MINIREVIEW

Alternative splicing: regulation of HIV-1 multiplication as a target for therapeutic action

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The retroviral life cycle requires that significant amounts of RNA remain unspliced and perform several functions in the cytoplasm. Thus, the full-length RNA serves both the viral genetic material that will be encapsulated in viral particles and the mRNA encoding structural and enzymatic proteins required for viral replication. Simple retroviruses produce one single-spliced *env* RNA from the full-length precursor RNA, whereas complex retroviruses, such as HIV, are characterized by the production of multiple-spliced RNA species. In this review we will summarize the current acknowledge about the HIV-1 alternative splicing mechanism and will describe how this malleable process can help further understanding of infection, spread and dissemination through splicing regulation. Such studies coupled with the testing of splicing inhibitors should help the development of new therapeutic antiviral agents.

Introduction

The HIV/AIDS epidemic is one of the primary health concerns worldwide [1]. Despite significant advances in anti-HIV chemotherapy, the treatment and/or prevention of the disease remains a largely unsolved problem. Current routine drug regimens, typically consisting of various combinations of compounds targeting the viral proteins reverse transcriptase, protease and gp120, have revolutionized the treatment of HIV/AIDS [2–4]. However, a number of problems with current therapies limit their usefulness. First, the cost of the drugs constitutes a significant burden to individuals and governments worldwide, and virtually eliminates their availability in developing countries. Additional problems include the inconvenient and complicated medication schedules, the lack of patient compliance, side-effects associated with the drugs, and, ominously, the development of drug-resistant HIV. For these reasons, alternative or adjuvant treatment strategies for HIV infection are being investigated. Understanding the mechanism of HIV replication in host cells will help to develop unexplored strategies for HIV therapy. This review will focus on alternative splicing, a key event for HIV replication.

HIV-1 alternative splicing mechanism

The HIV-1 DNA genome expresses a primary transcript of 9 kb that not only serves as genomic RNA

Abbreviations
3′ss, 3′ splice site; 5′ss, 5′ splice site; ESE, exonic splicing enhancer; ESS, exonic splicing silencer; ESSV, exonic splicing silencer of Vpr; hnRNP, heterogeneous nuclear ribonucleoprotein; ISS, intronic splicing silencer; PPT, polypyrimidine tract; RRE, Rev response element; snRNP, small nuclear ribonucleoprotein; SR protein, serine and arginine rich protein.

for progeny virus, but also as the mRNA that encodes the viral Gag and Gag-Pol proteins. Successful infection and production of new infectious viruses requires the balanced expression of seven additional viral proteins. To achieve this proteomic diversity, alternative or intron retention of the primary transcript and nuclear export of the unspliced transcript are regulated [5–7].

During replication of HIV-1, the viral (+)RNA genome is reverse transcribed and integrated into the host cell genome. Transcription of this provirus by the cellular RNA polymerase II generates a polycistronic pre-mRNA that contains multiple splicing sites that enable alternative splicing of more than 40 different mRNAs (Fig. 1). The process of HIV-1 RNA splicing is highly orchestrated. Several sequence motifs within the RNA are required for recognition by the cellular spliceosome: the 5′ splice site (5′ss) or splice donor (Fig. 1, D1–D4) and a branch point and a 3′ splice site (3′ss) or splice acceptor (Fig. 1, A1–A7). HIV-1 uses multiple alternative 5′ss and 3′ss to generate spliced mRNA species [8,9]. These spliced mRNAs can be divided into two classes: multiply spliced (~ 2 kb) and singly spliced (~ 4 kb) RNAs (Fig. 1).

In the early phase of HIV-1 gene expression, the five 3′ss (A3, A4c, A4a, A4b and A5) located in a small central part of the viral RNA are used for production of the completely spliced *tat*, *rev* and *nef* mRNAs [9], which are transported to the cytoplasm for translation of the Tat, Rev and Nef proteins (Fig. 2). All the *tat* mRNAs are spliced at site A3. The *rev* mRNAs are spliced at sites A4a, A4b or A4c, and the *nef* mRNAs are spliced at site A5 [9,10]. Nef mostly modulates the physiological status of the host cell to suit the needs of the virus.

As the Rev protein accumulates, nuclear export of the singly and unspliced mRNAs is facilitated [11,12]. These mRNAs express the Vif, Vpr, Vpu, Env proteins and the Gag and Gag-Pol polyproteins, respectively, and require Rev, which overcomes the restriction of nuclear export of intron-containing transcripts by accessing the CRM1 nuclear export pathway (Fig. 2). The 4.0 kb and nonspliced 9.0 kb transcripts include the *tat/rev* intron flanked by D4 and A7, which contains a complex secondary structure, i.e. the Rev response element (RRE), which functions as a high-affinity binding site for Rev (Fig. 2).

Regulation of HIV-1 alternative splicing occurs primarily because of the presence of suboptimal 5′ss, 3′ss polypyrimidine tracts (PPTs) and branch site sequences (Fig. 3), which decrease the recognition by the cellular splicing machinery of the splice signals [13–15]. Splicing at the viral splice sites is further regulated by the presence of exonic splicing enhancers (ESEs) and exonic/intronic splicing silencers (ESS/ISS) [15–20], which bind cellular factors and either promote or inhibit, respectively, splicing at neighbouring splice sites (Fig. 3) [10].

However, determination of the strength of a splice site is exacerbated by the fact that its intrinsic strength can be greatly modified, both positively as well as negatively, by these *cis*-acting splicing regulatory sequences (splicing enhancers and silencers). Several *cis*-acting elements, i.e. splicing silencer elements, have been identified in the HIV-1 genome. These serve as protein binding sites for members of the heterogeneous nuclear ribonucleoprotein (hnRNP) family by down-regulating splicing at the 3′ss A1 [21], A2 [16], A3 [18,22], the HXB2-specific A6 [17] and A7 [19] (Fig. 4B). Several ESE elements binding serine and arginine rich proteins (SR proteins) were also detected. and unexpectedly for inefficient splice sites, splicing enhancer sequences that bind SR proteins were mapped in exon 5 [23] and the HXB2-specific exon 6 [17]. Due to mutations that optimize its utilization in the HXB2 strain, exon 8 was only found to be used in this strain and up to now, this is the only case of an additional exon used in only one given HIV-1 strain (Fig. 4A) [19,24–26]. Binding of SR proteins downstream of a splice acceptor can increase the efficiency of U2AF binding to the PPT, either by displacement of hnRNP A1 protein that blocks access of spliceosomal components to the 3′ss or by direct interaction between the arginine serine (RS) domains of the SR protein and U2AF (Fig. 4A, B).

HIV-1 splicing is therefore regulated by both positive and negative *cis* elements within the viral genome that act to promote or repress splicing and their mechanisms of action were elucidated at the three most highly regulated HIV-1 3′ss.

Regulation of HIV-1 pre-mRNA splicing at different acceptor sites

Splicing acceptor site A1

Suboptimal splicing at 3′ss A1 is necessary for virus replication. Increased splicing at 3′ss A1 results in the accumulation of *vif* mRNA and increased inclusion of exon 2 within spliced viral mRNA species. A suboptimal 5′ss signal downstream of HIV-1 3′ss A1 is necessary for appropriate 3′ss utilization, accumulation of unspliced viral mRNA, Gag protein expression and efficient virus production [10].

Optimization of the 5′ss D2 signal results in increased splicing at the upstream 3′ss A1, increased inclusion of exon 2 into viral mRNA, decreased accumulation of unspliced viral mRNA and decreased virus production.

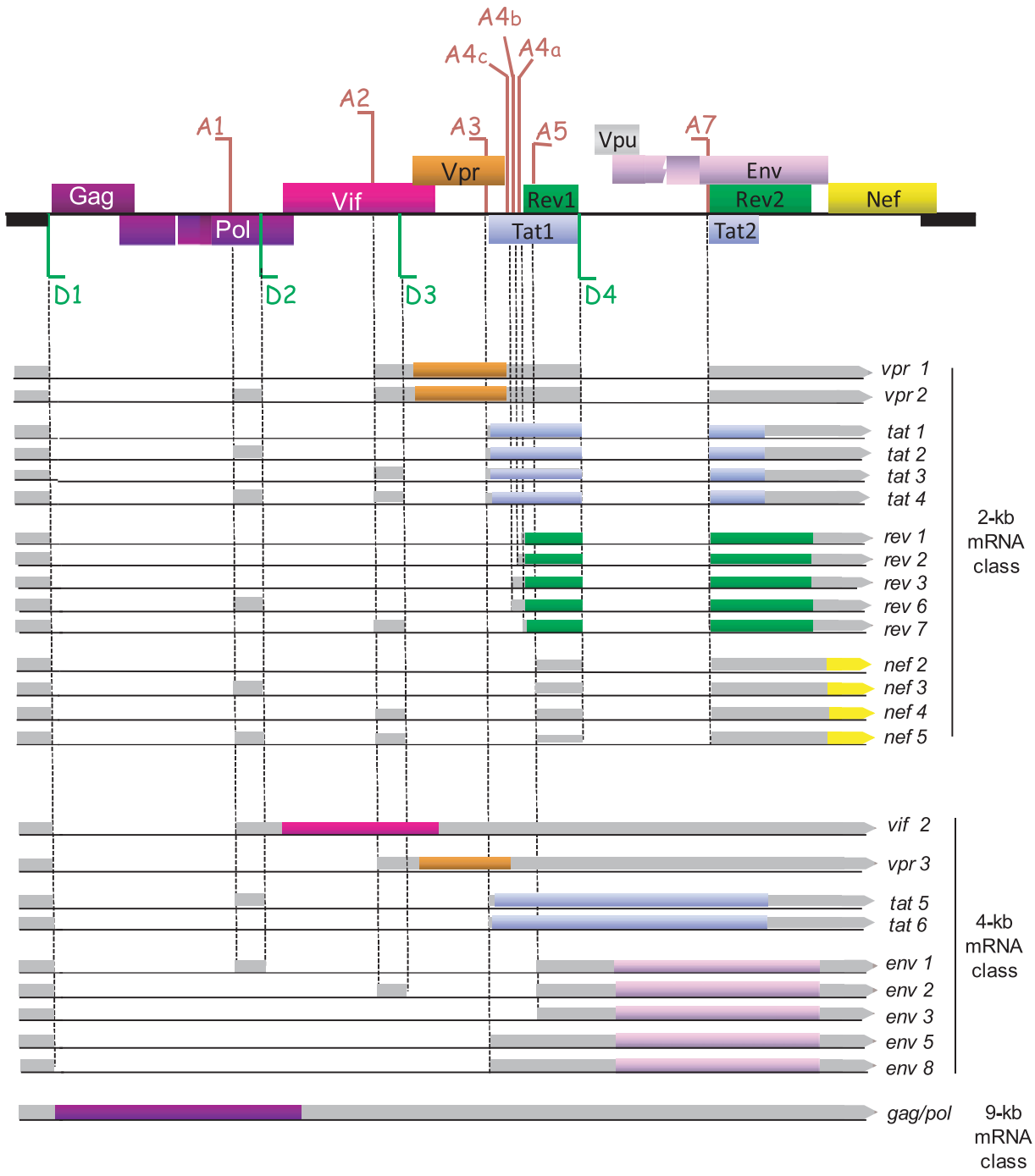

Fig. 1. Organization of HIV-1 genome and different mRNA splicing products. The 5′ss (D1–D4) and 3′ss (A1–A7) are indicated. ORFs of coding exons of each mRNA product are indicated with a different colour code alluding to the corresponding encoded proteins of the HIV genome. The noncoding exons are boxed in grey.

Splicing acceptor site A2

Splicing at HIV-1 3′ss A2 results in the accumulation of *vpr* mRNA and the inclusion of noncoding exon 3 when 3′ss A2 is spliced to the downstream 5′ss D3. This splicing event is repressed by exonic splicing silencer of Vpr (ESSV) and enhanced by the downstream 5′ss D3 signal. Disruption of ESSV results in increased *vpr* mRNA accumulation and exon 3 inclusion, decreased accumulation of unspliced viral mRNA and decreased

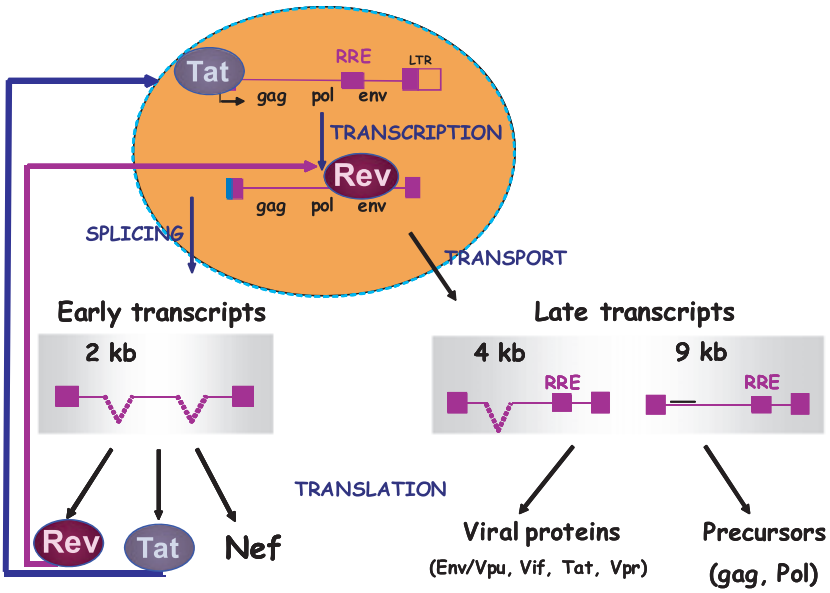

Fig. 2. Early and late transcripts derived from the viral HIV-1 genome. The integrated copy of the viral genome produces Rev and Tat proteins from the 2 kb early transcripts. Both Tat and Rev are RNA binding proteins that enter the nucleus and mediate transcription transactivation and export of 4 and 9 kb late transcripts, respectively. The late transcripts have an RNA binding site for Rev (RRE) allowing their export from the nucleus.

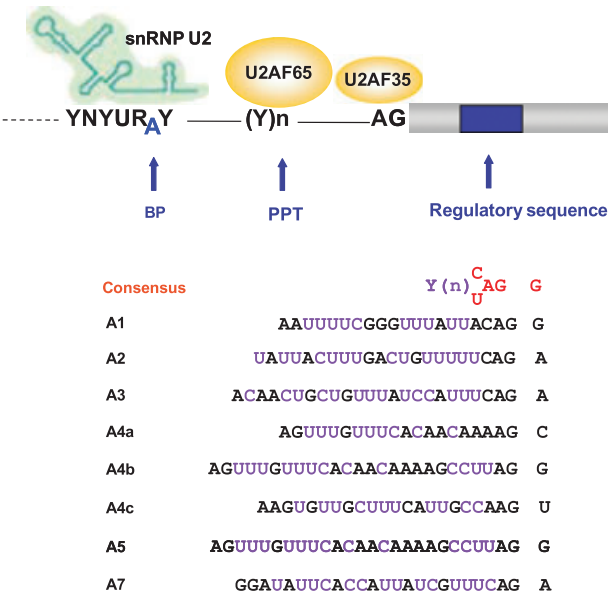

Fig. 3. Recognition of a weak 3′ss of the HIV precursors. The upper panel shows that the binding of U2 snRNP to the branch point (BP), where the first catalytic step takes place, is enhanced by the auxiliary factor U2AF (composed of two subunits 65 and 35 kDa). The regulatory element in the second exon can have either a positive or a negative effect on the binding of U2AF. The lower panel shows that most of the HIV-1 3′ss deviate from the consensus because of their low content of pyrimidine nucleotides.

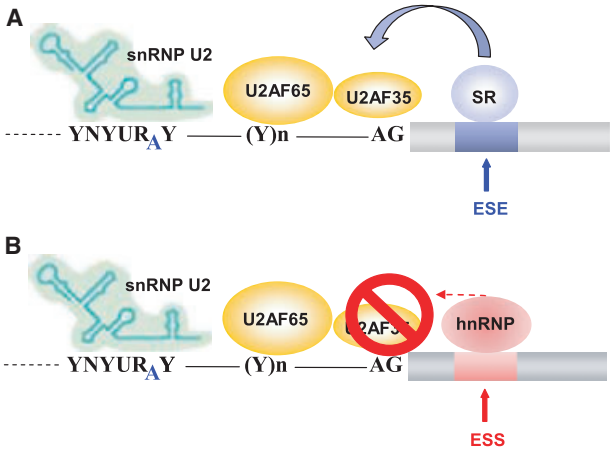

Fig. 4. Positive and negative regulation of HIV-1 3′ss. (A) Action of SR proteins as positive regulators. (B) Action of hnRNP proteins as negative regulators.

virus production [16,27] (Fig. 5, Table 1). HIV-1 replication is reduced by 95% when ESSV is inactivated by mutagenesis due to increased splicing at HIV-1 3′ss A2 and the resulting decrease in unspliced RNA accumulation. Second site mutations that either inactivate 3′ss A2 or 5′ss D3 can revert this replication defect [27].

Splicing at HIV-1 3′ss A2 is repressed by the hnRNP A/B-dependent ESSV, a 16 nucleotide element within HIV-1 exon 3 containing three (Y/A)UAG motifs. It has also been shown that 3′ss A2 utilization is repressed by inhibition of U2AF65 recognition of the 3′ss A2 PPT through the binding of cellular hnRNP A/B proteins to ESSV [16,28]. The maintenance of ESSV is necessary, not only for appropriate 3′ss utilization, but also for the accumulation of wild-type levels of unspliced viral mRNA, Gag protein production and production of virus particles.

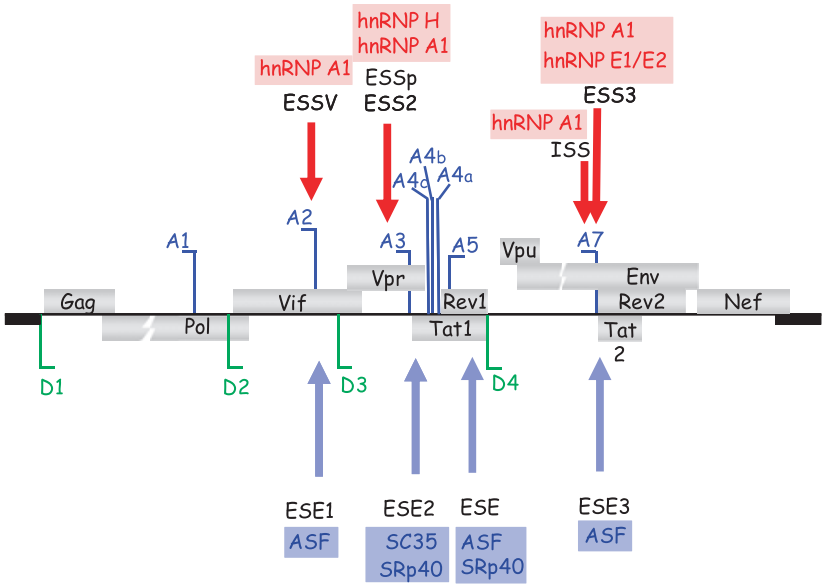

Fig. 5. Position of identified regulatory elements that act either as an enhancer (ESE) or a silencer (ESS) of the selection of different 3′ss.

Splicing at site A2 is also strongly activated by binding of the SR protein SF2/ASF, which competes with hnRNP A/B binding [18,29,30] (Fig. 5, Table 1). Among all HIV-1 3′ss, site A2 is the most strongly activated by SF2/ASF. Overexpression of SF2/ASF in HeLa cells leads to a strong increase in Vpr mRNAs at the expense of other mRNAs [29].

Vpr is an accessory gene product of HIV-1 and affects both viral and cellular proliferation by mediating long terminal repeat activation, cell cycle arrest at the G2 phase and apoptosis. It is also involved in nuclear localization [31,32] and regulation of transcription [33]. Vpr has also been found to play a novel role as a regulator of pre-mRNA splicing both *in vivo* and *in vitro* [34,35].

Splicing acceptor site A3

Surprisingly, despite its low efficiency, site A3 has the most optimized PPTs compared with the competitor sites [14]. One explanation for this apparent discrepancy is the presence of both an upstream (ESS2p) [18] and a downstream (ESS2) ESS acting on site A3. The proximal ESS2p element binds protein hnRNP H generating a steric hindrance at site A3 (Fig. 5, Table 1). In contrast, ESS2 is located far downstream from site A3 (69 nucleotides) [14]. It inhibits an early step of spliceosome assembly by initiating the recruitment of protein hnRNP A1 on a long stretch of RNA sequence that folds into a long irregular stem loop structure, SLS3 (Fig. 5, Table 1) [22]. This extensive multimerization of hnRNP A1 towards the A3 3′ss leads to the occlusion of the PPT and to site A3 inhibition [36].

Enzymatic and chemical probing revealed the occurrence of several SC35 and SRp40 binding sites in SLS3 and in agreement with the strong activation properties of these proteins on site A3 [29], several of their binding sites overlap the hnRNP A1 binding sites. However, SC35 binding on the SLS3 loop to a sequence named ESE2 seemed to only have a limited contribution to the activation of site A3 (25% of the overall activation). Therefore, the most important parameter of site A3 activation is expected to be the displacement of protein hnRNP A1 from ESS2 by SC35 or SRp40 proteins binding to ESE2 (Fig. 5, Table 1) [36].

In summary, hnRNP H and hnRNP A1 bind to the ESS2p and ESS2 elements, respectively, to repress activity at splice site A3. ESS2 initiates the multimerization of hnRNP A1 on the entire SLS3 stem loop structure. The SR proteins SC35 and SRp40 can out compete hnRNP A1 and activate splicing [36].

Production of the HIV-1 Tat protein depends upon A3 splicing site utilization and plays a key role in virus multiplication, as it is needed for the production of full-length HIV-1 transcripts by activating transcription from the HIV-1 promoter [37]. However, because of the apoptotic activity of this protein on both the infected cells and the neighbouring cells [38], HIV-1 strongly controls its production. In both lymphoid and nonlymphoid infected cells, the steady-state level of the doubly spliced *tat* mRNAs is considerably lower than the levels of doubly spliced *rev* mRNAs and singly spliced *env/vpu* mRNAs [9]. This seems to be due to the poor efficiency of the A3 splicing site as compared with the other downstream 3′ss [14].

Table 1. Summary of all data concerning regulatory sites, their position in the genome of HIV-1 BRU strain, factors that bind to them and references where they were described.

Acceptor site	Regulatory elements	Regulatory factors involved	Sequences	Positions	References
A2	ESSV	hnRNP A1	UUAGGACAUAUAGUUAGCCCUAGG	4995–5017	[5, 12, 38, 40]
	ESE1	ASF/SF2	unknown		
A3	ESSp	hnRNP H	UGGGU	5362–5366	[48, 41, 15, 17, 8]
	ESS2	hnRNP A1	CUAGACUAGA	5428–5437	
	ESE2	SC35, SRp40	CCAGUAGAUCCUAGACUAGA	5418–5437	
A5	ESE GAR	ASF/SF2, SRp40	GAAGAAGCGGAGACAGCGACGAAGA	5558–5582	[7]
A7	ESS3	hnRNP A1,	AGAUCCAUUCGAUUAG	8047–8062	[43, 50, 47, 46, 32]
		hnRNP E1/E2	unknown		
	ISS	hnRNP A1	UAGUGAAUAGAGUUAGGCAGGGA	7928–7950	
	ESE3	ASF/SF2	GAAGAAGAA	8016–8025	
		hnRNP A1	UAGAAGAAGAA	8018–8025	

The HIV-1 encoded proteins Tat, which acts as a transactivator of viral and cellular genes, and Rev, which is essential for nuclear export of incompletely spliced viral mRNAs, have also been shown to inhibit HIV-1 splicing by interacting with p32, a cofactor of ASF/SF2 [39].

Splicing acceptor sites A4a, A4b and A4c

Rev mRNAs are spliced at all three of these acceptor sites (Fig. 1). The RNA binding proteins Tat and Rev are key regulators for the expression of the other viral genes, for the synthesis of full-length genomic RNA and, ultimately, for the production of progeny virions (reviewed in [40]).

Rev channels the unspliced and partly spliced RNA forms into a nucleocytoplasmic export pathway (reviewed in [40]). Rev functions by forming multimers that interact directly with a *cis*-acting RRE. This complex is exported via an interaction with host cellular Crm1/Exportin 1 through a pathway normally used by snRNA [7]. Rev is crucial because it directs the export of the unspliced and single-spliced mRNAs from the nucleus to the cytoplasm, which permits their translation [41]. Fine tuning of splicing is then critical to ensure the balance between spliced versus unspliced viral RNAs.

Splicing acceptor site A5

Splice site A5 is used for the production of singly spliced *Env* mRNA and is followed by an ASF/SF2 protein-dependent ESE [23] (Fig. 5, Table 1).

Splicing acceptor site A7

Utilization of HIV-1 3′ss A7 by the spliceosome is negatively regulated by the ISS, ESS3 and ESE3 (Fig. 5, Table 1) [19,25]. These three splicing silencers bind hnRNP A1 synergistically.

Splicing of the tat intron is regulated by the combination of the above ESS elements, with ESE elements located in the third tat exon [25] as well as a purine rich ESE sequence (ESE2) located upstream of donor site D4 in the second tat exon [42]. In fact, ESE3 has both splicing silencer and enhancer activities, as it binds both hnRNP A1 and SF2/ASF [21,24,26]. The SR protein SF2/ASF is a *trans*-acting factor for the ESE3 sequence [25] and presumably also for the ESE sequence upstream of D4 [42]. It has been reported that ESE3 and ESS3 regulate the efficiency of A7 utilization by modulating the level of U2AF65 that is associated with the PPT.

In addition, hnRNP E1/E2 are also able to interact with an HIV-1 segment including the ESS3 element in tat/rev exon 3 of HIV-1 and modulation of hnRNP E1 expression alters HIV-1 protein synthesis. Overexpression of hnRNP E1 leads to a reduction in Rev transport activity, which cannot be fully accounted for by a reduced level of *Rev* mRNA, suggesting that hnRNP E1 might also act to suppress viral RNA translation [43].

In conclusion, the detailed analyses of regulations at HIV-1 splicing sites point out a major role of protein hnRNP A1 and the SR proteins SF2/ASF, SC35 and SRp40 in these regulations.

Targeting splicing as a novel antiretroviral therapy

As stated above, the RNA binding proteins Tat and Rev are key regulators for the expression of HIV-1 viral genes, for the synthesis of full-length genomic RNA and, ultimately, for the production of progeny virions (reviewed in [40]). Thus, it is not surprising that Tat, Rev and their respective RNA binding elements

have been selected as targets in several therapeutic studies. Most of these studies have made use of antisense nucleic acids, such as antisense RNA, oligonucleotides, ribozymes and, more recently, short interfering RNAs. Several of these strategies are being tested in clinical trials. However, as the outcome of these studies is difficult to predict and as HIV-1 treatment will probably require the use of multiple therapeutic principles, alternative methods are still required.

A novel strategy has been developed based on the combination of Vif deficiency with an antisense U7 snRNA approach that induces Tat/Rev exon skipping, which dramatically affects HIV-1 infection and may therefore be a powerful tool in the fight against HIV/AIDS [44]. In this approach, the antisense RNA sequence that targets HIV-1 is inserted in U7 snRNA, the RNA component of the U7 small nuclear ribonucleoprotein (snRNP) involved in histone RNA 3′ end processing [45]. This insertion converts the U7 snRNP from a mediator of histone 3′ end processing to an effector of alternative splicing by masking the specific HIV-1 splicing site [44]. Because HIV-1 regulatory proteins Tat and Rev are encoded by multiply spliced mRNAs that differ by the use of alternative 3′ss at the beginning of the internal exon, if these internal exons are skipped, the expression of these genes and, hence, HIV-1 multiplication, should be inhibited. This new approach targeting HIV-1 regulatory genes at the level of pre-mRNA splicing, in combination with other antiviral strategies, may be a useful new tool in the fight against HIV/AIDS.

More recently, a novel strategy using small molecules that inhibit splicing by specifically targeting individual SR proteins was developed [46]. After screening a collection of chemical compounds, one indole derivative (IDC16) was discovered to interfere with ESE activity of the SR protein splicing factor SF2/ASF. This compound suppresses the production of key viral proteins, thereby compromising subsequent synthesis of full-length HIV-1 pre-mRNA and assembly of infectious particles. IDC16 inhibits replication of macrophage- and T cell-tropic laboratory strains, clinical isolates and strains with high-level resistance to inhibitors of viral protease and reverse transcriptase.

The efficiency of IDC16 derivatives was also evaluated on an animal model of retroviral pathogenesis using a fully replication-competent retrovirus. In this model, all newborn mice infected with a fully replicative murine leukaemia virus (MLV) developed erythroleukaemia within 6–8 weeks of age. Several indole derivative compounds (IDC)16 selectively altered splicing-dependent production of the retroviral envelope gene, thus inhibiting early viral replication *in vivo* sufficiently to protect the mice from MLV-induced pathogenesis [47]. The apparent specificity and clinical safety observed here for IDC16 derivatives strongly support further assessment of inhibitors of SR protein splicing factors as a new class of antiretroviral therapeutic agents.

Concluding remarks

The various approaches aimed at reducing the viral load in patients infected by HIV utilize molecules intended to inhibit the enzymatic activity of viral reverse transcriptase or of the protease involved in virus protein maturation. The absence of cellular proteins resembling HIV integrase has also been exploited to develop novel anti-HIV molecules that inhibit this enzymatic activity. The only type of antiretroviral compound that targets cellular proteins is the one used for its ability to prevent viruses from entering the cell. These entry inhibitors can be either peptides that interfere with the fusion of viral glycoproteins gp41 or gp120 with the membrane of CD4 cells or molecules that target HIV cellular coreceptors CCR5 and CXCR4.

In this respect, alternative splicing offers many approaches for combating HIV-1 infection and even circumventing HIV-1 drug resistance through inhibition of cellular targets. As reported here, alternative splicing involves a flexible mechanism for selecting the HIV-1 splice site, based on regulatory sequences recognized by cognate *trans*-acting factors. These RNA·protein interactions provide two types of target for therapeutic manipulation. Masking regulatory RNA sequences with an antisense strategy is the most obvious. This approach includes the use of oligonucleotides or modified snRNA linked to antisense sequences to block the use of viral splice sites and, as mentioned above, encouraging results are beginning to accrue. The antisense molecules can also be designed as peptide nucleic acids or bifunctional oligos mimicking or recruiting SR proteins at specific sites [48,49] to modulate HIV-1 splicing.

Alternatively, the redundancy of SR protein activity for splicing of cellular endogenous genes but not for HIV-1 splicing can also be exploited in strategies aimed at modifying the expression level of a given SR protein or hnRNP protein. The one relying on RNA interference appears particularly interesting. Indeed, short interfering RNAs are not only an exciting new tool in molecular biology, but also represent the next frontier in molecular medicine [50]. Guaranteeing specificity and finding safe delivery systems will need further work, but the therapeutic promises of small RNA antiretroviral tools still remain important. The

discovery that several indole derivatives specifically inhibit ESE-dependent splicing through their direct and selective interactions with members of the SR protein family provides an attractive alternative to the use of short interfering RNAs. Furthermore, their specificity for a subset and possibly a single member of the SR protein family suggests that they could exhibit a low toxicity, therefore allowing their development as clinically usable drugs.

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