intron/exon 5 border ensures preferential selection of A5

RNA affinity chromatography assays indicated that inactivating the GAR ESE did not completely abolish binding of SF2/ASF to the *in vitro* transcript (data not shown). Analysis of the sequence upstream of the GAR

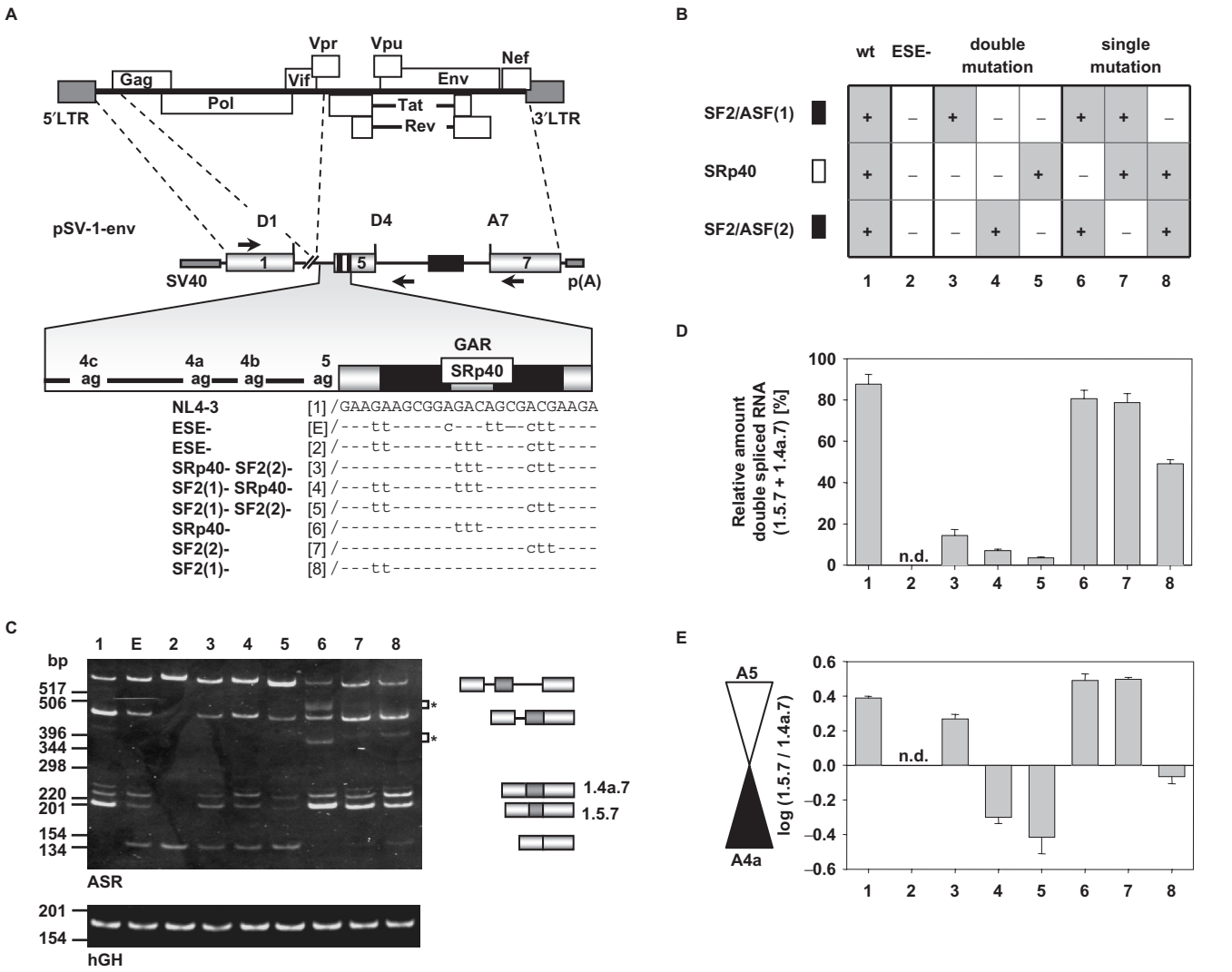

Figure 2. The GAR ESE in exon 5 ensures internal exon recognition and supports A5 usage. (**A**) Schematic representation of the HIV-1 2-intron RNA reporter construct: *gag*, *pol*, *vif* and *vpr*-coding regions were deleted from the genome of the isolate NL4-3 (upper panel) generating a 3-exon-2-intron minigene (middle panel). The GAR enhancer in the 5′ part of exon 5 consists of two SF2/ASF (black box) and an SRp40-binding site (white box). Positions of RT–PCR primers are indicated by arrows. An enlargement of the 5′ part of the internal exon 5 with the preceding intronic region illustrates the organization of the alternative 3′ ss cluster consisting of A4c, A4a, A4b and A5 and the mutations introduced in the GAR enhancer (lower panel). LTR: long terminal repeat, SV40: SV40 early promoter, p(A): SV40 polyadenylation signal. (**B**) Scheme of single and combinatorial SR protein-binding site mutations in the GAR ESE used in this assay. (**C**) RT–PCR analysis of the reporter transcripts and derivatives carrying mutations in the GAR ESE. HeLa-T4$^+$ cells were transiently transfected with the RNA reporter construct pSV-1-env or mutant derivatives [numbering according to (B)] and pXGH5. Total RNA was subjected to RT–PCR with the primers indicated in (A) (alternatively spliced RNA, ASR) or with a primer pair specific for hGH-mRNA as control for transfection efficiency (hGH). PCR products were separated on a non-denaturating polyacrylamide gel and stained with ethidium bromide. Asterisks denote heteroduplex PCR products as revealed by sequencing emerging due to non-denaturating conditions. (**D**) Quantification of internal exon recognition after SR protein-binding site mutations. PCR products were labelled using a Cy5-modified 5′ primer, separated on denaturating gels and detected by Automated Laser Fluorescence (ALF). Exon recognition was quantified as percentage of doubly spliced mRNA using either A4a or A5 relative to total mRNA (sum of spliced and unspliced mRNA isoforms) in each sample. Error bars indicate standard deviation from three independent experiments. (**E**) Quantification of 3′ ss usage in doubly spliced mRNA. Raw data resulting from the transfection experiments shown in (D) were analysed regarding 3′ ss selectivity, which is depicted as ratio of doubly spliced 1.5.7- to 1.4a.7-mRNA.

ESE using the ESEfinder algorithm (34) predicted a third SF2/ASF-binding site [SF2(3)] overlapping A5 (Figure 3A). To investigate whether this putative site additionally contributes to 3′ ss selection we mutated SF2(3) at position + 2 (A to C). Within the context of the wild-type GAR ESE, this mutation abrogated the preferential selection of A5 as evidenced by an equal activation of A5 and A4a (Figure 3B and C, cf. lanes 1 and 2). To clarify if this newly identified SF2/ASF-binding site fulfils a redundant function in the enhancer complex we analysed its influence on the splicing pattern in presence of a GAR ESE mutation (ESE-) leading to an increased ratio of A4a to A5 usage. To this end, we used the triple GAR ESE mutation that still facilitated residual exon

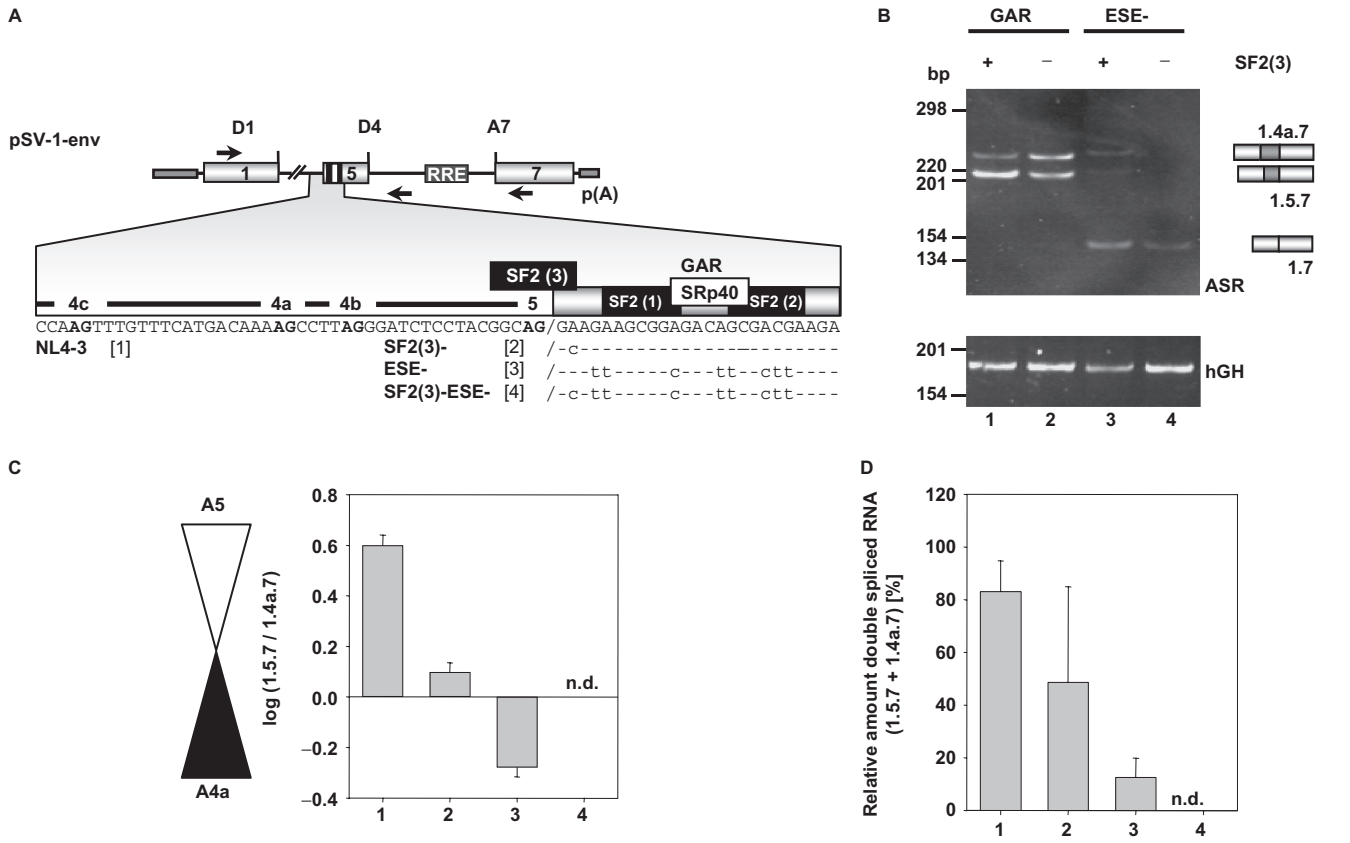

Figure 3. An additional SF2/ASF site overlapping A5 regulates 3′ ss selection. (**A**) An additional SF2/ASF-binding site is predicted upstream of the GAR ESE by the ESEfinder search algorithm (34). (**B**) RT–PCR analysis of RNA from HeLa-T4$^+$ cells transiently transfected with the 2-intron minigene construct pSV-1-env or mutant derivatives and pXGH5. RT–PCR products for alternatively spliced reporter RNA (ASR) and hGH as control for transfection efficiency were separated on non-denaturating polyacrylamide gels and stained with ethidium bromide. (**C**) Quantification of 3′ ss usage in doubly spliced mRNA. 3′ ss selectivity is shown as ratio of doubly spliced 1.5.7- to 1.4a.7-mRNA. Error bars indicate standard deviation from three independent experiments. (**D**) Quantification of exon inclusion. Raw data resulting from the transfection experiments shown in (C) were analysed regarding the amount of doubly spliced mRNA relative to total reporter mRNA isoforms in each sample. Error bars represent standard deviation.

recognition (Figure 2, lane E and Figure 3, lane 3), allowing the detection of increasing as well as decreasing amounts of exon inclusion. Mutating SF2(3) in this construct completely abolished exon recognition (Figure 3B and D, lane 4), suggesting the non-redundant function of the proximal SF2/ASF-binding site of the GAR ESE and the additional upstream SF2/ASF-binding site. These results indicate that SF2(3) is also part of the GAR ESE complex regulating splicing of the 3′ ss cluster preceding exon 5.

GAR ESE-mediated 3′ ss selectivity is augmented by U1 snRNP binding at D4

We previously showed that besides activating splicing at the upstream 3′ ss the GAR ESE also recruits U1 snRNP to the downstream 5′ ss D4 (22). Since U1snRNP binding at a downstream 5′ ss has been shown to assist exon recognition in general (35), we studied the role of cross-exon interactions between U1 snRNP bound at D4 and the 3′ ss cluster in the preferential activation of A5. To this end, we analysed, if increasing U1 snRNP-binding stability shifted the mutationally induced preferential activation of A4a back towards the dominant A5 usage observed in the wild-type splicing reporter construct. Preferential activation of A4a was again achieved by introducing the triple SR-protein binding site mutation into the GAR ESE, which still facilitated residual exon recognition (Figure 4A, lane 2). Cotransfection of an expression plasmid coding for an U1 snRNA with perfect complementarity to D4 solely increased recognition of internal exon 4a but failed to restore preferential 3′ ss A5 usage (Figure 4A–C, cf. lanes 2 and 4). This indicates that increased RNA duplex formation between U1 snRNA and D4 only advanced overall exon recognition, while preferential A5 selection was ensured by the GAR ESE. As control, we also analysed the effect of increasing U1 snRNP-binding stability on the ratio of A5 to A4a usage in the presence of the wild-type GAR ESE. Because exon recognition already predominates in the minigene construct containing the GAR ESE and D4, we first inactivated D4 by introducing a point mutation at position +3 from A to U. This mutation has been shown to lower the complementarity of D4 to U1 snRNA, thereby abrogating U1 snRNP binding, and can be

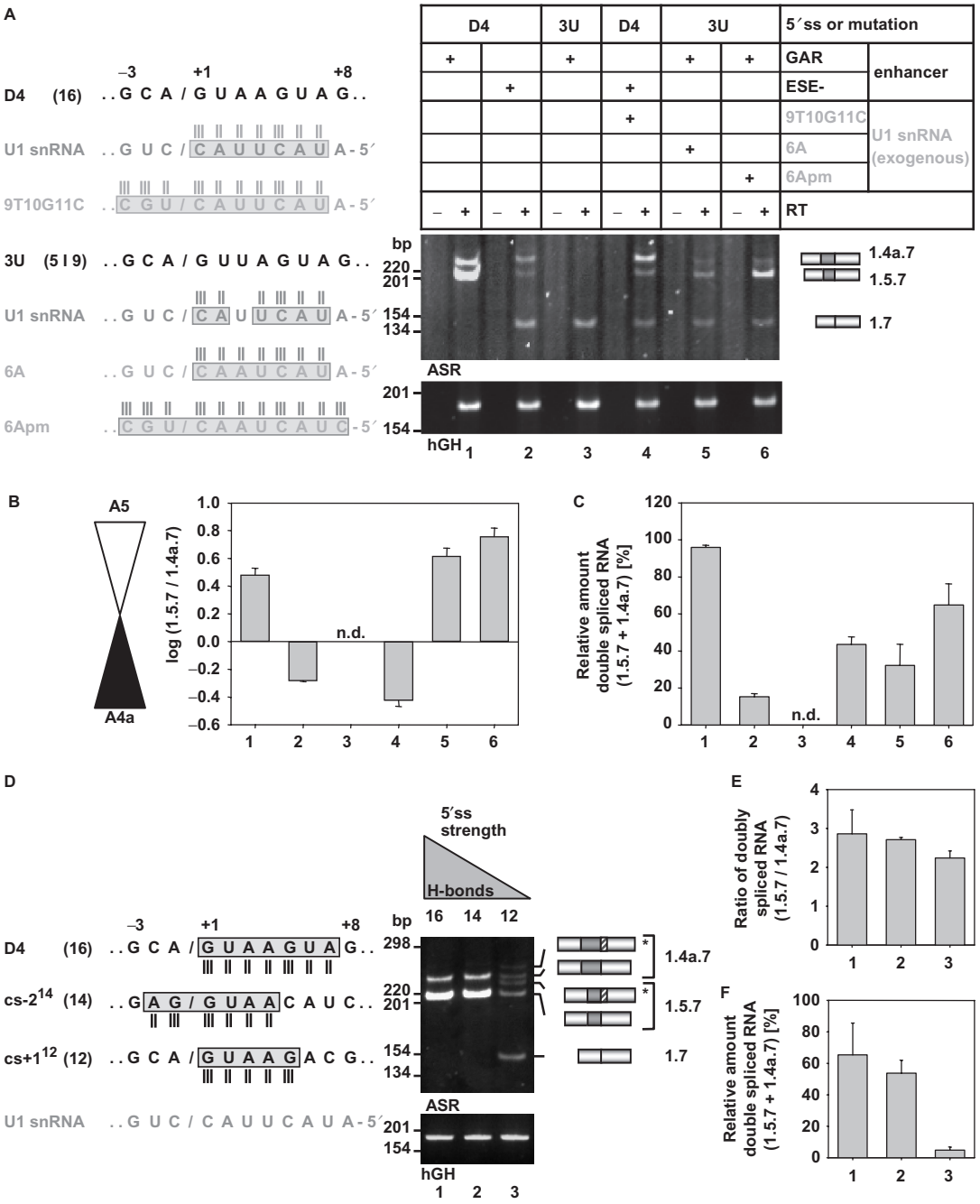

Figure 4. GAR ESE-mediated 3′ ss selectivity is augmented by increasing downstream U1 snRNP-binding stability. (**A**) Increasing U1 snRNP-binding stability at D4 enhances 3′ ss selectivity of the GAR ESE. RT–PCR analysis of RNA isolated from HeLa-T4$^+$ cells transiently transfected with the 2-intron reporter constructs pSV-1-env (lane 1, D4 GAR), the enhancer mutant pSV-1-env SF2(1)$^-$ SF2(2)$^-$ SRp40$^-$ (lanes 2 and 4, D4 ESE-), or a construct carrying a mutation in the 5′ ss D4, pSV-1-env 3U (lanes 3, 5 and 6, 3U GAR). Endogenous U1 snRNA forms a consecutive base pairing with 16 H-bonds to the 5′ ss in constructs containing D4 (left panel). Cotransfection of the U1 snRNA expression plasmid pUCBU1 6A harbouring a compensatory mutation (lane 5) reconstitutes the base pairing of a mutated 5′ ss compared to D4, while U1 snRNA expression plasmids pUCBU1 9T10G11C (lane 4) or pUCBU1 6Apm (lane 6) even extend the base pairing to neighbouring nucleotides. The exon structure of the alternatively spliced mRNAs (ASR) is indicated. RT–PCR analysis of hGH-mRNA was performed to monitor transfection efficiency. (**B**) Quantification of 3′ ss selectivity in doubly spliced mRNA. 3′ ss selectivity was calculated as ratio of 1.5.7- to 1.4a.7-mRNA. Error bars represent standard deviation from three independent experiments. (**C**) Quantification of exon inclusion. Raw data from the experiments in (B) were analysed regarding the percentage of doubly spliced mRNA relative to total mRNA isoforms per sample. Error bars indicate standard deviation. (**D**) Decreasing U1 snRNP-binding stability reduces 3′ ss selectivity. HeLa-T4$^+$ cells were transfected with the 2-intron minigene reporter carrying the wild-type 5′ ss D4 (lane 1, pSV-1-env) or mutant 5′ ss with reduced complementarity to the endogenous U1 snRNA (lane 2, pSV-1-env cs-2^{14}; lane 3, pSV-1-env cs+1^{12}) as depicted on the left side. The exon structure of the mRNA is indicated at the right. The asterisk marks the inclusion of 13-nt intronic sequence downstream of exon 5 due to activation of a cryptic 5′ ss downstream of D4. RT–PCR analysis of hGH-mRNA controlled equal transfection efficiency. (**E**) Quantification of 3′ ss selectivity as performed in (B). Error bars represent standard deviation from three independent experiments. (**F**) Quantification of exon inclusion. Raw data from the experiments in (E) were analysed as performed in (C). Error bars indicate standard deviation.

rescued upon cotransfection of a corresponding U1 snRNA expression plasmid carrying a compensatory mutation (8,28). Inactivating D4 eliminated internal exon recognition in the 2-intron minigene construct (Figure 4A and C, lane 3). As expected, cotransfecting plasmids expressing compensatory (high or full 5′ ss complementarity) U1 snRNAs rescued internal exon recognition (Figure 4A and C, lanes 5 and 6). However, as seen before with the mutated GAR ESE, increasing U1 snRNP binding solely augmented activation of the 3′ ss selected by the SR protein-binding sites of the GAR ESE (Figure 4B, cf. lane 1 with 5 and 6). This confirms that 3′ ss selection is exclusively determined by the GAR enhancer, while U1 snRNP binding at the downstream 5′ ss supports the overall level of 3′ ss selectivity by cross-exon interactions.

Although increased U1 snRNP binding did not account for 3′ ss selectivity, it generally supported GAR ESE-mediated 3′ ss activation (Figure 4A and C, cf. lane 2 with 4 and 5 with 6). Substituting D4 with a weaker 5′ ss of intermediate complementarity to endogenous U1 snRNA showed no effect on 3′ ss selection (Figure 4D, lane 2). Surprisingly, introducing a low complementarity 5′ ss activated the previously identified cryptic 5′ ss 13-nt downstream of D4 (36), leading to the inclusion of an additional intronic sequence into exons 4a and 5 (Figure 4D, lane 3). Introduction of this weak 5′ ss resulted in a moderate decrease in 3′ ss selectivity (Figure 4E, lane 3). This demonstrates that the degree of the GAR enhancer-mediated 3′ ss selectivity depends on the stability of U1 snRNP binding at the downstream 5′ ss.

Taken together, these results show that specific 3′ ss selection is conferred by the GAR ESE, rather than by U1 snRNP-binding stability. Nevertheless, U1 snRNP binding at 5′ ss D4 augments activation of the 3′ ss cluster and thereby enhances the 3′ ss selecting mechanism presumably through cross-exon interactions.

The 3′ part of exon 5 contains an additional enhancer activity

The finding that U1 snRNP bound to the downstream 5′ ss of exon 5 takes part in the GAR ESE-mediated 3′ ss activation raised the question of whether the exonic sequence located between the enhancer and D4, termed E42, might be involved in the formation of the cross-exon interactions. To investigate the impact of the E42 sequence on exon recognition we analysed the splicing pattern of the 2-intron minigene construct after either substituting the E42 fragment with an HIV-1 control sequence of identical length, which did not support U1 snRNP binding at D4 (HIV#18) (22), or deleting E42 (ΔE42). RT–PCR analysis of transiently transfected HeLa-T4$^+$ cells revealed that substituting the E42 sequence completely abolished internal exon recognition (1.4a.7; 1.5.7) (Figure 5B and D, cf. lanes 1 and 2) and, unexpectedly, also activation of D4 resulting in the loss of singly spliced RNA [Figure 5C, lane 2 (ss)]. This demonstrates that although E42 is insufficient to activate D4 by itself, the sequence is essential to connect the activating effect of the GAR enhancer to the 5′ ss. On the other hand, activation of D4 remained after deletion of E42, while exon recognition was nearly completely abolished resulting in the identification of mostly unspliced and singly spliced RNA using either splice site pair D1/A7 (1.7) or D4/A7 (ss) (Figure 5C and D, lane 3). This shows that the GAR ESE in proximity to the 5′ ss exclusively restored the enhancing function on D4 whereas internal exon recognition was only slightly recovered by use of A4c, the most upstream 3′ ss of the cluster. Therefore, the GAR ESE when positioned directly upstream of D4 enhances splicing at this 5′ ss but failed to efficiently activate any of the 3′ ss simultaneously. Although inhibitory elements in the substituted sequence or exon length constraints cannot be ruled out formally, we reasoned from this experiment that in addition to the GAR enhancer the E42 sequence is essential for establishing cross-exon interactions necessary for 5′ ss activation and efficient exon recognition in completely spliced mRNAs.

GAR ESE and binding of U1 snRNP at D4 are necessary for activation of the 3′ ss cluster in Rev-dependent mRNAs

While the early phase of HIV-1 gene expression is marked by complete intron removal from the RNA precursor, in the intermediate and the late phase splicing at 5′ ss D4 is restrained by the interaction of the viral regulatory protein Rev with the RRE. In order to examine the roles both of the U1 snRNP binding at D4 and of the GAR enhancer for activation of the 3′ ss cluster during the late phase, we used HeLa-T4$^+$ cells transiently cotransfected with the 2-intron splicing reporter construct and a Rev expression plasmid. The relative amount of unspliced versus spliced, but still intron-containing *vpu*/*env*-mRNA was detected by RT–PCR.

To examine the influence of U1 snRNP binding at D4 on activation of the 3′ ss cluster in the late phase, we compared the wild-type 5′ ss D4 with two mutations: 3U abolishing U1 snRNA binding and GTV 15A restitution of U1 snRNP binding but suppressing splicing at D4. The 3U-mutation led to complete loss of *vpu*/*env*-mRNA, while the amount of unspliced mRNA remained unchanged, demonstrating that none of the 3′ ss in the cluster upstream of exon 5 had been activated (Figure 6A, cf. 3U versus D4). To confirm that lack of detectability of the *vpu*/*env*-mRNA was caused by loss of U1 snRNP binding at D4, we reconstituted U1 snRNP binding by substituting D4 with a splicing inactive U1 snRNP-binding site (GTV) (28). The GTV sequence possesses perfect complementarity to the 5′ end of the U1 snRNA except for position +1, which was mutated from G to C to prevent splicing. After introduction of the GTV sequence, splicing from a cryptic 5′ ss 13-nt downstream of the inactivated 5′ ss (36) was observed (data not shown). To exclude splicing of the downstream intron, we additionally inactivated this cryptic 5′ ss (GTV 15A). Restitution of U1 snRNP binding rescued the expression of *vpu*/*env*-mRNA to the same extent as observed with D4 (Figure 6A, cf. GTV 15A versus D4). From this experiment we conclude that U1 snRNP binding at D4 is needed for 3′ ss activation for Rev-dependent mRNAs, although D4 is not spliced itself.

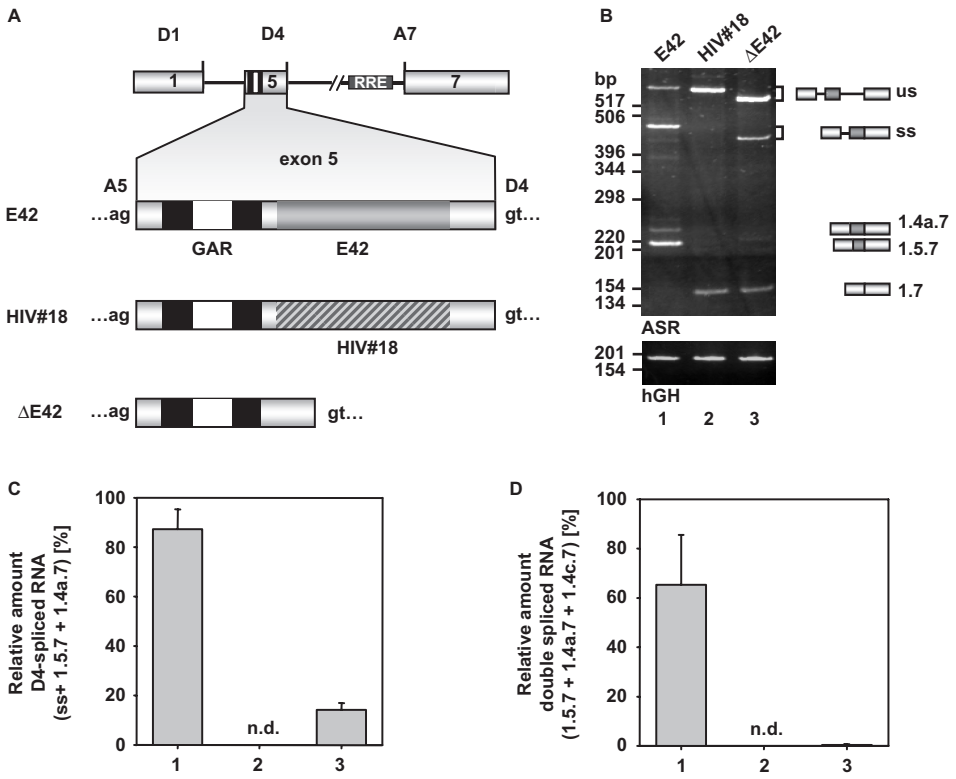

Figure 5. The 3′ part of HIV-1 exon 5 mediates an additional enhancer function. (**A**) The schematic depicts the elements in the central exon 5 of the 2-intron minigene reporter and mutant constructs. The E42 fragment in 3′ part of exon 5 has either been substituted for a control HIV-1 sequence (HIV#18) or deleted (ΔE42). (**B**) RT–PCR analysis of RNA from HeLa-T4$^+$ cells transiently transfected with the 2-intron reporter construct pSV-1-env (E42), the reporter construct with a substitution (HIV#18) or the deletion of the E42 sequence (ΔE42). RT–PCR was performed for alternatively spliced reporter RNA (ASR) and the control hGH-mRNA (hGH). us: unspliced, ss: singly spliced. (**C**) Quantification of D4 activation. The sum of doubly and singly spliced RNA using D4 (ss) was calculated relative to total mRNA per sample. Error bars indicate standard deviation from three independent experiments. (**D**) Quantification of exon inclusion. Raw data from the experiments in (C) were analysed regarding the percentage of doubly spliced mRNA using A5, A4a or A4c relative to total mRNA isoforms per sample. Error bars represent standard deviation.

To examine the influence of GAR on activation of the upstream 3′ ss cluster in the late phase, we compared the Rev-dependent splicing pattern of the subgenomic splicing reporter construct with differentially mutated GAR ESE versions. Simultaneous mutation of all three SR protein-binding sites of the GAR enhancer abolished expression of *vpu*/*env*-mRNA (data not shown). Since individual SR protein-binding sites of the GAR ESE were found to contribute differentially to its enhancer function in completely spliced RNA, we also examined this dependency in *vpu*/*env*-mRNA with constructs carrying mutations in either one of the SR protein-binding sites. Mutation of SF2(2) or the SRp40-binding site reduced the *vpu*/*env*-mRNA expression about 2-fold [Figure 6B, SF2(2)- and SRp40-], whereas mutating SF2(1) was already sufficient to eliminate its expression [Figure 6B, SF2(1)-]. In contrast, the amount of unspliced mRNA remained unchanged for mutation of the SRp40-binding site and even increased after mutation of any SF2/ASF-binding site. This result indicates that the individual SR protein-binding sites maintain their relative impact on 3′ ss activation in *vpu*/*env*-mRNA. Moreover, GAR enhancer dependency may be stronger than in completely spliced mRNA, since any single SR protein-binding site mutation clearly reduces *vpu*/*env*-mRNA expression.

In conclusion, both the GAR ESE and U1 snRNP binding at D4 are essential for activation of the 3′ ss cluster to generate *vpu*/*env*-mRNA in the intermediate and late phases of viral gene expression.

DISCUSSION

In HIV-1 exon 5a purine-rich multisite exonic splicing enhancer termed GAR ESE has been identified activating both an upstream 3′ ss as well as a downstream 5′ ss (22,28). In this work, we characterized the effect of the GAR ESE on simultaneous 3′ ss and 5′ ss activation, which was demonstrated to be additionally dependent on the interplay with downstream regulatory elements of exon 5. We found that the GAR ESE performed a dual splice regulatory function (i) by synergistically enhancing inclusion of the internal exons 4a and 5 through all identified SR protein-binding sites, and (ii) by specifically activating A5 of the 3′ ss cluster solely by the proximal SF2/ASF-binding sites. Analysing the interaction of the GAR ESE with U1 snRNP at the downstream 5′ ss D4 and the E42 sequence located in between, we showed it to be essential for activation and selectivity of the alternative 3′ ss cluster, which in turn is necessary for generation of *rev*-, *nef*- and *vpu*/*env*-mRNA in the early and late phases.

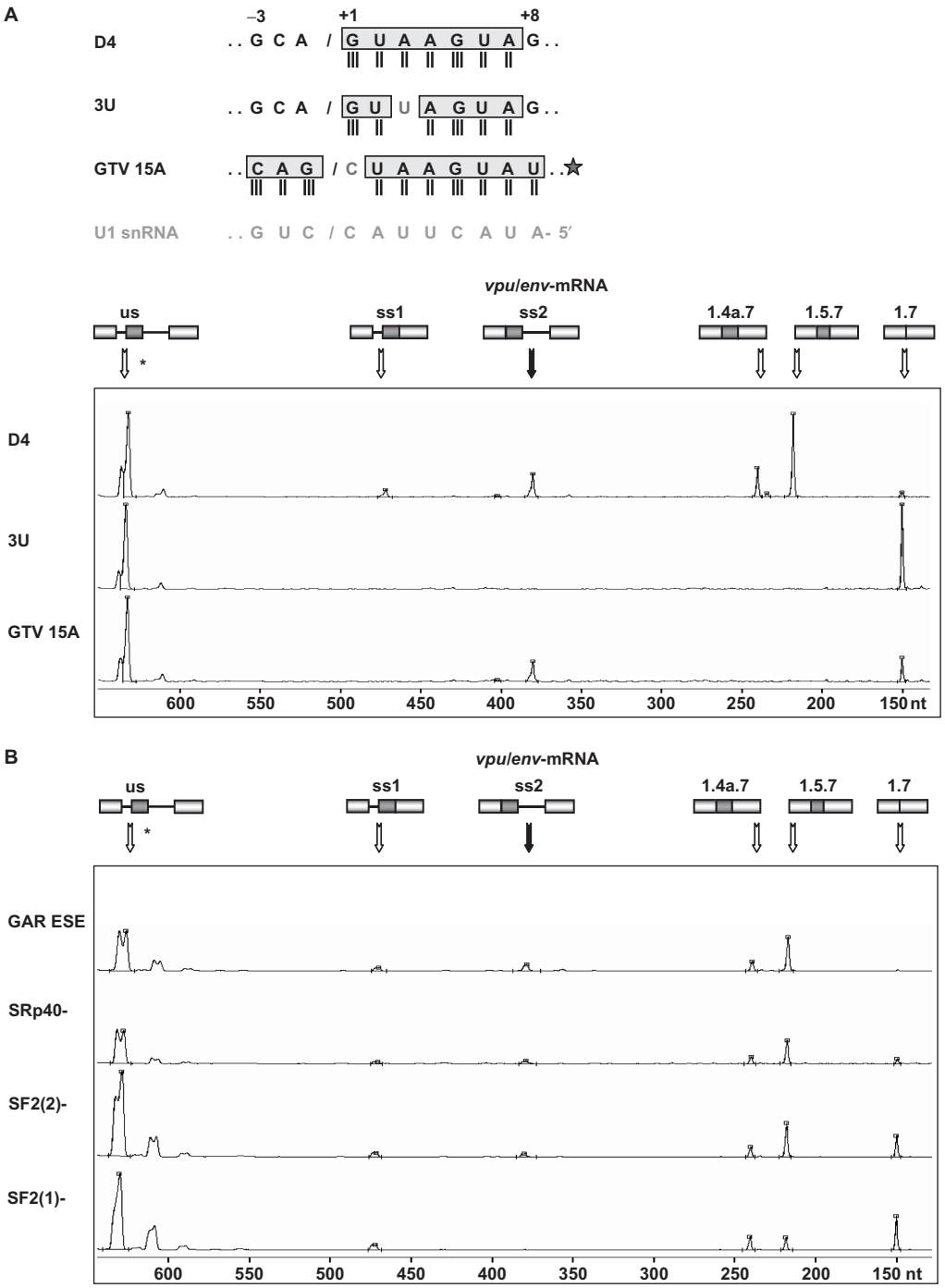

Figure 6. GAR ESE and U1 snRNP binding at D4 are necessary for activation of the 3′ ss cluster in *vpu*/*env*-mRNA. (**A**) RT–PCR analysis of RNA from HeLa-T4$^+$ cells transfected with the 2-inton reporter construct carrying wild-type D4 (D4), a mutant unable to stably bind endogenous U1 snRNA (3U) or a splicing-deficient U1 snRNP-binding site with the additional mutation of the cryptic 5′ ss downstream (GTV 15A). Cells were cotransfected with SVcrev, an expression plasmid for the viral regulatory protein Rev, allowing the export of intron-containing reporter mRNA into the cytoplasm, and with pXGH5. Base pairings between D4 and the splice site mutants 3U and GTV 15A are denoted at the upper panel. The star marks the additional mutation of the cryptic 5′ ss 13-nt downstream of D4 (36). RT–PCR was performed using a Cy5-labelled 5′primer, separated on denaturating gels and detected by (ALF). The RT-PCR products are shown as processed fluorescence curve data of the electrophoretic separation. The alternative spliced mRNA isoforms are depicted above the lanes. The asterisk marks an RNA signal, which was identified as unspecific signal by sequencing. (**B**) RT–PCR analysis of RNA from HeLa-T4$^+$ cells transiently transfected with the wild-type reporter construct or constructs carrying a single SR protein-binding site (cf. Figure 2A) as indicated in the respective lanes, the Rev-expression plasmid SVcrev and pXGH5. Cy5-labelled RT–PCR products were analysed by ALF.

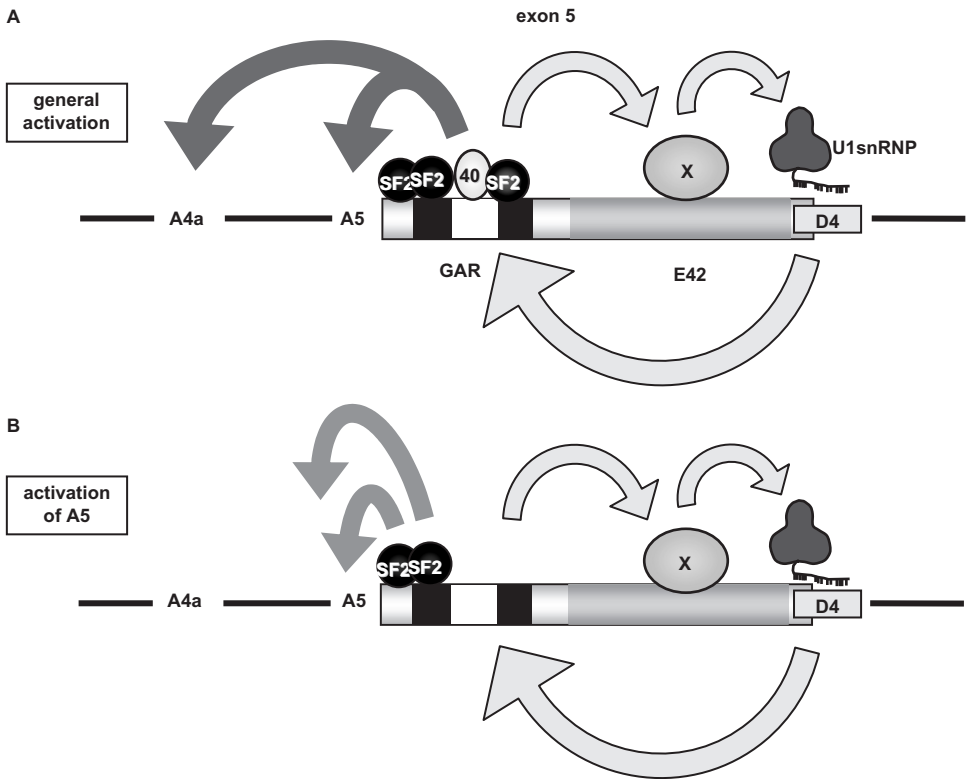

Figure 7. Model of interactions causing splice site activation in the internal HIV-1 exon 5. (**A**) The GAR ESE activates the 5′ ss presumably via a yet unknown factor x. Subsequent U1 snRNP binding in turn activates the GAR ESE, which mediates general activation of the 3′ ss cluster. (**B**) Besides the downstream interactions the GAR ESE specifically activates A5 by the proximal SF2/ASF-binding sites.

While most of the HIV-1 pre-mRNA sequence is removed by extensive splicing in the early phase of viral gene expression, the internal exons 4c, 4a, 4b and 5 have been described to be frequently included into the 1.8-kb mRNA class due to activation of the respective alternative 3′ ss and the 5′ ss D4 (7). Our results evidence that the three SF2/ASF and SRp40-binding sites of the GAR ESE (22) individually contribute to the recognition of the internal exons 4a and 5 in a 2-intron minigene reporter whose splicing pattern was comparable to the respective splicing pattern of HIV-1 NL4/3-infected PM1 cells. The individual SR protein-binding sites differed in their extent of activating exon recognition [SF2(1) > SF2(2) > SRp40], albeit at a rather low overall level. However, the combination of two SR protein-binding sites in the GAR enhancer complex synergistically activated exon recognition way beyond the sum of the individual contributions, while addition of a third binding site only slightly increased exon recognition. Two SF2/ASF protein-binding sites mediated the inclusion of the internal exons 4a and 5 to a similar extent as an SRp40- and an SF2/ASF-binding site, which is in line with the finding that the RS domains are interchangeable between different SR proteins for some enhancer functions (37,38).

Synergy in regulatory processes, like e.g. alternative splice site activation, has been proposed to be mediated either by cooperative binding of regulatory factors to enhancer sequences or by enhancement of two or more inefficient steps of the regulated process by direct interactions (39). Analysing the function of the GAR ESE, we found that any single SR protein-binding site was already sufficient for sole activation of 5′ ss D4, but not for exon inclusion, suggesting downstream directed cross-exon interactions of the GAR enhancer. Since we did not observe any synergistic enhancement of splicing at D4 in the presence of two or more SR protein-binding sites of the GAR ESE, it seems highly unlikely that its synergistic effect on exon recognition is mediated through cooperative binding of SR proteins, as has been found in the *Drosophila doublesex* repeat element (40,41). In our previous work, the individual SR protein-binding sites of the downstream GAR ESE showed no synergistic effect on activation of a heterologous 3′ ss (22). We thus hypothesize the synergy found in exon recognition to stem from simultaneous interactions with the 3′ ss and 5′ ss, emanating from the enhancer into opposite directions, and thereby promoting different steps in the spliceosome assembly at the respective upstream and downstream intron (cf. Figure 7). Such synergistic action is not automatically associated with every bidirectional enhancer (42) and may hint to different mechanisms than present in cases of additive enhancer action. Synergy in complex assembly has been implicated as molecular switch for regulatory processes (39). The synergistic activation of internal exon inclusion by the three distinct binding sites for the SR proteins SF2/ASF and SRp40 of the GAR ESE might allow the virus to switch from the shortest *nef1*-mRNA isoform, consisting only of the terminal

exons 1 and 7 of the HIV-1 pre-mRNA, to internal exon-containing mRNAs coding for the regulatory proteins Rev or Nef.

Our model of activation through oppositely directed interactions originating from the GAR ESE is supported by the additional requirement of the E42 sequence, located between the enhancer and D4, for internal exon recognition in the subgenomic 2-intron minigene construct. As E42 failed to initiate U1 snRNP binding at the 5′ ss in the absence of the GAR enhancer (22), we assumed that it recruits a yet unidentified factor to mediate the GAR-dependent activation of D4. This factor might either act indirectly by facilitating cross-exon interactions between the GAR ESE-bound SR proteins and U1 snRNP, or establish an independent additional weak interaction with the U1 snRNP providing the stabilization necessary to activate D4. The importance of this element for recognition of the short HIV-1 exon 5, flanked by long introns, supports the model that recognition is predominantly promoted by interactions of proteins across the exon (43–45).

Unbalanced activation of the competitive 3′ ss A4a and A5 has also been observed during viral gene expression and revealed a considerably higher expression of mRNAs using A5 than any other 3′ ss of the cluster (7), leading to Nef-encoding mRNA required in the early phase of viral gene expression. Nef fulfils a number of regulatory functions during viral replication, e.g. cellular surface receptor down-modulation (46), which are executed through mostly weak interactions of distinct Nef domains with a variety of cellular proteins (47). An elevated Nef concentration in the infected cell might thus contribute to efficient viral replication. Increased inclusion of the alternative internal exon 5 compared to exon 4a, as detected in our experiments, may thus lend a further functionality to the GAR enhancer for viral replication.

SR proteins are likely to mediate the function of the GAR ESE through their RS domains, which are known to interact with proteins and have more recently been shown to also stabilize double-stranded pre-mRNA-U snRNA intermediates during spliceosomal assembly (26,27). Both protein–protein and RNA–protein interactions might be involved in the 3′ ss selectivity of the GAR ESE. An interaction of an RS domain with double-stranded RNA might depend on their stereo-specific arrangement, and might therefore be restricted to the proximal SF2/ASF-binding sites. In contrast, protein–protein interactions between the RS domains and proteins of the splicing machinery might be less reliant on stereo-specific requirements, and thereby might act more uniformly on the whole 3′ ss cluster.

The extent of 3′ ss selectivity mediated by the GAR enhancer was shown to be decreased by weakening the interaction of U1 snRNP with the 5′ ss D4, leading to more uniform 3′ ss activation. This additionally supports that U1 snRNP binding participates in 3′ ss activation through cross-exon interactions, presumably by stabilizing a specific 3′ ss selecting SR protein configuration at the GAR enhancer.

Besides its function in the intronless 1.8-kb mRNA class, we present evidence that U1 snRNP binding at D4 is absolutely required also for activation of the 3′ ss cluster in Rev-dependent 4-kb *vpu*/*env*-mRNAs, which are not spliced at D4. Since we detected equal amounts of unspliced RNA for several mutations of D4, we assume that in the context of the additional upstream 5′ ss D1, U1 snRNP binding at D4 is needed for 3′ ss activation rather than for transcript stability (28,48).

In the *vpu*/*env*-mRNAs the 3′ ss activation ratio of A5 over A4a exceeded that of the 1.8-kb mRNAs, as has been described for viral infection (7). Since U1 snRNP is not displaced from D4 in the 4-kb mRNA class, its upstream-directed cross-exon interactions are likely to persist longer, mediating the pronounced 3′ ss selectivity of the GAR ESE. Thereby the GAR ESE might contribute to the preferential generation of the *vpu*/*env*-mRNA isoforms efficiently expressing Vpu (1), which has been shown to support viral replication.

This work demonstrates that the GAR ESE constitutes a complex substantial splicing regulatory element throughout HIV-1 gene expression. The GAR ESE has recently been successfully targeted to inhibit HIV-1 multiplication (49), and further insights regarding the mechanism of GAR ESE-mediated splice site activation and selectivity might create new strategies to specifically inhibit generation of essential regulatory HIV-1 mRNAs.

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