Control of Alternative Splicing by the Differential Binding of U1 Small Nuclear Ribonucleoprotein Particle

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Cellular factors controlling alternative splicing of precursor messenger RNA are largely unknown, even though this process plays a central role in specifying the diversity of proteins in the eukaryotic cell. For the identification of such factors, a segment of the rat preprotachykinin gene was used in which differential expression of neuropeptides γ and K is dependent on alternative splicing of the fourth exon (E4). Sequence variants of the three-exon segment, (E3-E4-E5) were created, resulting in a sensitive assay for factors mediating the splicing switch between E4-skipping and E4-inclusion. A dinucleotide mutation in the 5′ splice site of E4 that increases base-pairing of this site to U1 small nuclear RNA resulted in uniform selection of E4, whereas a control mutation that destroyed base-pairing resulted in uniform E4-skipping. Affinity selection of spliceosomes formed on these functionally distinct substrates revealed that the extreme difference in splicing was mediated by differential binding of the U1 small nuclear ribonucleoprotein particle (snRNP) to the 5′ splice site of E4. These data show that, apart from its established role in selecting 5′ splice sites, U1 snRNP plays a fundamental role in 3′ exon selection and provides insight into possible mechanisms of alternative splicing.

ALTERNATIVE SPLICING OF PRECURSOR MESSENGER RNA (pre-mRNA) is a naturally occurring in vivo mechanism for generating protein diversity and affecting gene control. Yet little is known about how the splicing machinery discriminates between alternative splice sites and normal sites that are used uniformly (*1*). In the basic splicing process, noncoding, intervening sequences (IVS's) are removed and exons are joined by two cleavage-ligation events. Essential to this process is the precise recognition of 5′ and 3′ splice sites, which occurs by assembly of a spliceosome complex involving interactions between the pre-mRNA and the small nuclear ribonucleoprotein particles (snRNP's) U1, U2, U5, and the particle containing both U4 and U6 small nuclear RNA's (*2–8*), as well as, other factors, (*9*). Because the spliceosome is the structure that specifies the splice sites to be joined, it is important to determine the role of the spliceosome in specifying alternative splicing mechanisms.

From biochemical and genetic studies in the selection of splice sites in eukaryotic pre-mRNA's (*10*), we now know that recognition of 5′ splice sites occurs by the binding of U1 snRNP as a result of direct base-pairing between nucleotides of the 5′ splice site and the 5′ end of U1 RNA (*11–14*). Recognition of 3′ splice sites appears to be more complex since binding of the protein U2AF (U2 auxiliary factor) to the polypyrimidine tract is a prerequisite for efficient binding of U2 snRNP at the branch site (*15*). Other proteins that bind 3′ splice site regions have been characterized in somewhat less detail and appear also to be important in recognition of that site (*16*). Subsequent to the binding of U2 snRNP, the complex containing U4, U5, and U6 RNA's binds to complete the assembly of the spliceosome (*7*).

Factors that influence selection of alternative splice sites include the strength of competing 5′ splice sites (*17*), unusually positioned or multiple branch sites (*18*), polypyrimidine tract sequences (*19*), and other sequences within exons and introns (*20*). We have now used site-directed mutagenesis to examine the role of the downstream 5′ splice site in governing exon selection. Affinity chromatography of splicing complexes demonstrates that the ubiquitous U1 snRNP can modulate alternative splicing because of its fundamental influence on 3′ exon selection.

Sequence elements controlling exon selection. To elucidate the biochemistry of factors controlling alternative splicing, we studied three-exon substrates derived from the rat preprotachykinin gene. This gene encodes substance P and related tachykinin peptides by alternative splicing of a common pre-mRNA (*21*). Of the three mRNA's produced from this gene, the most abundant, γ mRNA, is specified by alternative splicing, which joins exons 1, 2, 3, 5, 6, and 7, but removes exon 4 (E4) with its flanking introns, IVS3 and IVS4. Previously, we demonstrated that this is an interesting model system since selection of the optional exon, E4, is dependent on a downstream 5′ splice site (*22*). A particularly striking enhancement of E4 selection resulted from a dinucleotide mutation in the 5′ splice site of E4 that increased base complementarity to U1 RNA in a two-exon substrate containing E3 and E4.

We have pursued the question of how the dinucleotide mutation in the 5′ splice site of E4—(AUG:GUAAAC to AUG:GUAAGU; the colon indicates the exon-intron junction)—affects alternative splicing by constructing a three-exon substrate with the dinucleotide change (Fig. 1A, bottom). This structural variant, RP57, in which base complementarity to U1 RNA has been improved, results in the uniform selection of E4 (Fig. 1A, RP57). In contrast, the control variant RP58, in which this complementarity has been destroyed by a three-nucleotide mutation, (AUG:GUAAAC to AUG:CAAAUC), results in uniform E4-skipping (Fig. 1A, RP58). Analysis of the final products of reactions of Fig. 1A, which are more clearly resolved in less porous gels confirmed that the RP57 substrate produced only

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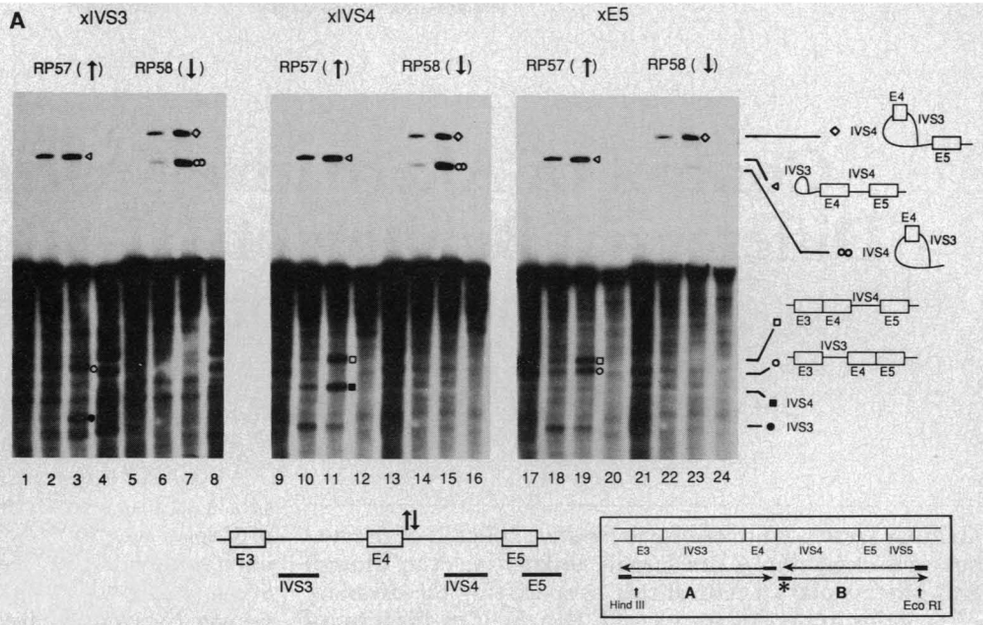


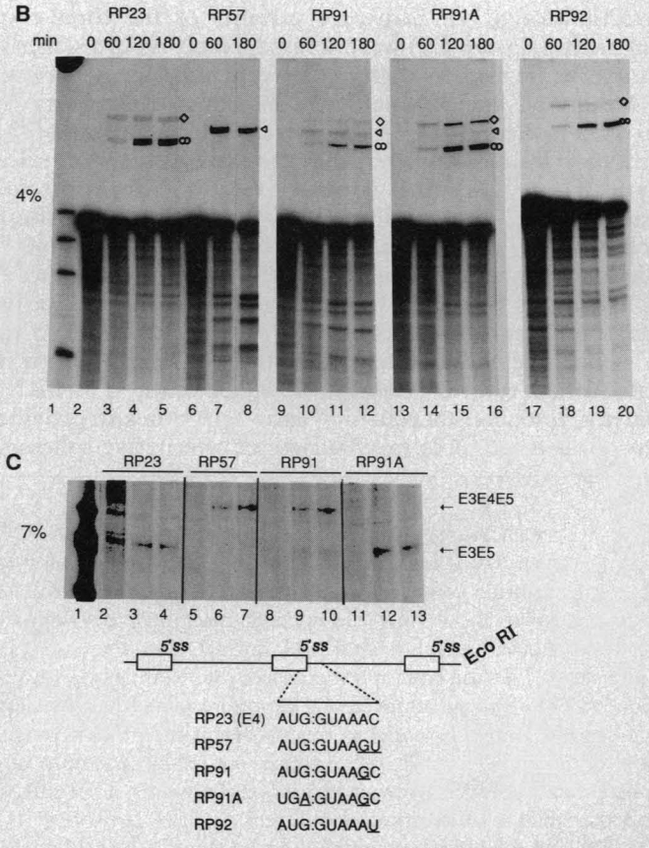
Fig. 1. Alternative splicing is modulated by base pairing to U1 RNA. Site-specific mutagenesis of the 5′ splice site of E4. (**Inset**) Substrates were constructed by ligation of fragments A and B (*37*), generated by polymerase chain reactions containing four oligonucleotides, one of which contained the mutation (asterisk). RNA substrates, 1200 nucleotides in length, were prepared (*22*) from Eco RI–cleaved DNAs and contained E3, 97 nucleotides (nt); IVS3, 438 nt; E4, 45 nt; IVS4, 464 nt; E5, 24 nt; and IVS5, 96 nt. (**A**) Northern (RNA) blot analysis, (4 percent polyacrylamide–7 M urea gel), of splicing reactions incubated for 0, 60, 120, and 120 minutes minus ATP, tested sequentially with IVS3, IVS4, and E5 DNA probes as indicated. (Lanes 1 to 4, 9 to 12, 17 to 20), substrate RP57; (lanes 5 to 8, 13 to 16, 21 to 24), substrate RP58. RNA splicing and Northern blotting was as described (*22*). (Bottom) arrow pointing up, RP57 mutation; down, RP58 mutation; bars, DNA probes (**B** and **C**) Single-nucleotide mutations. Splicing reactions containing ^{32}P-labeled pre-mRNA were prepared as in (A) and resolved on 4 percent (B) and 7 percent (C) polyacrylamide–7 M urea gels.

the E3E4E5 RNA product, whereas the RP58 substrate produced only the E3E5 RNA product (Fig. 1C, lanes 5 to 7) (*23*).

To unambiguously identify the lariat intermediates derived from substrates RP57 and RP58 (Fig. 1A), we performed a branch point analysis. In agreement with the assignments made in Fig. 1A, branch points were found upstream of E4 and E5 for substrate RP57, whereas only one branch point was found for substrate RP58, upstream of E5 (Fig. 2). Analysis of the E4 branch point revealed a striking increase in the normal branch point because of the dinucleotide mutation in the (downstream) 5′ splice site of E4 (Fig. 2A, lanes 9 and 10, substrate RP57; and lanes 2 and 3, substrate RP23 wild type, see below). The normal E4 branch point is found at the adenosine residue, 22 nucleotides upstream of the 3′ splice site (A-22). To a lesser extent, two novel branch points (A-17 and A-27), were activated by the dinucleotide mutation in RP57 (Fig. 2A, lanes 9 and 10). The E5 branch point, in contrast, was not significantly increased by the RP57 mutation (Fig. 2B).

Further analysis was performed to determine the contribution of individual nucleotide changes at IVS4 positions 5 and 6 of substrate RP57. The single-base change at IVS4 position 5, substrate RP91, results in an intermediate effect on E4 selection, whereas the single change at position six, substrate RP92, shows a product distribution essentially like that of the wild-type substrate, RP23 (Fig. 1, B and C). The characteristic pattern of splicing for the RP23 substrate is predominantly E4-skipping (*22*). Thus, the IVS4 mutation at position five gives the dominant effect, resulting in the enhanced selection of E4. Also tested was a substrate containing, in addition to the single-base change at IVS4 position five, a single-base insert at the exon-intron junction. This latter substrate, RP91A, gives a product distribution very much like that of the wild-type molecule (Fig. 1B, lanes 13 to 16); E4 selection is only slightly better than that of wild-type and is much reduced compared to the RP91 substrate, which contains only the change at position 5. Thus, it is the continuous stretch of base pairs and not simply the total number of base pairs with U1 RNA that governs the selection of E4 (Fig. 5).

Affinity selection of alternative spliceosomes. In that the extreme effects on alternative splicing of E4 shown above are due to nucleotide changes in the 5′ splice site of E4, it is possible that U1 snRNP could be the factor mediating these effects on splicing. To



test for the involvement of U1 snRNP, we used affinity selection of spliceosomes formed on the three-exon substrates RP57 and RP58, which give clear-cut differences in splicing (Fig. 3, schematic). Spliceosomes were resolved by velocity sedimentation on sucrose gradients and sedimented at 80*S* for both the RP57 and RP58 substrates because of the substantial length of these substrates, 1200 nucleotides (Fig. 3C) (*24*).

When biotinylated substrate RNA's were used, gradient fractions enriched in spliceosomes were affinity-selected on immobilized

streptavidin under high salt conditions, for example, 0.3 M KCl. This analysis showed an enrichment of U1 snRNP in the spliceosomes formed on the RP57 substrate, which uniformly selects E4, whereas only a background amount of U1 snRNP was detected for the RP58 substrate, which uniformly excludes E4 (Fig. 3, A and B, lanes 2 to 4). The other snRNP's, U2, U5, and the particle containing U4 and U6 snRNA's were present at similar levels in the RP57 and RP58 spliceosomes (*25*). Both end-labeling and Northern blot analysis indicated that U1 snRNP was present at essentially stoichiometric levels compared to U2 snRNP in the RP57 spliceosome (Fig. 3A, lane 2) (*26*). Affinity selection of the slower sedimenting RNP region (Fig. 3C), which contains the presplicing complex, shows that, while both substrates contain U2 snRNP, only substrate RP57 contains stably bound U1 snRNP (Fig. 3, A and B, lanes 5 to 7).

The differential U1 snRNP binding for the 5′ splice site variants, RP57 and RP58, shown by affinity selection (Fig. 3, A and B), is due to the unusually stable binding of U1 snRNP to the 5′ splice site of E4 in substrate RP57 and not to the additional 5′ splice sites of E3 and E5 present in the same substrates. Most compelling is the argument that substrate RP58 shows only a background level of U1 snRNP binding at 0.3 M KCl but is identical in structure to RP57, except for the nucleotide changes in the 5′ splice site of E4, which render this site nonfunctional. This observed difference in U1 snRNP binding is condition-dependent and is most likely explained by the release of U1 snRNP from the flanking 5′ splice sites of E3 and E5 at monovalent salt concentrations greater than or equal to 0.3 M (*27*). We attribute the unusually stable binding of U1 snRNP, in the case of substrate RP57, to the dinucleotide mutation which increases the number of contiguous base pairs that can be formed with U1 snRNA from five to nine (Fig. 5). In comparison, the 5′ splice sites of E3 and E5 can form only six contiguous base pairs with U1 RNA. In further support for the idea of differential

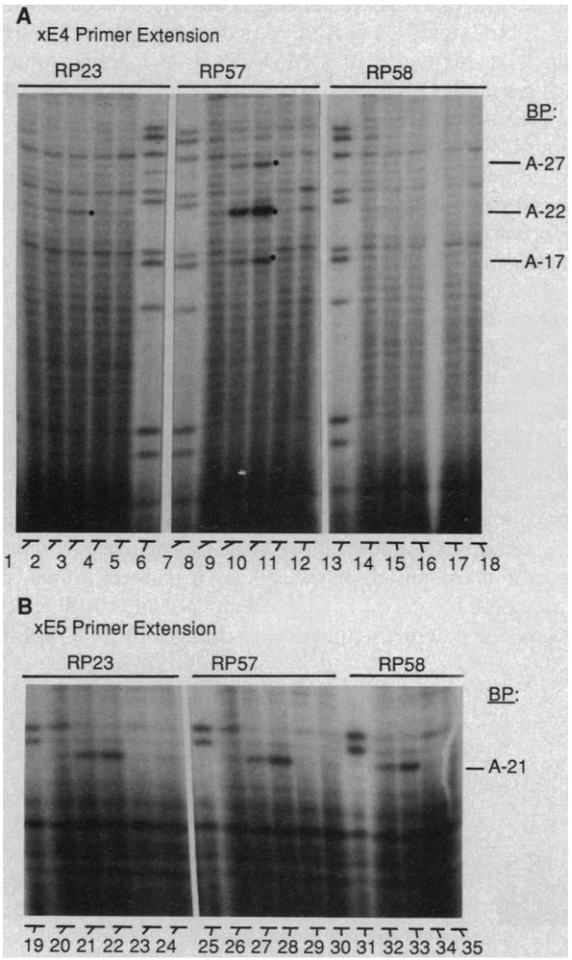


Fig. 2. Localization of branch points by primer extension analysis. Three-exon substrates, RP23, wild type, RP57, and RP58, were prepared by in vitro transcription with the use of unlabeled nucleoside triphosphates and spliced in a HeLa nuclear extract as in Fig. 1. Splicing reactions were 0 minute (lanes 1, 8, 14, 20, and 26), 60 minutes (lanes 2, 9, 15, 21, 27, and 32), 120 minutes (lanes 3, 10, 16, 22, 28, and 33). Control reactions were, 120 minutes without ATP (lanes 4, 11, 17, 23, 29, and 34), or 120 minutes with ATP followed by debranching (lanes 5, 12, 18, 24, 30, and 35). Debranching reactions were performed as described (*38*). Branch points were detected by primer extension of total RNA from splicing reactions with an E4-specific (lanes 1 to 18), or E5-specific (lanes 19 to 35) primer, ^{32}P-labeled at its 5′ end (*39*). Reaction products were separated by electrophoresis on 10 percent polyacrylamide–7M urea gels and then subjected to autoradiography. Sequencing ladders of plasmid DNA's containing dideoxythymidine triphosphate demarcate adenosine residues (lanes 6, 7, 13, 19, 25, and 31). Location of branch points (BP) are specified at nucleotide positions relative to the 3′ splice site (right). Reverse transcriptase stops 1 nucleotide downstream of the branch point residue.

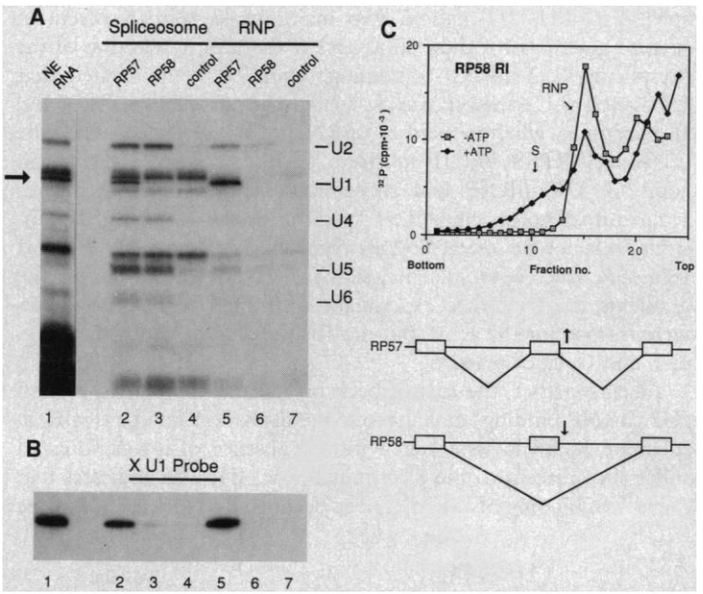


Fig. 3. Affinity selection of spliceosomes formed on three-exon substrates. Spliceosome (*S*) and RNP (RNP) complexes were first localized by resolving RNA species from sucrose gradients on denaturing polyacrylamide gels. The spliceosome peak contained the intermediates of splicing. Schematic of substrates and characteristic splicing pattern (bottom, right). (**A**) Analysis of snRNP components from affinity-selected splicing complexes of (C). Biotin and ^{32}P-labeled substrate RNA's were prepared as described (*8*). Two peak fractions from the spliceosome and RNP regions were adjusted to 0.3 M KCl and bound to streptavidin-biotin-cellulose columns prepared as described (*40*). Binding conditions were 2 hours, +4°C, in 1× binding buffer (*41*). RNA's were 3′ end-labeled as described (*8*); half of the eluted material was used. Labeled RNA's were resolved on a 10 percent polyacrylamide–7 M urea gel. The presence of 5.8*S* and 5*S* ribosomal RNA's, just above U1 RNA and U5 RNA, respectively, in lanes 2 to 4, is due to the presence of endogenous 80*S* ribosomes in the spliceosome region of the gradients. Ribosomes interact with the streptavidin column and serve as internal controls for the evenness of sample treatment. Lanes 2 to 7 contain equivalent amounts of sample loaded, except for lane 6, which contains one half that amount. RNA components eluted from the spliceosome, or RNP regions of gradients containing three-exon substrates (RP57), (RP58), or no substrate (control). RNA extracted from Hela nuclear extract (NE RNA) serves as marker for the small nuclear RNA's (right). (**B**) Half of each eluted sample from (A) was resolved on a 10 percent polyacrylamide–7 M urea gel, blotted onto GeneScreen membrane and probed with a labeled (5×10^6 cpm) U1 DNA probe (7). (**C**) Graph of ^{32}P-labeled complexes formed under splicing conditions, 45 minutes (+ATP), or under conditions that prevent splicing (−ATP). Because the gradient profiles of substrates RP57 and RP58 showed such similar patterns, only one is shown.

binding at high salt, affinity selection of splicing complexes formed on the RP91 substrate (which can form six contiguous base pairs with U1 RNA) shows no stable U1 snRNP binding at ≥0.3 M KCl (*26*).

Differential binding of U1 and U2 snRNP's to two-exon variants. To test the hypothesis that U1 snRNP binding was fundamentally involved in the mechanism of 3′ exon selection, we used two-exon substrates containing E3 and E4, but not E5. A convenient Bgl II restriction enzyme site located 74 nucleotides downstream of E4 was used to prepare two-exon substrates containing one 3′ splice site from the RP57 and RP58 sequence variants (Fig. 4A). The substrate, RP57 Bgl II, showed strong splicing activity, whereas the substrate, RP58 Bgl II, was entirely defective in splicing (*28*).

In that substrate RP58 Bgl II was defective in splicing, it was necessary to determine at which stage the process of splicing was blocked as a result of the defect in the 5′ splice site of the 3′ exon, E4. The binding of U2 snRNP was assessed because its recognition of the branch site of the pre-mRNA is an early and essential step in spliceosome assembly (*29*). Under conditions that maximized early splicing complex formation, two major peaks were resolved on sucrose gradients for these substrates (*24*). Affinity selection of the presplicing (U2-snRNP–containing) complex, demonstrated that U2 as well as U1 snRNP was associated with the (active) RP57 Bgl II substrate, whereas neither snRNP was associated with the (defective) RP58 Bgl II substrate (Fig. 4A, lanes 2 to 4). In addition, U1 snRNP was substantially enriched in the slower sedimenting, nonspecific "RNP" region for the RP57 Bgl II substrate, but not for the analogous complex containing RP58 Bgl II (Fig. 4A, lanes 5 to 7). Analysis of the RNA species across the gradients from another experiment confirmed that spliceosomes were formed for the active but not for the defective substrate (Fig. 4, B and C, lanes 4 and 6).

Taken together, the lack of both presplicing complex formation (U2 snRNP binding) and spliceosome formation for the two-exon substrate RP58 is associated with the absence of a functional 5′ splice site adjacent to the 3′ terminal exon, E4. This indicates that the lack of binding of U1 snRNP adjacent to E4 blocks spliceosome assembly at an early stage, at least prior to the binding of U2 snRNP.

Exon selection is finely tuned to the differential binding of U1 snRNP. Multiple mechanisms must exist to account for the considerable variety of alternative splicing pathways that have so far been documented. Here we have used a simple model system derived from the preprotachykinin gene to show that the mechanism responsible for exon-skipping is sensitive to the binding of U1 snRNP to the 5′ splice site of the internal exon.

The novel finding that stems directly from our work is that increased U1 snRNP binding adjacent to an internal exon stimulates the rate of splicing of not only the intron in which it is bound but also the intron immediately upstream. The rate stimulation for the upstream intron, IVS3, is informative and surprising (substrate RP57, Fig. 1 and 2). The strength of these findings rests in showing that the extreme difference in splicing is directly associated with the differential binding of U1 snRNP. Resident binding of U1 snRNP is retained at high salt (0.3 M KCl) in the spliceosome and presplicing complex for the substrate that uniformly selects E4, but not for the substrate that uniformly skips E4 (Fig 3). Under these conditions U1 snRNP is normally released from functional 5′ splice sites (*5, 30*), and only exceptionally stable U1 binding interactions survive (substrate RP57, Fig. 5). These extreme effects underscore the importance of U1 snRNP in governing exon selection and the potency with which U1 determines the resulting pathway of splicing.

Our data support the exon definition model of Berget and co-workers (*31, 32*) whereby exons are recognized as individual units by the interaction of factors bound simultaneously at the 5′ and 3′ splice site of a single exon. This model addresses the question of how, for genes containing multiple exons, the splicing machinery operates so that exons are not inadvertently skipped. The model also addresses the question of what mechanisms direct the elimination of specific exons when genes are alternatively spliced. Our experiments, with three-exon substrates, show that the degree to which an internal exon is recognized by the splicing machinery is finely tuned to the binding interaction between U1 snRNP and the 5′ splice site of the same exon. For E4, nucleotide changes in its 5′ splice site

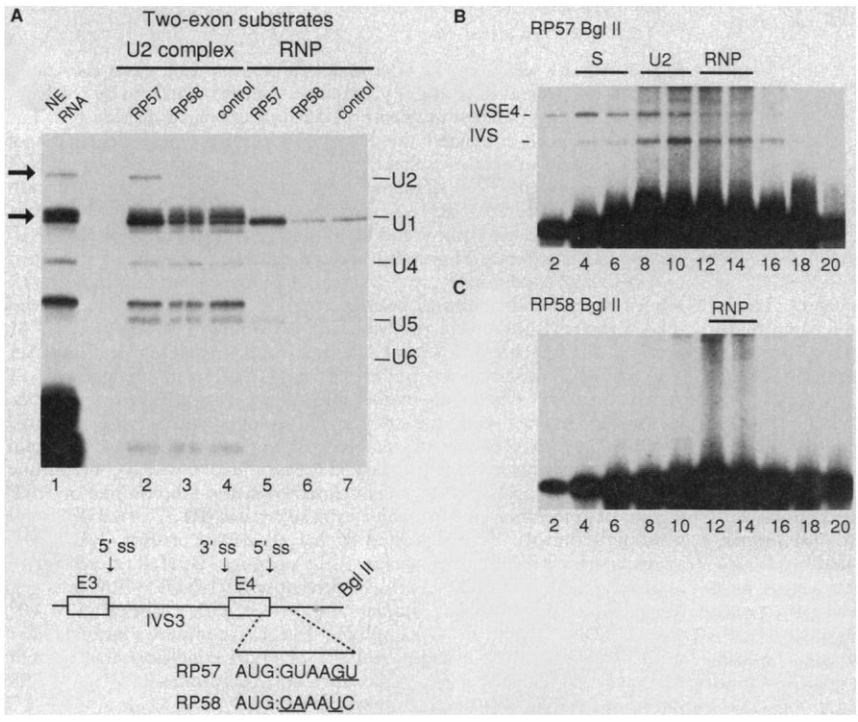


Fig. 4. Affinity selection of complexes formed on two-exon substrates. Two-exon sequence variants, 700 nucleotides, were prepared from Bgl II–cleaved DNA's. (**A**) Splicing reactions, 45 minutes, containing biotin and ^{32}P-labeled RP57 Bgl II (RP57) and RP58 Bgl II (RP58) substrates, or no substrate (control), were sedimented on 10 to 20 percent sucrose gradients as described above (*41*) except that the salt concentration was substantially lower (25 mM KCl, 1 mM $MgCl_2$). Two fractions were affinity-selected as in Fig. 3A from each peak (U2 complex and RNP). Small nuclear RNA's were detected as in Fig. 3A. (**B** and **C**) Profile of RNA products and intermediates purified from gradients containing RP57 Bgl II (B) or RP58 Bgl II (C) substrates. RNA species purified from even-numbered fractions were resolved on 7 percent polyacrylamide–7 M urea gels. (S), spliceosome region; (U2), region containing pre-splicing complex; (RNP), ATP-independent complex containing substrate RNA associated with ribonucleoproteins. (IVSE4), intermediate RNA containing the lariat IVS3 linked to the 3′ exon, E4; (IVS), IVS3 RNA product.

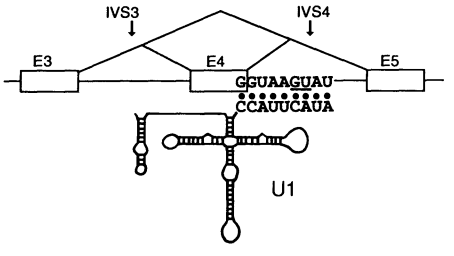
show that E4-inclusion can be effectively titrated from 0 to 100 percent by increasing the number of contiguous base pairs that can be formed with U1 RNA (Fig. 5). In support for the generality of these findings, analysis of nucleotide changes in the 5′ splice site of the adjacent exon (E5) show that, like E4, selection of this exon is dependent on its adjacent U1 snRNP binding site (*23*).

Alternative splicing must operate by a combination of cis-acting signals in the pre-mRNA and cellular factors that recognize these signals. Thus, as a basis for understanding the various alternative splicing mechanisms, it is important to elucidate the role of ubiquitous factors, such as snRNP's, as well as factors that are cell type specific that may regulate snRNP function. Questions about the role of such cell type specific factors must await the outcome of studies in systems subject to biological regulation.

One question raised by our work is how resident binding of U1 snRNP modulates the rate of splicing of the upstream intron. Clues to this puzzle are apparent from experiments demonstrating that U2 complex formation at a given 3′ splice site either requires (our data) or is stimulated by (*31*) U1 snRNP binding at the immediate downstream 5′ splice site. A simple mechanism could be explained by the interaction of one of the U1-specific proteins, 70K, U1-A, or U1-C, with one of the protein components of U2 snRNP resulting in the stable binding of U2 snRNP. One of these proteins, the 70K protein, has a highly charged carboxyl-terminal domain which may perhaps behave as a surface for some interaction with another protein (*33*). Alternatively, U1 snRNP may interact directly with the 3′ splice site region through a protein-RNA interaction. The 70K and U1-A proteins have RNA binding domains that, in principle, could enable either of these proteins to serve as a bridge to connect U1 snRNP and the pre-mRNA 3′ splice site region (*33*). More complicated models than these can also be envisioned involving interactions with other factors.

Regardless of the details of the mechanism for U1 snRNP action, our concept about spliceosome assembly has changed. Our data strongly suggest that, in addition to U1 snRNP binding at the (upstream) 5′ splice site of E3, a second U1 snRNP must interact at the (downstream) 5′ splice site of E4 in order for spliceosome assembly to occur. We propose that two such distal U1 snRNP-5′ splice site interactions define the boundaries of a functional splicing unit and that these interactions are fundamental in determining whether a pre-mRNA will be constitutively or alternatively spliced.

As our data show, mutations in 5′ splice sites of unrelated genes are known to cause exon-skipping when these mutations decrease base complementarity to U1 RNA. Mutations of this type in 5′ splice sites of the human collagen gene have been shown to cause exon-skipping and lead to a defective collagen protein product (*34*). A mutation in the human β-globin gene provides an example of the opposite effect. A single base change within IVS2 of this gene has been shown to produce a prominent β-globin RNA product with an additional exon (*35*). In this case, a C to G change generates a cryptic 5′ splice site which has the potential to form six contiguous base pairs with U1 RNA. Just upstream of this cryptic site in the same intron is a 9-nucleotide polypyrimidine stretch followed by an AG dinucleotide that is utilized as a 3′ splice site as a direct result of this mutation. Without the mutation this 3′ splice site-like sequence is silent. These data and our own lend support for the hypothesis that new exons might arise from intron sequences by a single mutational event that generates a sequence that stably interacts with U1 snRNP (*36*).



			Base pairs Contiguous	Base pairs Total	% Alt	% Const	Spliceosome
RP57	E4 U1	AUG:GUAAGUAU GUC CAUUCAUA	9	9	0	100	→ U2+U4,U5,U6 + U1
RP91	E4 U1	AUG:GUAAGCAU GUC CAUUCAUA	6	8	40	60	
RP91A	E4 U1	UGA:GUAAGCAU GUC CAUUCAUA	5	9	80	20	
RP92	E4 U1	AUG:GUAAAUAU GUC CAUUCAUA	5	8	80	20	
RP23 (wt)	E4 U1	AUG:GUAAACAU GUC CAUUCAUA	5	7	80	20	
RP58	E4 U1	AUG:CAAAUCAU GUC CAUUCAUA	2	5	100	0	→ U2+U4,U5,U6
Consensus		NAG:GURAGU GUC CAUUCA	8/9	8/9			
		N=C, A R=A, G					

Fig. 5. Summary of results. (Top) Schematic of three-exon sequence variant, RP57, with U1 snRNP shown base-paired to the 5′ splice site of E4. (Bottom) Potential base pairs formed between each 5′ splice site variant and the 5′ end of U1 RNA (filled circles). Colon indicates exon-intron junction. Overall effect of mutations (underlined) on alternative, (%Alt, E4-skipped), or constitutive, (%Const, E4-included), splicing. These percentages having an associated error of 12 percent were obtained by densitometric scanning of the autoradiograms shown in Fig. 1. (Arrows) Spliceosome components detected by affinity chromatography at 0.3 M KCl with the use of three-exon substrates.

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24. Resolution of splicing complexes formed on these substrates could not be achieved by the more commonly used technique of native polyacrylamide gel electrophoresis (*7*).
25. Northern (RNA) blot analysis of material from the same reactions shown in Fig. 3A verified the identity of each small nuclear RNA (Fig. 3B) (*26*).
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27. Differential U1 snRNP binding for the RP57 and RP58 substrates is also reproducibly observed when total splicing reactions are selected on immobilized streptavidin and washed at even higher concentrations, 0.5 M KCl (*26*). In addition, U1 snRNP binding to substrate RP57 occurs in the absence of adenosine triphosphate (ATP), consistent with the established ATP independence of binding (*12*).
28. The two-exon substrates tested previously (*22*) were 29 nucleotides shorter at the 3′ end compared to RP57 and RP58 Bgl II but gave essentially the same results in a standard splicing assay.
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37. A one-step ligation reaction involved joining the Bluescript vector to the Hind III site of DNA fragment A and the Eco RI site of fragment B; fragments A and B were joined in the same step by blunt-end ligation. Correct clones were identified by DNA sequence analysis of all splice site regions.
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41. ^{32}P-labeled RNA substrates were incubated under splicing conditions as described (*22*). Complete reaction mixtures were size-fractionated through 15 to 30 percent sucrose gradients containing 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 20 mM Hepes (pH 7.5), and 5 percent (v/v) glycerol. Sedimentation was for 2 hours, (40,000 rpm; SW50.1 rotor) at +4°C. Binding buffer (1×) contained 0.3 M KCl, 2 mM $MgCl_2$, 20 mM Hepes (pH 7.5), 0.1 mM EDTA, 5 percent (v/v) glycerol, and at 0.14 mg/ml each, glycogen and bovine serum albumin (as carrier). Selected material was washed four times in 1 ml of 1× binding buffer at +4°C. Components were eluted by proteinase K treatment (5 mg/ml at 30°C for 10 minutes). Under these conditions the ^{32}P-labeled substrate RNA was not eluted from the column.
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" Here it is in Genesis: ' He took one of Adam's ribs, and made the rib into a woman.' Cloning, if I ever heard it. "