and tunnel junction devices that could withstand more rigorous chemical and temperature treatments. IETS also shows great promise in the study of corrosion and in chemical sensing because of its extreme sensitivity.

Finally, we wish to mention a fascinating microscopy based on tunneling which was recently described by Bining *et al.* (*21*). Scanning tunneling microscopy is capable of showing three-dimensional surface topography with a resolution orders of magnitude better than that of scanning electron microscopy. This microscopy is based on elastic tunneling. It is possible, however, that the technique could be extended to IETS.

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22. The work described in this article was carried out by the Jet Propulsion Laboratory, California Institute of Technology, and was sponsored under contract DE-IA01-82ER13007 by the Office of Basic Energy Sciences, Engineering Research Programs, U.S. Department of Energy, through an agreement with the National Aeronautics and Space Administration.

RESEARCH ARTICLE

RNA Splice Site Selection: Evidence for a 5′→3′ Scanning Model

Katharine M. Lang and Richard A. Spritz

Abstract. *Human G_γ-globin genes containing tandem duplications of the donor (5′) or acceptor (3′) RNA splice sites of the second intervening sequence were constructed in order to ascertain the directionality of RNA splice site selection. These genes were introduced into cultured monkey cells, and their transcripts were analyzed. Transcripts of these duplication variants were spliced only at the proximal copy of the duplicated splice sites. These data are consistent with a 5′→3′ model of splice site selection.*

Intervening sequences (IVS) are a feature of a large number of eukaryotic genes (*1*). These sequences are included in the initial nuclear RNA transcripts, but are excised during maturation into cytoplasmic RNA, a process referred to schematically as splicing. RNA splicing is a precise process with highly accurate recognition of appropriate coding-intervening sequence boundaries. However, relatively little specific sequence information appears required to define a splice site (*2, 3*), and the factors that influence the selection of appropriate splice sites are largely unknown. Many messenger RNA (mRNA) precursor molecules are complex, with multiple intervening sequences and large numbers of potential splice sites. Therefore, it is likely that selection of splice junctions is a highly ordered process.

Several models for the appropriate selection of splice sites have been proposed (*4*), the simplest of which is that splice site selection occurs by a directional "scanning" process. In such a model the recognition of specific sequences on a nucleic acid macromolecule by a protein is facilitated by "linear diffusion," the "sliding" of the protein along the length of the nucleic acid chain (*5*). This type of process probably mediates the recognition of the *Escherichia coli* lac operator sequence by the repressor protein (*6*). A lateral diffusion process might either begin at one end of a linear macromolecule or it might initiate internally. If internal, the initial binding of protein and nucleic acid might either be site-specific (that is, mediated by a defined sequence or structure) or nonspecific (random). Futhermore, a scanning process of splice site recognition and IVS excision might either be processive (the splicing protein remaining on the RNA after a splice event and sliding to the next available splice sites) or distributive (the protein dissociating from the mRNA precursor after each splice event and reassociating at random with the same or another molecule). Therefore, a directional component to the splice site selection process does not imply that excision of intervening sequences from a multi-IVS precursor occurs in an obligate or even preferred order. In addition, a directional process of splice site selection could be complicated by a number of modifying factors. These types of models describe an essentially passive process of splice site selection. In some biological systems apparent alternative pathways of RNA splicing and transcript termination or polyadenylation (*7, 8*) might reflect some form of active regulation that modifies or even supersedes any passive mechanisms of splice site selection.

To determine the role of directionality in selection among potential splice junctions, we used the second IVS (IVS-2) of the human G_γ-globin gene as a test system since there is no evidence that alternative splicing of G_γ-globin transcripts ever occurs in normal individuals. Variant G_γ-globin genes containing small tandem duplications which included either the donor (5′) or acceptor (3′) splice sites of IVS-2 were constructed and introduced into cultured mammalian cells with the use of prokaryote-eukaryote "shuttle vectors" capable of replication and expression of cloned eukaryotic genes in monkey cells (*9*). Transcripts of the normal human G_γ-globin gene are appropriately processed in this system.

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Because the duplicated splice sites and their surrounding regions have identical sequences, analysis of spliced transcripts from the G_γ-globin duplication variants could provide information regarding the directionality of splice site utilization. The structures of the spliced transcripts of these duplication variants were consistent with a 5′→3′ directional process of splice site selection.

SV40 plasmids replicate and express the human G_γ-globin gene in cultured monkey cells. Plasmids containing an SV40 origin of replication undergo efficient extrachromosomal replication in COS cells (*9, 10*), a monkey kidney cell line transformed by an origin-defective mutant of SV40 (*11*), although transcription may depend on the presence of certain *cis*-acting "enhancer sequences" (*9, 12*). We constructed a similar plasmid, pSVd, containing portions of SV40 *dl*2005 (*13*) and pBRd (*9*) (Fig. 1A). In order to construct the γ-globin plasmid pSVγ, we inserted into the Cla I site of pSVd a 3.1-kb Msp I fragment of the human G_γ-globin gene extending from 51 bases 5′ to the cap site of G_γ-globin mRNA to 1483 bases 3′ to the polyadenylation site (*14*). Although the TATA (T, thymine; A, adenine) box (*15*) is included in this G_γ-globin segment, the CCAAT (C, cytosine) box (*15*) is not; therefore, we expected that transcription of the G_γ-globin gene would initiate at SV40 late mRNA 5′ termini. The G_γ-globin duplication variant plasmids pSVγ5′ and pSVγ3′ also contained tandem duplication of an 85-base Bam HI–Sau 3A or a 181-base Sac I fragment containing the donor or acceptor splice sites of IVS-2, respectively (Fig. 1B). Limited DNA sequence analyses (*16*) confirmed the presence and stability of these duplications.

COS cells were transfected (*17*) with 2 μg of pSVγ or pdγ per 100-mm plate; pdγ is similar to pSVγ but lacks an SV40 replication origin. After 48 hours, low molecular weight DNA (*18*) or total RNA (*19, 20*) was extracted from the transfected cells. Southern blotting analysis (*21*) demonstrated that most of the recovered pSVγ DNA was sensitive to cleavage by both Sau 3A and Mbo I (not shown), indicating a loss of adenine methylation from the plasmid by replication in COS cells (*9, 22*). In contrast, recovered pdγ DNA was cleaved by Sau 3A but not by Mbo I (not shown), confirming that plasmid replication in COS cells is dependent on the SV40 replication origin.

Blot hybridization analysis (*23*) of total RNA from COS cells transfected with pSVγ demonstrated a single diffuse band of approximately 960 bases with maximal hybridization 48 to 72 hours after transfection when a probe specific for the coding strand of G_γ-globin RNA was used (not shown). The size of this globin RNA band suggests that globin gene transcription does initiate in the late region of SV40, and that the SV40-globin hybrid RNA has been appropriately processed.

Transcripts of pSVγ have SV40 5′ termini and are appropriately processed. To map the 5′ termini of pSVγ transcripts we carried out S1 nuclease analysis (*24, 25*) using a 5′ end-labeled 330-base Sph I–Bgl I fragment of pSVγ.

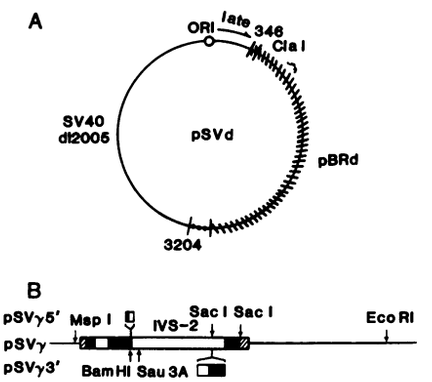


Fig. 1. Structures of SV40–G_γ-globin recombinants. (A) Vector pSVd. The solid line represents SV40 sequences, the hatched line plasmid sequences, and the dotted line adaptor sequences. (B) Structures of the G_γ-globin portions of pSVγ, pSVγ5′, and pSVγ3′. Restriction endonuclease cleavage sites used to construct these plasmids are indicated.

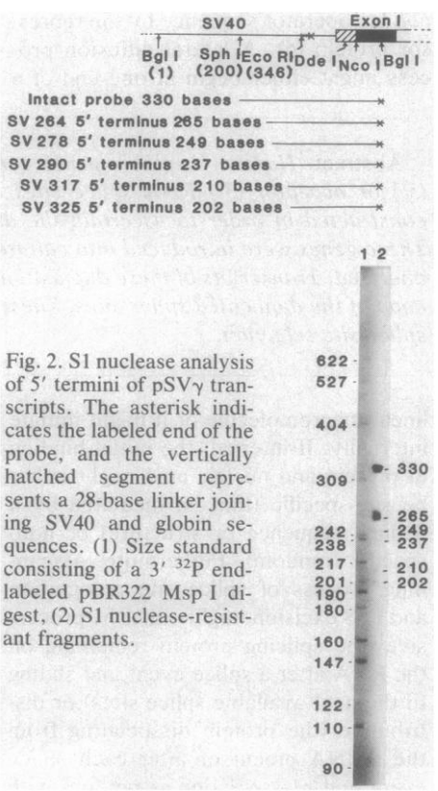


Fig. 2. S1 nuclease analysis of 5′ termini of pSVγ transcripts. The asterisk indicates the labeled end of the probe, and the vertically hatched segment represents a 28-base linker joining SV40 and globin sequences. (1) Size standard consisting of a 3′ ^{32}P end-labeled pBR322 Msp I digest, (2) S1 nuclease-resistant fragments.

Total polyadenylated RNA (*26*) protected a major product of approximately 265 bases (Fig. 2), as well as four additional minor products of 249, 237, 210, and 202 bases, corresponding to apparent 5′ termini at SV40 nucleotides 264, 278, 290, 317, and 325, respectively (*27*). Primer extension analysis (*28*) with a 5′ end-labeled 33-base Dde I–Nco I primer derived from the G_γ-globin 5′ untranslated sequence identified these same 5′ termini, as well as an additional minor terminus at SV40 nucleotide 168 (not shown). This 5′ terminus would not be demonstrated by the Sph I–Bgl I probe described above, but was observed with a similar but longer 527-base 5′ end-labeled single-stranded Bgl I–Bgl I probe that extended into the early region of SV40 (not shown). Thus, the 960-base pSVγ transcripts described above actually consist of at least six discrete RNA species with 5′ termini corresponding to the known 5′ termini of SV40 late mRNA's (*8, 27*).

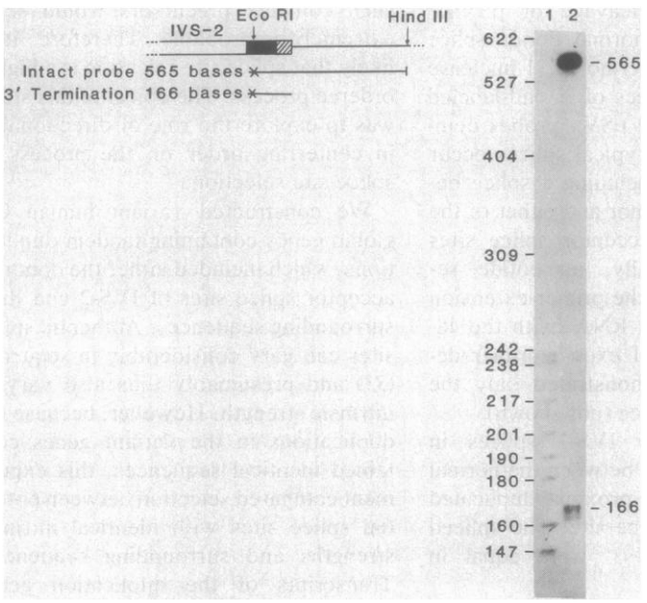
The site of polyadenylation of pSVγ RNA was determined by S1 nuclease analysis with a 3′ end-labeled 565-base Eco RI–Hind III G_γ-globin DNA probe. As shown in Fig. 3, pSVγ RNA protected a 166-base DNA segment, indicating that at least some pSVγ transcripts are polyadenylated at the same site as normal G_γ-globin mRNA (*29*).

Splicing of the G_γ-globin segment of pSVγ RNA was assessed by S1 nuclease mapping of all four coding-intervening sequence junctions. To assay splicing at the donor splice site of the first IVS (IVS-1), we used a 3′ end-labeled 551-base Eco RI–Bam HI probe derived from pSVγ. As shown in Fig. 4A, pSVγ RNA protected a 224-base segment of this probe, consistent with cleavage of pSVγ transcripts at the normal IVS-1 donor splice site. Similarly, pSVγ RNA protected a 207-base segment of a 5′ end-labeled 343-base Mbo II–Bam HI probe (Fig. 4B), consistent with cleavage of pSVγ transcripts at the normal acceptor splice site of IVS-1. Splicing at the donor splice site of IVS-2 was assessed with the use of a 3′ end-labeled 399-base Nco I–Xba I fragment of pSVγ. The pSVγ RNA protected a 203-base segment of this probe (Fig. 5), consistent with cleavage at the normal donor splice site of IVS-2. A 5′ end-labeled 399-base Hind III–Sfa NI DNA probe derived from pSVγ3′ yielded a 137-base protected fragment on S1 nuclease analysis (Fig. 6), consistent with cleavage of pSVγ transcripts at the normal acceptor splice site of IVS-2.

The overall size of the pSVγ transcripts was assessed by primer extension

analysis with a 5′ end-labeled 48-base Bst NI–Eco RI primer derived from exon 3 of the G_γ-globin gene. This yielded a major extension product of approximately 592 bases and a series of other minor extension products (not shown), corresponding to completely spliced transcripts with 5′ termini as described above. Thus, pSVγ globin transcripts have 5′ termini from the late region of SV40 and are appropriately processed at the normal G_γ-globin splice and polyadenylation sites in COS cells.

Transcripts of pSVγ5′ are spliced only at the 5′ copy of the duplicated IVS-2 donor splice sites. To assess the utilization of the duplicated IVS-2 donor splice sites in pSVγ5′ RNA, total polyadenylated RNA from COS cells transfected with pSVγ5′ was hybridized to the Nco I–Xba I IVS-2 donor splice site probe described above. As shown in Fig. 5, Sl nuclease digestion of the hybrids produced a 203-base protected fragment, identical to that protected by pSVγ RNA. Prolonged exposure of this autoradiograph failed to demonstrate an Sl-resistant fragment of 273 bases, which would be protected by pSVγ5′ transcripts cleaved at the distal (3′) copy of the duplicated IVS-2 splice sites. When the 399-base Hind III–Sfa NI probe described above was used to map the acceptor splice site of IVS-2, pSVγ5′ RNA protected a 137-base fragment (not shown), identical to that protected by pSVγ RNA. Primer extension analysis of pSVγ5′ RNA, with the Bst NI–Eco RI exon-3 primer described above, demonstrated a series of extension products indistinguishable from those from pSVγ RNA (not shown). Thus, there are no apparent differences in the structures of the spliced transcripts of pSVγ and pSVγ5′, indicating that, within the limits of detection, all pSVγ5′ transcripts are spliced at the proximal (5′) copy of the duplicated IVS-2 donor splice sites. In general, the abundances of pSVγ and pSVγ5′ transcripts were similar, indicating that the apparent absence of pSVγ5′ transcripts spliced at the distal IVS-2 donor splice site does not result from their preferential degradation.



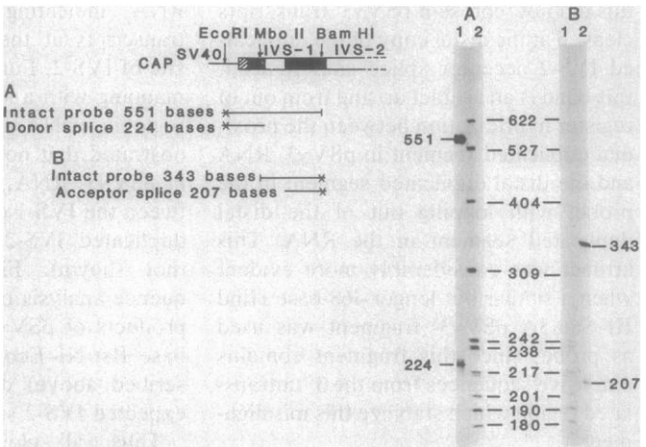
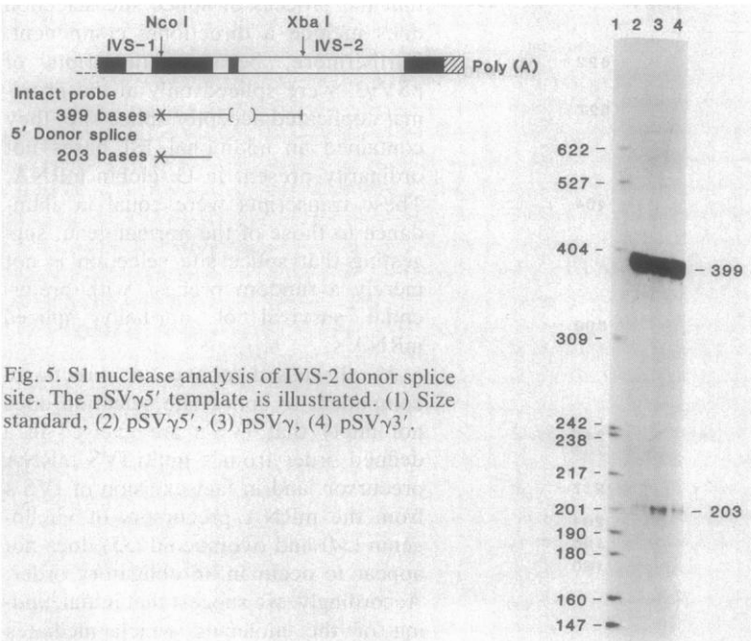


Fig. 3 (left). S1 nuclease analysis of 3′ terminus of pSVγ transcripts. (1) Size standard, (2) S1 nuclease-resistant fragments. Fig. 4 (right). S1 nuclease analyses of IVS-1 donor and acceptor splice sites in pSVγ transcripts. (A) Donor splice site analysis; (1) S1 nuclease-resistant fragments, (2) size standard. (B) Acceptor splice site analysis; (1) size standard, (2) S1 nuclease-resistant fragments.



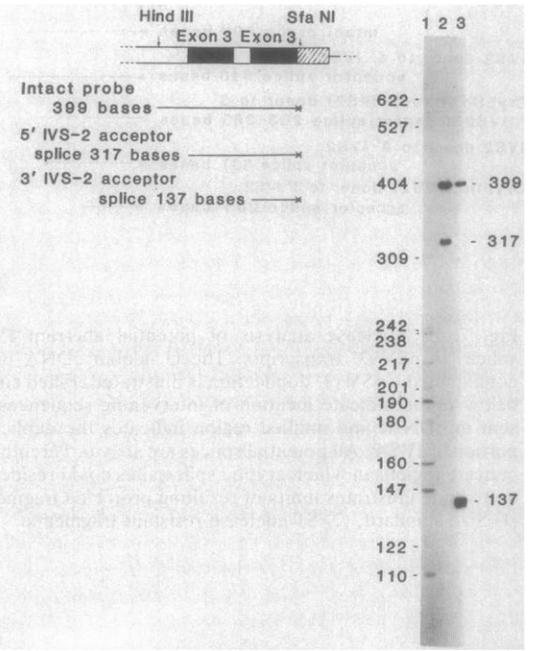


Fig. 5. S1 nuclease analysis of IVS-2 donor splice site. The pSVγ5′ template is illustrated. (1) Size standard, (2) pSVγ5′, (3) pSVγ, (4) pSVγ3′.

Transcripts of pSVγ3′ are spliced only at the 5′ copy of the duplicated IVS-2 acceptor splice sites. To assess the utilization of the duplicated IVS-2 acceptor splice sites in pSVγ3′ RNA, S1 nuclease mapping was performed with the Hind III–Sfa NI IVS-2 acceptor splice site probe described above. As shown in Fig. 6, this probe extended from within IVS-2 through the duplicated region and into the 3′ untranslated sequence. The pSVγ3′ RNA protected a 317-base segment of this probe, corresponding to cleavage of pSVγ3′ transcripts at the proximal copy of the tandemly duplicated IVS-2 acceptor splice sites. Although not easily seen in Fig. 6, pSVγ3′ RNA also protected a trace amount of the same 137-base fragment which pSVγ RNA protected (see above). However, this did not represent pSVγ3′ transcripts cleaved at the distal copy of the duplicated IVS-2 acceptor splice site. Instead, this band is an artifact arising from out of register hybridization between the proximal duplicated segment in pSVγ3′ RNA and the distal duplicated segment in the probe, with looping out of the distal duplicated segment in the RNA. This artifact was considerably more evident when a similar but longer 468-base Hind III–Sau 3A pSVγ3′ fragment was used as probe, since this fragment contains extensive sequences from the 3′ untranslated region which stabilize this misalignment.

To prove that the 137-base artifact band did not result from cleavage of pSVγ3′ transcripts at the distal duplicated IVS-2 acceptor splice site, S1 nuclease mapping was performed with a 3′ end-labeled 890-base Nco I–Msp I probe consisting of G_γ-globin (complementary DNA) (thus not containing IVS-2) containing the same duplication as pSVγ3′ (Fig. 7). This resulted in a single protected fragment of 610 bases, indicative of a splice between the IVS-2 donor and the proximal duplicated IVS-2 acceptor splice sites in pSVγ3′ RNA. No 337-base fragment that would result from a splice between the IVS-2 donor and the distal duplicated IVS-2 acceptor splice sites was observed. Furthermore, there was no evidence for splices utilizing cryptic splice sites within IVS-2 or exon 3. S1 nuclease mapping with the Nco I–Xba I IVS-2 donor site probe described above demonstrated a 203-base fragment (Fig. 5), identical to that protected by pSVγ RNA, indicating cleavage of pSVγ3′ transcripts at the normal donor splice site of IVS-2. Furthermore, S1 nuclease mapping with a series of 3′ end-labeled γ-globin cDNA and pSVγ probes demonstrated that no atypical splices occur in pSVγ3′ RNA, including a splice between the IVS-1 donor and either of the duplicated IVS-2 acceptor splice sites (not shown). Finally, nucleotide sequence analysis of the primer extension products of pSVγ3′ RNA (with the 48-base Bst NI–Eco RI exon-3 primer described above) demonstrated only the expected IVS-2 splice (not shown).

Thus, all globin IVS-2 splices in pSVγ3′ RNA occur between the normal IVS-2 donor and the proximal duplicated IVS-2 acceptor splice site. The spliced transcripts of pSVγ3′ were equal in abundance to those of pSVγ, both in nuclear and cytoplasmic RNA fractions (*30*) (not shown). Therefore, persistence of a portion of an intervening sequence in spliced transcripts does not lead to preferential degradation or defective compartmentalization of these transcripts.

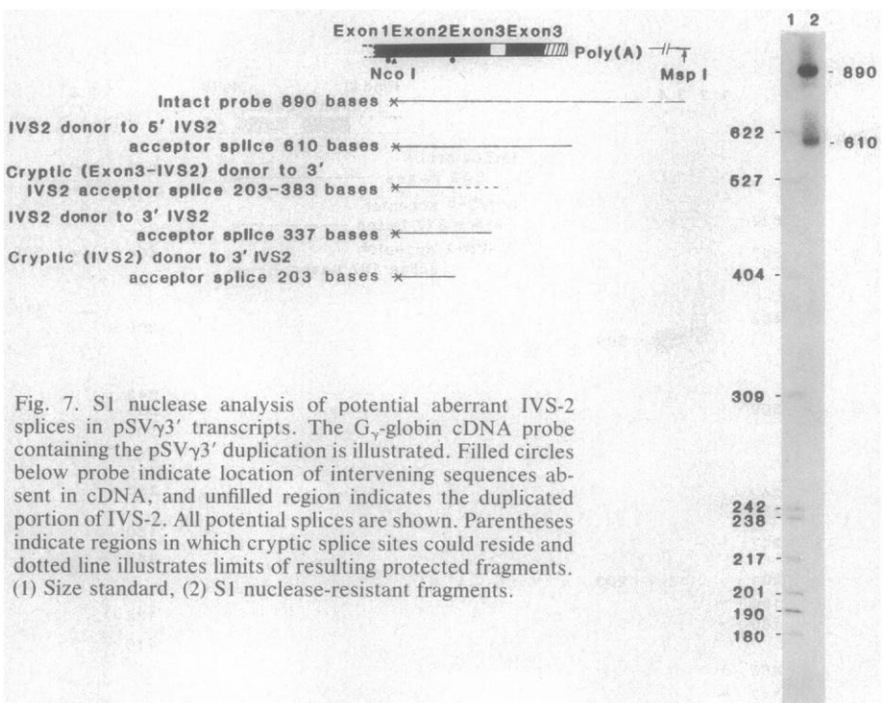


Fig. 7. S1 nuclease analysis of potential aberrant IVS-2 splices in pSVγ3′ transcripts. The G_γ-globin cDNA probe containing the pSVγ3′ duplication is illustrated. Filled circles below probe indicate location of intervening sequences absent in cDNA, and unfilled region indicates the duplicated portion of IVS-2. All potential splices are shown. Parentheses indicate regions in which cryptic splice sites could reside and dotted line illustrates limits of resulting protected fragments. (1) Size standard, (2) S1 nuclease-resistant fragments.

Interpretation

Some mRNA precursors, such as that encoding chicken α2 (type I) collagen (*31*), contain as many as 50 intervening sequences, and some appear to be processed through a series of partial stepwise splice events (*32*). If splice site selection were a stochastic process, the maturation of functional mRNA's from such complex precursors would seem extremely improbable. Therefore, it is likely that splice site selection is a highly ordered process. The object of this study was to explore the role of directionality in conferring order on the process of splice site selection.

We constructed variant human G_γ-globin genes containing tandem duplications, which included either the donor or acceptor splice sites of IVS-2 and their surrounding sequences. Authentic splice sites can vary considerably in sequence (*33*) and presumably thus also vary in intrinsic strength. However, because the duplications in the variant genes contained identical sequences, this experiment compared selection between potential splice sites with identical intrinsic strengths and surrounding sequences. Transcripts of the duplication genes were spliced only at the proximal copies of the duplicated splice sites, suggesting that the process of splice site selection does include a directional component. Furthermore, because transcripts of pSVγ3′ were spliced only at the proximal duplicated acceptor splice site, they contained an additional 181 bases not ordinarily present in G_γ-globin mRNA. These transcripts were equal in abundance to those of the normal gene, suggesting that splice site selection is not merely a random process with preferential survival of normally spliced mRNA's.

As discussed above, a directional component to splice site selection does not imply that IVS's are excised in a defined order from a multi-IVS mRNA precursor, and in fact excision of IVS's from the mRNA precursors of vitellogenin (*34*) and ovomucoid (*35*) does not appear to occur in an obligatory order. Accordingly, we suggest that initial binding of the molecule which mediates

splice site recognition to the mRNA precursor might be internal, followed by 5′→3′ lateral diffusion to the first available donor and acceptor splice sites. Whether this hypothetical recognition factor is actually an integral part of the splicing enzyme itself, whether binding is site-specific or nonspecific, and whether splicing is distributive or processive, cannot be determined from the available data.

Considerable biological evidence tends to support a 5′→3′ directional component to splice site selection. Two different human growth hormone (hGH) polypeptides differing in length by 15 amino acids apparently result from utilization of two alternative acceptor splice sites for IVS-B in hGH RNA (*36*). Since the shorter hGH polypeptide amounts to only 10 percent of total hGH (*37*), there is apparent preferential selection of the proximal alternative splice acceptor. Similarly, in the most common type of Mediterranean β^+-thalassemia a single base change in IVS-1 of the β-globin gene creates an alternative splice acceptor upstream from the normal acceptor of IVS-1 (*38*), with resultant preferential splicing at the novel alternative acceptor splice site (*39*). In one form of β^0-thalassemia, a mutation abolishes the donor splice site of the β-globin IVS-2 (*40, 41*), and a novel cryptic donor splice site within IVS-2 itself is utilized efficiently (*41*). However, several instances in which apparently acceptable splice sites are bypassed have also been described (*3, 41, 42*). Although it is difficult to reconcile all of the biological examples with any single model of splice site selection, many of these represent minor or aberrantly spliced RNA species in abnormal situations and may therefore be misleading.

In addition, any basically directional process of splice site selection is probably subject to modification by a number of factors. These might include variability of intrinsic splice site strength (depending on the specific sequence), masking of otherwise acceptable splice sites by secondary structure or the binding of exogenous molecules to the mRNA precursor, and inaccessibility of some potential splice sites due to the overall tertiary structure of the mRNA precursor molecule. In fact, local secondary structure has already been shown to affect the rate of processing of a eukaryotic transfer RNA precursor (*43*). A complete understanding of the process of splice site recognition and utilization will require the elucidation of the relationships among these and probably other features of the RNA splicing mechanism.

Note added in proof: In recent experiments, similar to those reported here, Kuhn *et al.* (*44*) have observed apparent preferential utilization of a distal duplicated acceptor splice site. We are attempting to clarify the basis for this discrepancy.

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45. We thank T. Maniatis, O. Smithies, D. Hamer, J. Mertz, and J. Ross for providing plasmids pSVOd, pG$_\gamma$R1.6 and pA$_\gamma$0.56, and p28c, COS-7 cells, and S1 nuclease, respectively, and J. Ross for critical evaluation of this manuscript. Supported by NIH grant AM28598 and Basil O'Connor Starter grant 5-341 from the March of Dimes Birth Defects Foundation. This is paper No. 2598 from the Laboratory of Genetics, University of Wisconsin.

24 March 1983