Inefficient Spliceosome Assembly and Abnormal Branch Site Selection in Splicing of an HIV-1 Transcript in Vitro*

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Continuous replication of human immunodeficiency virus type I (HIV-1) requires balanced expression of spliced and nonspliced mRNAs in the cytoplasm. This process is regulated post-transcriptionally by the viral encoded Rev protein. An important prerequisite for Rev responsiveness is the presence of weak splice sites in the viral mRNA. We have investigated the splicing of the second intron of the HIV-1 Tat/Rev transcript in vitro and show that the 3' splice site region is responsible for the inefficient splicing of the HIV-1 transcript. In contrast, the HIV-1 5' splice site is highly functional in combination with a heterologous 3' splice site. Incubation of the HIV-1 transcript in nuclear extract leads to a rapid accumulation of 50 S nonproductive pre-spliceosome complexes. These complexes contain mainly U1 and U2 small nuclear ribonucleoproteins and are formed independently of the presence of the downstream 3' splice site. The HIV-1 transcripts, which do proceed through the first splicing step, utilize primarily a uridine as the branch acceptor nucleotide. Sequence comparison with the first splicing step, utilize primarily a uridine as the site. The HIV-1 transcripts, which do proceed through U4/U6 small nuclear ribonucleoproteins (snRNPs)1 on the intron prior to nuclear transport to the cytoplasm. This is a served sequences near the two splice sites and removes the intron. The cellular splicing machinery recognizes conserved sequences near the two splice sites and removes the intron prior to nuclear transport to the cytoplasm. This is a complex process involving the assembly of U1, U2, U5, and U4/U6 small nuclear ribonucleoproteins (snRNPs)1 on the pre-mRNA (reviewed in Refs. 1 and 2). Initially, U1 snRNP interacts with a conserved sequence at the 5' splice site, U2 snRNP then binds to the branch point region just upstream from the 3' splice site, and the remaining U5 and U4/U6 snRNPs enter this complex as a tri-snRNP particle to form the complete spliceosome. In addition to the snRNPs, a number of other auxiliary protein factors play important roles in spliceosome assembly. In particular, ASF/SF2 and U2AF assist the binding of U1 and U2 snRNPs to conserved sequences at the 5'-splice site and the branch point region, respectively, and SC-35 is critical for the recognition of the 3'-splice site (reviewed in Ref. 1). The RNA splicing process is generally very efficient, leading to the export of only one major mRNA species to the cytoplasm. However, the expression of a substantial number of mRNAs is tissue-specific and developmentally regulated by alternative splicing. Differentiated splicing products may be obtained by a number of different mechanisms including the utilization of alternative 5'- and 3'-splice sites, exon and intron inclusion or skipping, and mutually exclusive exons, all of which have been found in biological systems (3). The complexity of the splicing reaction makes it a well suited target for regulation of gene expression, and the mechanisms involved in biological systems appear to be diverse (3).

Retroviruses have evolved a post-transcriptional regulatory system based on intron retention, in order to express multiple proteins from the same promoter. Crucial for the life cycle of all retroviruses is a balanced expression of an unspliced mRNA of about 9 kb and a singly spliced mRNA of about 4 kb, encoding the Gag, Pol and Env proteins, respectively. Most of our knowledge about elements controlling this differential splicing comes from studies of avian sarcoma viruses. In this group of viruses the ratio between genomic and singly spliced mRNAs appears to be constitutive and mainly regulated by a suboptimal 3'-splice site (4-7) and a negative regulator of splicing (NRS element), which acts in cis to decrease the splicing efficiency of the viral transcript (8, 9).

In complex retroviruses, which include lentiviruses (e.g. HIV-1), spumaviruses, and human T-cell leukemia virus, the post-transcriptional regulation of splicing appears to be more complex. In addition to the two classes of mRNA found in all retroviruses, the complex viruses express a major class of approximately 2 kb long mRNAs, which encode a number of small regulatory proteins. Best studied are the HIV-1 regulatory proteins Tat and Rev, both of which are essential for virus propagation. Tat is a transcriptional activator, whereas Rev appears to function only at a post-transcriptional level, up-regulating the appearance of unspliced and singly spliced mRNAs in the cytoplasm (reviewed in Ref. 10). There has been some controversy about what level of gene expression is subject for Rev regulation. Rev may function directly at the level of mRNA splicing (11-15), mRNA stability and transport (16-20), and/or translation (21, 22). Although some of these functions may be closely coupled, it suggests that Rev is a multifunctional protein.

The specificity of Rev relies on direct binding to the Rev response element (RRE) and the presence of cis-acting repressive sequences within the transcript. In contrast to the RRE, which is a well defined RNA element located at the start of the env gene, the identity of cis-acting repressive sequences is less well understood. These elements may constitute nuclear retention or instability elements and have been mapped to both the Gag, Pol, and Env regions (23-27).
Splicing of HIV-1 Transcripts

Based on the observation that the introduction of weak splice sites into an RRE-containing β-globin gene renders the mRNA Rev responsive, it has been suggested that weak splice sites may function as nuclear retention elements (11). This interpretation is supported by a recent study showing that the integrity of the splice sites in HIV-1 mRNA is necessary for Rev regulation of HIV-1 gene expression (14). This implies that Rev-mediated regulation of HIV-1 gene expression requires an intrinsically inefficient splicing process. A search for splicing regulatory elements have identified a cis-acting repressor element within the Tat-coding exon that suppresses splicing of the upstream intron in vitro and in vivo (28). This element is position-dependent, but works in the context of a heterologous intron. cis Elements controlling the splicing of the second intron of the Tat/Rev transcript have been investigated in vivo, and it is concluded that a nonoptimal 3′-splice site splicing signal is the main determinant for inefficient splicing (29). We have studied the splicing of the same HIV-1 intron and show that the 5′-splice site region directs a rapid accumulation of a 50 S complex, containing U1 and U2 snRNPs, and that the 3′-splice site is inefficiently recognized by the splicing apparatus. A possible role for the 50 S complex could be to retain the mRNA in the nucleus in the absence of Rev protein.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—In the following, an asterisk (*) denotes that the restriction site has been blunt-ended with Klenow enzyme prior to ligation. The cloning scheme is indicated in Fig. 1. The pPIP7A plasmid contains an artificial transcription unit including optimal splice signals (30), pTat-CMV contains a 2.7-kb Taq fragment including the Tat gene of the HIV-1 strain HXB3 (nt 5392–8039; numbered according to Ratner et al. (31)) behind a CMV promoter (11). The pgTat-CMV3 and pgTat-CMV4 are derived from pgTat-CMV by deleting the Spal–BglII* (nucleotides 5737–7198) and Spal–HindIII* (nucleotides 5737–7118) fragments within the intron, respectively (11). pTAT3 and pTAT4 were constructed by deleting the BamHI–XhoI fragment of pgTat-CMV3 and pgTat-CMV4, respectively, into the BamHI–SalI sites of pBS (Stratagene). pTAT3 and pTAT4 were constructed by replacing the BamHI–XhoI fragment of pTAT4 containing the 5′-splice site. pTAT5 was constructed by deleting the HindIII–HindIII fragment of pTat-CMV by deleting the Spal–BglII* (nucleotides 5737–7198) and Spal–HindIII* (nucleotides 5737–7118) fragments within the intron, respectively (11). pTAT4 was constructed by deleting the BamHI–XhoI fragment of pgTat-CMV3 and pgTat-CMV4, respectively, into the BamHI–SalI sites of pBS+ (Stratagene). pTAT5 was constructed by replacing the HindIII–HindIII fragment of pTAT4 containing the 5′-splice site. pTAT5 was constructed by replacing the HindIII–HindIII fragment of pTat-CMV by deleting the Spal–BglII* (nucleotides 5737–7198) and Spal–HindIII* (nucleotides 5737–7118) fragments within the intron, respectively (11). pTAT5 was constructed by deleting the BamHI–XhoI fragment of pgTat-CMV3 and pgTat-CMV4, respectively, into the BamHI–SalI sites of pBS+ (Stratagene). pTAT5 was constructed by replacing the HindIII–HindIII fragment of pTAT4 containing the 5′-splice site. pTAT5 was constructed by replacing the HindIII–HindIII fragment of pTat-CMV by deleting the Spal–BglII* (nucleotides 5737–7198) and Spal–HindIII* (nucleotides 5737–7118) fragments within the intron, respectively (11). pTAT5 was constructed by replacing the BamHI–XhoI fragment of pgTat-CMV3 and pgTat-CMV4, respectively, into the BamHI–SalI sites of pBS+ (Stratagene). pTAT5 was constructed by replacing the HindIII–HindIII fragment of pTAT4 containing the 5′-splice site. pTAT5 was constructed by replacing the HindIII–HindIII fragment of pTat-CMV by deleting the Spal–BglII* (nucleotides 5737–7198) and Spal–HindIII* (nucleotides 5737–7118) fragments within the intron, respectively (11). pTAT5 was constructed by replacing the BamHI–XhoI fragment of pgTat-CMV3 and pgTat-CMV4, respectively, into the BamHI–SalI sites of pBS+ (Stratagene).
MgCl₂, 4 mM spermidine, 10 mM dithiothreitol, 40 units of RNasin, 0.7 mM ATP, 0.7 mM UTP, 0.7 mM GTP, 0.7 mM CTP, 0.07 mM biotin-11-UTP (10% of total UTP), 1 μCi of α-³⁵S-UTP (Amersham Corp., 3000 Ci/mmol), and 200 units of T7 RNA polymerase. The RNAs were separated from unincorporated nucleotides on a Sephadex G-50 spin column and purified on 4% polyacrylamide denaturing gels. The final concentration of the RNA was calculated from the specific activity of incorporated ³⁵S label.

In Vitro Splicing and Complex Gel Analysis—In vitro splicing was performed essentially as described previously (12). For denaturing gel analysis, the mRNA was incubated for 90 min at 30°C, whereas for native gel runs and sucrose gradient centrifugations, samples were incubated for only 20 min at 30°C. To protect the RNA from 3'-exonucleases in the nuclear extract, 10 μg of tRNA were included in each splicing reaction. Splicing products were analyzed on denaturing gels, containing 6% polyacrylamide, 8 M urea, and 50–100 mM Tris borate (pH 8.3), and splicing complexes were analyzed by loading 5 μl of the splicing reactions onto native gels containing 2.5% 80:1 acrylamide/ bisacrylamide and 50 mM Trisglycine (pH 8.8).

Purification of Splicing Complexes and Northern Blotting—Streptavidin affinity purification of biotinylated RNA and probing for U1, U2, U4, U5, and U6 snRNPs were performed as described by Kjems and Sharp (32). U11 and U12 snRNA antisense RNAs were prepared as described by Wassarman and Steitz (33).

Debranching, Primer Extension, and Sequencing of RNA Products—Debranching of lariats was done as described in Ruskin and Green (34) by recircularizing the phenol-extracted splicing reaction in a 25 μl mixture, containing 20 μl of debranching buffer (50 mM HEPES (pH 7.8), 100 mM KCl, 0.1 mM EDTA), and 5 μl of the S100 fraction of HeLa cell extract (35) for 30 min at 30°C, followed by phenol extraction and precipitation. Primer extension analysis was performed essentially as described in Kjems et al. (36), using 1 pmol of 5'-end-labeled primer (5'-GTGGGTCCCCTCGGG-3'), complementary to position 15–30 downstream from the 3'-splice site in TAT4 and gel-purified RNA templates. To obtain sequence information 1 mM ddTTP or ddATP, or 10 μM of ddGTP or ddCTP, was included in each of the respective sequencing reactions. The TATPIP exon-exon product was purified and identified by the following procedure: 1 μg of nonradioactive TATPIP was incubated in a 200 μl splicing reaction and electrophoresed together with a hot sample on a denaturing gel. RNA co-migrating with the exon-exon band was purified, and the sequence across the ligation site was determined by primer extension as described above. Preparation of lariat TAT3 and TAT4 RNAs for primer extension was done similarly.

RESULTS

A Nonoptimal HIV-1 3'-Splice Site Renders Splicing An Inefficient Process in Vitro—Controlling the cytoplasmic appearance of non-, single-, and double-spliced mRNAs is an important aspect of the HIV-1 life cycle. To determine elements important for this regulation, we have investigated the splicing of HIV-1 mRNAs in vitro using a number of chimerical constructs between the PIP7A, a construct optimized for splicing, and TAT4 RNAs were constructed (Fig. 1). When the 5'-half of PIP7A including the intron (TAT3) had no effect on splicing efficiency compared to that observed using TAT4 RNA (result not shown).

To study the efficiency of the 5'- and 3'-splice sites of HIV-1 in vitro a number of chimerical constructs between the PIP7A, a construct optimized for splicing, and TAT4 RNAs were constructed (Fig. 1). When the 5'-half of PIP7A including the

![Fig. 2. In vitro splicing analysis of TAT4 and chimerical constructs. A, autoradiograms showing the splicing products of TAT4 (lanes 1 and 2), PIPTAT (lanes 3 and 4), TATPIP (lanes 5 and 6), PIPTAT (lanes 7 and 8), TATPIP (lanes 9 and 10), and TAT5'5's (lanes 11 and 12). — and + denote lanes with samples that have been incubated at splicing conditions for 90 min in the absence and presence of ATP, respectively. The bands were identified as follows. The intermediate lariat RNA (IL) and lariat RNA (L) products were identified by their abnormal behavior on gels containing different salt and acrylamide concentrations. In addition, gel analysis of debranced splicing reaction was performed on TATPIP derived lariats (see Panel B). The linear splicing products including the unprocessed precursor RNA (P), ligated exon-exon RNA (EE), and 5'-exon RNA (SE) were identified on the basis of apparent size as compared to molecular size markers, and the TATPIP derived exon-exon product was sequenced by primer extension (data not shown). The samples were loaded on a 6% polyacrylamide gel containing 75 mM of Tris borate (pH 8.3). B, lariat identification of TATPIP splicing products on a 6% gel containing 100 mM Tris borate. D denotes untreated splicing products; D denotes splicing products treated with debranching extract. M denotes the lane containing single-stranded DNA size marker, numbers on the left indicate the molecular sizes in base pairs. Identities of individual bands are indicated. The 5'-exon of TATPIP generally migrates as two bands of variable intensities. Sequence analysis of the TATPIP specific exon-exon product showed no sign of alternative 5'-splice site usage, suggesting that the lower 5'-exon band may result from partial RNA degradation of the upper 5'-exon band.
5'-splice site was substituted with the 5'-half of HIV-1 mRNA (TATPIP; Fig. 1), splicing became highly efficient, yielding more than 70% splicing products (Fig. 2A, lanes 5 and 6). The identities of the branched splicing products were confirmed both by a change in mobility when altering the ionic strength of the gel and by debranching (Fig. 2B). Purification and direct sequencing of the exon-exon RNA product confirmed that the normal 5'-splice site of the HIV-1 RNA was correctly joined to the 3'-splice site of the PIP7.A RNA (result not shown).

When substituting the 3'-half of PIP7.A with the 3'-half of TAT4 (PIPTAT; Fig. 1) splicing was as inefficient as observed for TAT4 (Fig. 2A, lanes 3 and 4). Similar results were obtained when shorter regions of the HIV-1 transcript, containing the 5'- or 3'-splice site regions, were inserted into PIPT7.A to replace the corresponding splice site (ΔTATPIP and PIPTAT, respectively; Figs. 1 and 2A, lanes 7-10), although a slight increase in splicing of PIPTAT RNA was observed as compared to PIPTAT and TAT4 (compare Fig. 2A, lanes 2, 4, and 8). These data imply that the region containing the 3'-splice site of the HIV-1 transcript is responsible for the inefficient splicing in vitro.

It has previously been shown that an element positioned downstream of the 3'-splice site of the first Tat intron inhibits the splicing of the upstream intron (28). To investigate the possibility that sequences within the 3'-exon flanking the second Tat/Rev intron function as inhibitory elements, the splicing efficiency of the TAT4 transcripts truncated at different positions within the 3'-exon was analyzed. No significant differences in splicing efficiency were detected using constructs containing 87, 80, 33, and 19 nucleotides of the 3'-exon, implying that no cis-acting inhibitory elements are present in the 3'-exon of the TAT4 transcript (results not shown).

Identification of the Branch Point Sequence in the HIV-1 Intron—Examination of the sequence upstream from the 3'-splice site in the HIV-1 intron revealed no obvious branch site consensus. To identify the branched nucleotide, utilized for the inefficient lariat formation in vitro, 1 μg of low specifically labeled TAT3 and TAT4 transcripts was incubated under splicing conditions for an extended period of time to increase the yield of splicing products. Approximately 2% of the radioactive label incorporated in TAT3 or TAT4 transcripts appeared in bands corresponding to branched RNAs. The bands were excised from the gel, extracted, and annealed to a primer complementary to a region within the common 3'-exon of TAT3 and TAT4. When extended by reverse transcriptase, specific stops were observed as compared to a control reaction containing a template of unspliced TAT4 RNA. This suggests that the observed splicing product corresponds to an intermediate lariat. Surprisingly, the reverse transcription was almost completely terminated at a cytidine, located 47 nucleotides upstream from the 3'-splice site in TAT3 and TAT4 (Fig. 3A, lanes 1 and 2), whereas no termination was observed at this position in the control reaction (Fig. 3A, lane 3). Since reverse transcription generally is arrested one nucleotide 3' to a branched nucleotide, this strongly suggests that the sequence UACUUUC is recognized as the branch site and that the underlined U is branched to the 5'-end of the intron (Fig. 3B). In addition, weaker bands were observed at nucleotides more proximal to the 3'-splice site which may represent alternative branch site nucleotides.

Splicing of the HIV-1 Intron Leads to Accumulation of a 50 S Pre-spliceosome Complex Containing Mainly U1 and U2 snRNPs—The splicing process requires a stepwise assembly of the snRNPs and other auxiliary splicing factors on the pre-mRNA in a highly ordered fashion. The splicing complexes formed on PIPT7.A, TAT4 RNA, and chimerical constructs, when incubated in nuclear extract, were analyzed by native gel electrophoresis. As expected, PIPT7.A and TAT4P7.A, which both splice efficiently, formed pre-spliceosomes (complex A) and spliceosomes (complex B) very efficiently in the presence of ATP (Fig. 4A). In contrast, both TAT4 and PIPTAT formed only one complex, independent of ATP (Fig. 4A).

To investigate the content of these complexes in more detail, biotinylated mRNAs were incubated under splicing condition and fractionated on sucrose gradients. The PIPT7.A RNA sedimented as a 40 S peak, corresponding to the A complex, and a 60 S ATP-dependent peak, corresponding to the B complex. (The 40 S peak was visible only as a shoulder on the 40 S peak of the sucrose gradient profile shown in Fig. 4B due to excess mRNA in this type of preparative gradient.) In contrast, the TAT4 mRNA sedimented in a peak at around 50 S (Fig. 4B). This peak formed independently of ATP (data not shown). Specific splicing complexes were purified from individual fractions of the sucrose gradient by streptavidin affinity chromatography and tested for the content of snRNA by Northern blotting, probing with a mixture of antisense snRNAs (Fig. 4C). As expected, the 40 S complex of the PIPT7.A construct contained mostly U1 snRNP and some U2 snRNA, and the 60 S
snRNAs were electrophoresed on an 8% polyacrylamide-8M urea gel. Recovered complexes were denatured and the eluted binding of snRNA to streptavidin beads and derived from fractions unbiotinylated pre-mRNA (these fractions is approximately 40 S to 60 S as indicated above (32). The affinity purified splicing complexes of the individual fractions shown in the Fig. 4. Northern blot analysis was performed on chimerical mRNA constructs (Fig. 6, A and B). The snRNP content of individual fractions of the TATPIP gradient looked very similar to that of PIP7.A except that the 40 S complex of the chimerical construct contained relatively more U2 snRNP (compare Fig. 4 C, left panel, and Fig. 6A). In contrast, incubation of PIPTAT pre-mRNA in nuclear extract produced a complex containing mainly U1 snRNP and relatively less U2 snRNP as compared to TAT4 (compare Fig. 4C, right panel, and Fig. 6B). This suggests that the 5'-half of the HIV-1 transcript does not only bind U1 snRNP, but also U2 snRNP. To analyze the complexes formed on a construct containing the HIV-1 5'-splice site region alone, Northern blot analysis was performed using TAT5'ss, which lacks the 3'-exon and most of the intron (Fig. 1). This construct does not produce any detectable splicing products when incubated in nuclear extract (TAT5'ss, Fig. 2A). An ATP-independent peak, sedimenting at around 40 S, was detected (results not shown), and Northern blot analysis of this peak revealed the presence of U1 and U2 snRNPs (Fig. 6C). Considering the differences in length of the transcripts, this 40 S peak may correspond to the 50 S peak observed for the TAT4 transcript, suggesting that the 3'-splice site region of TAT4 is dispensable for stable U1 and U2 snRNP interactions with the HIV-1 transcript.
Splicing of HIV-1 Transcripts

Balanced expression of differentially spliced mRNAs is an evolutionary conserved feature among retroviruses. Studies of two distantly related retroviruses, Rous sarcoma virus and HIV-1, have revealed that the mechanisms controlling this balance are strikingly similar. Both viruses apparently use a combination of suboptimal 3'-splice site signals (4-7, 29) (this report) and cis-acting negative regulators of splicing (8, 9, 28). The elements controlling the splicing of the second intron in the HIV-1 Tat/Rev transcript have been investigated previously in vivo (29). In agreement with that report, we found that the 3'-splice site region of the HIV-1 Tat intron contains inefficient splicing signals for in vitro splicing, whereas the 5'-splice site was highly efficiently spliced to a heterologous 3'-splice site. Our analysis also suggests that the inefficiency of splicing is not controlled by cis-acting repressive sequences in the downstream exon, as observed for the 3'-splice site of the first Tat intron (28).

A mammalian 3'-splice site consensus is composed of a highly conserved AG immediately upstream of the splice site, a continuous stretch of 7 or more pyrimidines (preferably uridines), just upstream from the AG nucleotides, and a branch point sequence YNYURAY (Y = pyrimidine, R = purine, N = any nucleotide), in which the highly conserved A is used as the branch point. During splicing the branch point sequence base pairs with GUAGUA in U2 snRNP, bulging out the branch point adenosine from the helix (reviewed in Ref. 1). Analysis of cellular genes in general shows that a long U-rich polyuridimidine tract can compensate for a poor branch site, and vice versa. However, inspection of the 3'-splice site of the second Tat/Rev intron reveals a very irregular polyuridimidine tract and no obvious branch point candidate. Improvement of the polyuridimidine tract, or introduction of a consensus branch site 29–35 nucleotides upstream from the 3'-splice site, increased the splicing efficiency in vivo, suggesting that both elements play an important role in maintaining a suboptimal 3'-splice site (29).

Surprisingly, we found that the uridine, underlined within the sequence UACUUUC, functioned as the major branch site (29). Surprisingly, we found that the uridine, underlined within the sequence UACUUUC, functioned as the major branch site (29). Surprisingly, we found that the uridine, underlined within the sequence UACUUUC, functioned as the major branch site (29).

The downstream exon, as observed for the 3'-splice site consensus is composed of a highly conserved AG immediately upstream of the splice site, a continuous stretch of 7 or more pyrimidines (preferably uridines), just upstream from the AG nucleotides, and a branch point sequence YNYURAY (Y = pyrimidine, R = purine, N = any nucleotide), in which the highly conserved A is used as the branch point. During splicing the branch point sequence base pairs with GUAGUA in U2 snRNP, bulging out the branch point adenosine from the helix (reviewed in Ref. 1). Analysis of cellular genes in general shows that a long U-rich polyuridimidine tract can compensate for a poor branch site, and vice versa. However, inspection of the 3'-splice site of the second Tat/Rev intron reveals a very irregular polyuridimidine tract and no obvious branch point candidate. Improvement of the polyuridimidine tract, or introduction of a consensus branch site 29–35 nucleotides upstream from the 3'-splice site, increased the splicing efficiency in vivo, suggesting that both elements play an important role in maintaining a suboptimal 3'-splice site (29).

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auxiliary splicing factors will interact with the RNA. The RRE within the HIV-1 transcript probably also binds Rev protein at this stage. A logical reason for an inefficient second step of spliceosome assembly may be to accumulate high levels of nuclear unspliced HIV-1 mRNA partially assembled into spliceosomes, which are capable of interacting with Rev. Interestingly, several other lines of evidence suggest that Rev functions within the HIV-1 transcript probably also binds Rev protein at auxiliary splicing factors will interact with the RNA. The RRE within the HIV-1 transcript probably also binds Rev protein at this stage. A logical reason for an inefficient second step of spliceosome assembly may be to accumulate high levels of nuclear unspliced HIV-1 mRNA partially assembled into spliceosomes, which are capable of interacting with Rev. Interestingly, several other lines of evidence suggest that Rev functions within the HIV-1 transcript probably also binds Rev protein at

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