Overlapping Cis Sites Used for Splicing of HIV-1 env/nef and rev mRNAs*

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(Received for publication, August 19, 1998, and in revised form, October 8, 1998)

Alternative splicing is used to generate more than 30 human immunodeficiency virus type 1 (HIV-1) spliced and unspliced mRNAs from a single primary transcript. The abundance of HIV-1 mRNAs is determined by the efficiencies with which its different 5′ and 3′ splice sites are used. Three splice sites (A4c, A4a, and A4b) are upstream of the rev initiator AUG. RNAs spliced at A4c, A4a, and A4b are used as mRNAs for Rev. Another 3′ splice site (A5) is immediately downstream of the rev initiator. RNAs spliced at A5 are used as mRNAs for Env and Nef. In this report, primer extension analysis of splicing intermediates was used to show that there are eight branch points in this region, all of which map to adenosine residues. In addition, cis elements recognized by the cellular splicing machinery overlap; the two most 3′ branch points overlap with the AG dinucleotides at rev 3′ splice sites A4a and A4b. Competition of the overlapping cis sites for different splicing factors may play a role in maintaining the appropriate balance of mRNAs in HIV-1-infected cells. In support of this possibility, mutations at rev 3′ splice site A4b AG dinucleotide dramatically increased splicing of the env/nef 3′ splice site A5. This correlated with increased usage of the four most 3′ branch points, which include those within the rev 3′ splice site AG dinucleotides. Consistent with these results, analysis of a mutant in which three of the four env/nef branch points were inactivated indicated that use of splice site A5 was inhibited and splicing was shifted predominantly to the most 5′ rev 3′ splice site A4c with preferential use of the two most 5′ branch points. Our results suggest that spliceosomes formed at rev A4a-4b, rev A4c, and env/nef A5 3′ splice sites each recognize different subsets of the eight branch point sequences.

Splicing of mRNA precursors in the nucleus of metazoan cells occurs by the cleavage and joining of 5′ and 3′ splice sites. This is mediated by recognition of conserved cis-acting sequences near the two splice sites that removes the intron between these splice sites. The splicing process is catalyzed by the cellular splicing machinery within spliceosome complexes (for reviews, see Refs. 1–3). The assembly of spliceosomes involves an initial interaction with U1 snRNP at the 5′ splice site. The 3′ splice site is then recognized by factor U2AF binding to a greater than 12-nt polypyrimidine tract upstream of the AG dinucleotide, which borders the site of 3′ cleavage. Another cis element, the branch point sequence, is located 18–40 nt upstream of the AG dinucleotide with the loosely conserved consensus sequence YNYURAY (the underlined residue is the branch point). The branch point sequence is recognized by U2 snRNP, whose binding is facilitated by the protein U2AF. This step is followed by binding of U5 and U4/U6 snRNPs as a triple snRNP to form the complete spliceosome.

The splicing process for most cellular mRNA precursors is normally efficient. However, processing of some pre-mRNAs occurs by tissue-specific or developmentally regulated alternative splicing pathways, resulting in multiple mRNAs produced for a single pre-mRNA. This involves the use of alternative 5′ or 3′ splice sites, exon skipping, intron inclusion, and mutually exclusive exons. Such regulated splicing results in the production of different proteins from the same gene and is an important mechanism for expanding the repertoire of cellular gene expression (1).

HIV-1 uses the cellular splicing machinery to express its genes. Over 30 different HIV-1 mRNAs are derived by alternative splicing from a single RNA precursor. In addition, approximately half of the RNA transcripts remain unspliced. These primary transcripts are packaged into progeny virions and are used as mRNA for the gag and pol gene products. It has been shown that HIV-1 RNA splicing efficiency is primarily determined by the relative strengths of the various 3′ splice sites in the viral RNA (4–6). Several cis elements have been shown to affect HIV-1 3′ splice site usage. These include suboptimal splice sites (polypyrimidine tracts and branch point sequences) as well as enhancer and silencer elements mapping within the exons downstream of the splice sites (4–12).

The levels of tat, rev, nef, and env mRNAs in HIV-1-infected cells are determined in part by splicing. Several alternative 3′ splice sites are present in a 300-nt region in the middle of the HIV genome (splice sites A3, A4a, A4b, A4c, and A5; Fig. 1). In addition, a 5′ splice site (D4) downstream of these alternative 3′ splice sites is spliced to 3′ splice site A7 within the env coding sequence (Fig. 1). When both splicing events occur, multiply spliced mRNAs are created. tat mRNAs are spliced at site A3, whereas rev mRNAs are spliced at either A4a, A4b, or A4c. Most env and nef mRNAs are spliced at site A5; however, env mRNA remains single-spliced (13). Splicing between sites D4 and A7 creates a noncoding exon downstream of the nef reading frame. In addition to alternative splicing, the levels of single-spliced and unspliced RNA are also selectively regulated by the HIV-1 Rev protein (for a review see Ref. 14). This small basic protein binds to an RNA element in the env gene called the Rev-responsive element within the env gene and facilitates transport of the unspliced and single-spliced RNA from the nucleus to the cytoplasm.

We have previously shown that the efficiency of splicing at...
splicing, we identified branch points in the 80-nt region containing splice acceptors at each of these AGs. To distinguish these corresponding to different binding sites for U2 snRNP would be spliceosomes may form upstream of each AG. This model predicts that only one branch point sequence or one set of branch point sequences determined by competition between the AGs based on their position and sequence context (15, 16). This predicts that only one branch point sequence will be used by the single splicing machinery.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Plasmid pHIS1-X (9), which contains HIV-1 sequences derived from the infectious clone pNL4–3 (GenBank™ accession no. M19921), was used to construct plasmids to synthesize RNA substrates for splicing (Fig. 1). In order to facilitate cloning, plasmid pHIS1-ΔSac was constructed in which a Sac I site in the vector sequence of pHIS1-X was removed by blunt end ligation to create a unique Sac I site at nt 5999 of the pNL4–3 sequence. Mutant plasmids 4B-S, 4B-/5-, and 5-, which inactivate splice acceptors A4b and/or A5, were created by replacing the region between the ESS and AGs (13) and p4BSAM (17) were generously provided by D. Purcell (Macfarlane Burnet Center for Medical Research; Fairfield, Victoria, Australia) and J. Guatelli (University of California, San Diego), respectively. Plasmids 4B-G and 4B-P containing mutations AG

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**Fig. 1. Diagram of constructs and HIV-1 splice sites.** A, map of known 5′ (D) and 3′ (A) splice sites in the NL4–3 strain of HIV-1. Open reading frames of HIV-1-encoded proteins are shown. The locations of the RNA initiation site (Cap) and poly(A) site (pa) are shown. B, minigene construct pHS1-ΔSac contains the indicated regions of pNL4–3. The locations of 5′ and 3′ splice sites are shown by vertical arrows. The AUG initiation codons for Tat and Rev are also shown. The plasmid pHIS1-ΔSac was linearized with XhoI and used as template to synthesize RNA substrates for in vitro splicing assays. Location of the antisense primer PED133 used in the primer extension localization of branch points is indicated. C, sequence of the 80-nt region containing the rev and env/nef 3′ splice sites. Branch points determined by primer extension analysis carried out in Fig. 2C are shown. D, sequence of human β-globin construct pHS1-Δ6 showing the location of the branch point previously determined by structural analysis.
Plasmid pSP64-Hb\(\beta\)G6 containing human \(\beta\)-globin downstream of an SP6 RNA polymerase promoter has been previously described (18).

**RNA Substrate Synthesis—**In vitro run-off RNA transcripts labeled with \(\alpha\)-\[^{32}\]P\]UTP (Amersham Pharmacia Biotech) were carried out essentially as described (9). To prepare templates, pHS1-\(\Delta\)Sac and its derivatives were linearized with XhoI, and pSP64-Hb\(\Delta\)6 was linearized with BamHI. In vitro transcription reactions (20 \(\mu\)l) were carried out in a reaction mixture containing 40 mM Tris-\(\text{HCl}\) (pH 7.5), 6 mM MgCl\(_2\), 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 40 units of RNA-guard RNase inhibitor (Amersham Pharmacia Biotech), 500 \(\mu\)M rATP, 500 \(\mu\)M rGTP, 250 \(\mu\)M rUTP, 50 \(\mu\)M rCTP, 500 \(\mu\)M diguanosine triphosphate, 80 nCi of \(\alpha\)-\[^{32}\]P\]UTP, 40 units of T3 or SP6 RNA polymerase, and approximately 1 \(\mu\)g of DNA template.

**In Vitro Splicing—**Splicing reactions were carried out essentially as described previously (9). In brief, a 25-\(\mu\)l reaction mixture containing 60\% (v/v) nuclear extract in Dignam's buffer D (19), 20 mM creatine phosphate, 3 mM MgCl\(_2\), 0.8 mM ATP, and 2.6\% (v/v) polyvinyl alcohol was incubated with approximately 8 fmol of radiolabeled RNA substrate for 2 h at 30 °C. In order to increase the yield of branched lariat intermediate product for subsequent primer extension analysis, the splicing reaction was stopped at 1 h, and approximately 100 fmol of RNA substrate was used. Except where noted, RNA splicing products were electrophoresed on 6\% (w/v) polyacrylamide, 7 \(\mu\)m urea gels for 19 h at 300 V.

**Primer Extension—**Primer extension analysis was carried out essentially as described previously (20). Branched lariat intermediate and unspliced RNA bands were excised from the in vitro splicing gels and eluted in a solution containing 0.2\% (w/v) SDS and 0.3 \(\mu\)M NaOAc (pH 5.2) at 4 °C. For pHS1-\(\Delta\)Sac and derivatives, primer PED133 (\(\text{5'}-\text{ACCTGCTAATGCGTGGATATT-3'}\)) was used instead of primer PED133. Branched lariat-exon intermediates were isolated and a primer, complementary to the exon (PED 133) was used to extend a DNA product to the branch points using avian myeloblastosis virus reverse transcriptase. As a control for the specificity of the stops, we isolated linear RNA precursor, annealed it to the same primer, and used it as substrate for reverse transcriptase.

**Multiple Branch Points Are Present in the rev and env nef 3' Splice Site Region—**We first performed primer extension analysis to identify branch points in the 80-nt region containing the four rev and env 3' splice sites (A4a, A4b, A4c, and A5). The products of a splicing reaction of an RNA substrate transcribed from minigene template pHS1-\(\Delta\)Sac (see Fig. 1B) were separated on a denaturing polyacrylamide gel (Fig. 2A). Branched lariat-exon intermediates were isolated and a primer, complementary to the exon (PED 133) was used to extend a DNA product to the branch points using avian myeloblastosis virus reverse transcriptase. As a control for the specificity of the stops, we isolated linear RNA precursor, annealed it to the same primer, and used it as substrate for reverse transcriptase.
from the 3′ splice site. In the case of the HIV-1 substrate, specific stops corresponding to eight branch points were detected, and all of these branch points were mapped to adenosine residues (Figs. 1C and 2C). Examination of the sequences containing these branch points indicated that there was a correspondence to the mammalian consensus branch point sequence YNYURAC ranging from three out of seven to six out of seven nucleotides (Table I). Interestingly, the most 3′ branch point (branch point 8) mapped to the adenosine residue within the AG dinucleotide of the rev A4b 3′ splice site. In addition, a minor amount of branching occurred at the rev A4a 3′ splice site (branch point 7).

Mutations in the rev A4b 3′ Splice/Branch Point Site Enhance Splicing at the env/nef A5 3′ Splice Site and Increase Usage of Downstream Branch Points—The fact that branch points 7 and 8 overlapped with the rev AG dinucleotides strongly suggested that these branch points were used in splicing at the env/nef 3′ splice site A5. To further test this possibility, we correlated the use of the different branch points with the use of specific splice sites. It has been previously reported that certain mutations of the rev A4b AG dinucleotide cause a significant inhibition of virus replication and reduced usage of tat and the remaining rev 3′ splice sites (17). We tested four different mutant substrates: AG ↓ G → GG ↓ G (mutant 4B-G), AG ↓ G → AC ↓ G (mutant 4B-S), AG ↓ G → GA ↓ G (mutant 4B-P), and AG ↓ G → CG ↓ C (mutant 4B-W). Fig. 3 shows that, as expected, splicing at 3′ splice site A4b was blocked with all mutant substrates. Splicing at 3′ splice site A5 of substrates with AG to GG, AC, and CG mutations was dramatically increased compared with wild type. On the other hand, splicing of the AG to GA mutant substrate was elevated only slightly compared with wild type. In each case, there was a reduction in splicing at the rev A4a 3′ splice site; this was most pronounced for the 4B-S substrate. Concomitant with the increase in splicing at A5 there was a shift in the distribution of lariats and lariat-exon intermediates to the slowest migrating bands (Fig. 3). This shift occurred to a lesser extent with the AG to GA mutant. Since RNA intermediates containing larger lariats would be expected to migrate more slowly on such gels, this result suggested that the branch points used for splicing at the env/nef 3′ splice site A5 are farther downstream than those that are used for splicing at the rev 3′ splice sites A4a, A4b, and A4c.

To determine the locations of the branch points used in splicing of the rev A4b mutants, we carried out primer extension analysis of the mutant lariat-exon intermediates. It can be seen in Fig. 4 that enhanced splicing at the A5 3′ splice site was correlated with a relative increase in lariat-exon intermediate products of the four most 3′ branch point sites (branch points 5–8) in substrate 4B-S and three of the four distal branch point sites (branch points 5–7) in substrates 4B-G and 4B-W. The 4B-G and 4B-W substrates, in which the AG dinucleotide was mutated to GG and CG, respectively, did not branch at the mutated A4b 3′ splice site (branch point 8). Branching did occur at this site with mutant substrate 4B-S, in which the AG dinucleotide was mutated to AC. Thus, the presence of the AG adenosine is necessary for branch point formation at the A4b 3′ splice site. The increase in the use of branch points 5–8 relative

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**Table I**

Branch point sequences in the region of HIV-1 rev and env/nef 3′ splice sites

| Branch point | Sequence* | Consensus matchb |
|--------------|-----------|------------------|
| Consensus    | nt-YNYURAC-nt | YNYURAC (7/7) |
| 1            | 5901-UUGCUAU-5907 | YN–A– (3/7) |
| 2            | 5907-UUGCUAA-5913 | YN–URA– (5/7) |
| 3            | 5922-CUUCUAU-5928 | YNY–A– (5/7) |
| 4            | 5928-CUUCUAU-5934 | YN–A– (3/7) |
| 5            | 5939-GUUUCAU-5945 | NYU–A– (4/7) |
| 6            | 5942-UCUACUG-5948 | YN–URAC (6/7) |
| 7            | 5947-ACAAGG-5953 | N–RA– (3/7) |
| 8            | 5953-GCUAGG-5959 | NYU–A– (4/7) |

* Nucleotide numbers (nt) in NL4–3 sequence are shown on the left and right.

* Y, pyrimidine; N, any nucleotide; R, purine; numbers of matches are shown in parentheses.

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**Fig. 3.** Mutations within the rev A4b 3′ splice site (branch point 8) enhances splicing at the env 3′ splice site A5. Substrates with the rev A4b mutations (AG ↓ G → GG ↓ G (mutant 4B-G), AG ↓ G → AC ↓ G (mutant 4B-S), AG ↓ G → GA ↓ G (mutant 4B-P), and AG ↓ G → CG ↓ C (mutant 4B-W)) were spliced in vitro. Products of the reaction were analyzed by gel electrophoresis as described under "Experimental Procedures." The positions of splicing precursors, intermediates, and products are shown on the left.
to the use of branch points 1–4 correlated with increased use of 3' splice site A5, suggesting that these branch points are used for splicing at this site. On the other hand, with the 4B-P mutant in which the AG dinucleotide was mutated to GA, the shift to splicing at A5 was less pronounced (see Fig. 3), and there was a more uniform usage of all eight branch points. The strong correlation that existed between the enhanced splicing to the A5 splice site of the A4b mutants and the increased use of downstream branch points in the lariat-exon intermediates suggested that the four most 3' branch points were used for splicing to A5.

**Mutation of env A5 Branch Points Shifts Splicing to the rev 3' Splice Site A4c**—To further confirm the role of the branch points identified above in A5 splicing, we used substrate EB124 in which the candidate branch points for A5 were mutated from adenosine to other nucleotides. EB124 combines the mutation present in substrate 4B-G (with the A4b and branch point 8 mutation AG → GA) with mutations of the remaining two major A5 branch points (branch points 5 and 6). The minor branch point 7 within the A4a AG dinucleotide was not changed. If these three branch points were necessary for splicing to the A5 3' splice site, we expected that the splicing pattern of substrate EB124 should resemble that of 4B-S substrate. Interestingly, mutant substrates EB124 and 4B-S-5' both showed an increased amount of product spliced at the rev splice site A4c compared with wild type. As shown in Fig. 5B for EB124, this correlated with a relative increase in the usage of branch points 1 and 2. This result suggested that these branch points may be used preferentially for splicing at A4c. There was also a residual amount of splicing at 3' splice site A5 (Fig. 5A), and this was correlated with a small amount of branching at the rev A4a 3' splice site (branch point 7), which remains intact in EB124 (Fig. 5B). The shift to increased splicing at A4c required mutations of both the A4b AG dinucleotide and either the A5 AG dinucleotide or the A5 branch points. Mutation of the A5 AG dinucleotide alone (mutant 5') did not show the preferential use of the A4c 3' splice site. The AG to GG mutation at A4b caused the expected increase in splicing at A5 (Fig. 5A).

**DISCUSSION**

In this study, we showed that different sets of branch points are used for splicing at the different HIV-1 rev and env/nef 3' splice sites. This implies that separate spliceosome complexes used for splicing at the rev and the env/nef mRNAs form upstream of the different AG dinucleotides. It further implies that there are a number of different binding sites for U2 snRNPs. From the locations of the branch points, we can deduce the locations of the polypyrimidine tracts used for assembling spliceosomes at the different 3' splice sites (Fig. 6). The polypyrimidine tract is positioned downstream of the branch points used for splicing at a given site. Thus, the polypyrimidine tract used for the env/nef 3' splice site A5 at nt 5975 is almost certainly the sequence spanning nt 5963–5970 (5'UCUCCUAU-3'). Consistent with this, branch points 5–8, which are used for splicing at site A5, are all upstream of this sequence. A second polypyrimidine tract located between nt 5922 and 5929 (5'UUGCUUUC-3') is upstream of branch sites.
points 3 and 4 but downstream of branch points 1 and 2. Thus, branch points 1 and 2 may be used for splicing at rev 3' splice site A4c. This is in agreement with the data shown in Fig. 5, where an increase in product spliced at site A4c correlated with the increased use of branch points 1 and 2. It is likely that the rev 3' splice sites A4a and A4b use a common set of branch points, since mutation of the A4a AG in the context of HIV-1 infection has been shown to result in a compensatory increase in splicing at the A4b splice site but with little or no change in splicing at A5 (17). It follows that a likely polypyrimidine tract for rev 3' splice sites A4a and A4b is the sequence from nt 5936 to 5944 (5'-UUUGUUUC-3'), which is downstream of branch points.

Fig. 5. Mutagenesis of env/nerf branch points 5, 6, and 8 shifts splicing to the rev A4c 3' splice site. A, in vitro splicing assays of substrate EB124 with mutations in three of the four A5 branch points. Also shown are the products of mutants in the A5 AG dinucleotide (5-) and the A5 plus A4b AG dinucleotide (5/-4b-). B, primer extension analysis of branched lariat-exon intermediates and substrate derived from EB124.

Fig. 6. Model showing locations of splicing elements in the 80-nt region containing the rev and env/nerf 3' splice sites. Branch points are grouped and labeled with the appropriate splice sites. rev branch points are circled; env branch points are circled and shaded; 3' splice sites are boxed. Polypyrimidine tracts predicted to be used for spliceosome assembly at each of the sites are indicated.
points 1–4 but upstream of branch points 5–8. Interestingly, all of these potential polypuridine tracts are shorter than the eukaryotic consensus (greater than 12 pyrimidines) and have interspersed purine residues. Thus, as has been shown with other HIV 3′ splice sites, the polypuridine tracts of the rev and env/nef splice sites also appear to be suboptimal (4–6).

We also showed in this study that, although most of the branch point sequences differ significantly from the metazoan consensus sequence, all of the branch points in the region of the HIV-1 rev and env/nef 3′ splice sites map to adenosine residues. Previous studies of several other HIV-1 branch points showed that nucleotides other than adenosine residues are used. Dyhr-Mikkelsen and Kjems found that the major branch point for the tat/nef splice site maps to a uridine residue within the sequence 5′-UACUUUC-3′ (11). Damier et al. mapped the branch point for the tat/nef 3′ splice site A7 maps to a uridine residue within the sequence 5′-UAGCAGA-3′ (12). Our results show that the use of nonadenosine branch points is not universal for all HIV-1 3′ splice sites. Interestingly, the adenosine residues within the AG dinucleotides of the rev 3′ splice sites A4a and A4b also both serve as branch points for splicing at env/nef 3′ splice site A5. To our knowledge, there is no precedent for overlap of these two essential cis elements within two adjacent alternative 3′ splice sites. We also showed that each of the HIV-1 splice sites appears to use multiple branch points. This has previously been shown for a number of other alternatively spliced viral and cellular mRNAs (22–25).

We showed above that substrates with different mutations of the rev A4b AG dinucleotide behave differently. Mutations in which the AG was changed to GG, AC, or CG resulted in dramatic increases in splicing at the env/nef 3′ splice site A5, whereas a mutation in which the AG was changed to GA does not show this increase. Interestingly, these differences in splicing can be correlated with previous results in which the effects of these mutations were studied in the context of HIV-1 infection. Two of these mutants (AG to GG and AG to CG) are Rev-deficient and replication-defective (17, 26). In the case of the AG to GG mutant, this was shown to involve a relative decrease in levels of mRNAs spliced at the remaining rev 3′ splice sites as well as the tat 3′ splice site A3 (17). In contrast, the AG to GA mutant has wild type Rev function and is replication-competent (13). Our results suggest that the Rev-deficient HIV-1 phenotypes may be caused by an unbalanced splicing of the viral RNA, resulting in an increase in splicing at the env/nef 3′ splice site A5 and a consequent failure to splice efficiently at the remaining rev 3′ splice sites. In the in vitro system, the 3′ splice site A4b mutations do not exhibit the same inhibitory effect on rev and tat splicing as they do in the context of virus infection. We do not yet understand this difference in the effects in the two systems. One possibility is that in infected cells the alternative 3′ splice sites may compete for limiting splicing factors. An increase in splicing efficiency at the env/nef 3′ splice site may cause a diminution in splicing at the tat and rev 3′ splice sites. In the in vitro splicing system, these factors may not be limiting. Further studies comparing splicing in vivo and in vitro will be necessary to resolve this issue.

Our results suggest that the rev A4b 3′ splice site AG dinucleotide contributes to the maintenance of balanced HIV-1 RNA splicing by inhibiting splicing at the env/nef 3′ splice site A5. The only A4b mutation tested that blocks splicing but fails to significantly increase splicing at 3′ splice site A5 is AG G to GA G. In both this mutant and the AG G to AC G mutant, env/nef branch point 8 adenosine was used (see Fig. 4), indicating that the ability to branch within this AG dinucleotide is not correlated with repression of splicing at 3′ splice site A5. Furthermore, the use of branch point 8 was abolished by the AG G to GG G and AG G to GC G mutations, yet the inhibition of splicing at 3′ splice site A5 was relieved. Interestingly, the AG G to GA G mutation is unique in that a new AG dinucleotide is created one nucleotide downstream. Several different protein factors that interact with the AG dinucleotide both early and late in spliceosome assembly have been identified by UV cross-linking experiments (27, 28). Thus, it is possible that factors bound at or near the rev A4b 3′ splice site may interfere with assembly of spliceosomes, leading to splicing at env/nef 3′ splice site A5. Alternatively, the three A4b AG mutations that activate 3′ splice site A5 splicing may elicit a change in the RNA secondary structure that allows more efficient formation of spliceosomes at the A5 splice site.

Acknowledgments—We thank Stanley Perlman and Mark Stinski for critical comments and suggestions on the manuscript.

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