A sequential splicing mechanism promotes selection of an optional exon by repositioning a downstream 5' splice site in preprotachykinin pre-mRNA

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To explore the structural basis of alternative splicing, we have analyzed the splicing of pre-mRNAs containing an optional exon, E4, from the preprotachykinin gene. This gene encodes substance P and related tachykinin peptides by alternative splicing of a common pre-mRNA. We have shown that alternative splicing of preprotachykinin pre-mRNA occurs by preferential skipping of optional E4. The competing mechanism that incorporates E4 into the final spliced RNA is constrained by an initial block to splicing of the immediate upstream intervening sequence (IVS), IVS3. This block is relieved by sequential splicing, in which the immediate downstream IVS4 is removed first. The structural change resulting from the first splicing event is directly responsible for activation of IVS3 splicing. This structural rearrangement replaces IVS4 sequences with E5 and its adjacent IVS5 sequences. To determine how this structural change promoted IVS3 splicing, we asked what structural change(s) would restore activity of IVS3 splicing-defective mutants. The most significant effect was observed by a 2-nucleotide substitution that converted the 5' splice site of E4 to an exact consensus match, GUAAGU. Exon 5 sequences alone were found not to promote splicing when present in one or multiple copies. However, when a 15-nucleotide segment of IVS5 containing GUAAGU was inserted into a splicing-defective mutant just downstream of the hybrid exon segment E4E5, splicing activity was recovered. Curiously, the 72-nucleotide L2 exon of adenovirus, without its associated 5' splice site, activates splicing when juxtaposed to E4. Models for the activation of splicing by an RNA structural change are discussed.

[Key Words: Alternative splicing; pre-mRNA; optional exon; splice site activation]

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Alternative splicing of pre-mRNA molecules is a process of widespread importance in which structurally distinct, but related, proteins are produced from a single gene. This process occurs by a variety of mechanisms and results in the exclusion of exon sequences and/or retention of intervening sequences (IVSs) in the spliced mRNA (Leff et al. 1986; Breitbart et al. 1987). In addition to increasing the diversity of proteins produced from a limited DNA sequence, another important consequence of alternative splicing is the regulation, in some cases, of mRNA levels in response to developmental or tissue-specific signals. Although it is known that a large number of genes (>50) are subject to alternative splicing, a detailed molecular understanding of the process has not yet been achieved for any system.

For a given pre-mRNA, it is the change in the pattern of splice site selection that is the basis of alternative splicing. Thus, the molecular interactions and/or structures that dictate the selection of splice sites observed is a key problem to be solved. For simple splicing mecha-

nisms, the molecular interactions that specify splicing involve the binding of small nuclear ribonucleoprotein particles (snRNPs) to conserved sequences on the pre-mRNA (Green 1986; Padgett et al. 1986; Maniatis and Reed 1987; Sharp 1987). U1 snRNP interacts with the 5' splice site (Mount et al. 1983) and is believed to promote the ATP-dependent binding of U2 snRNP to the branch site (Black et al. 1985; Ruby and Abelson 1988; Ruskin et al. 1988). Subsequently, the binding of a complex containing U4, U5, and U6 snRNAs completes the subunit assembly of the spliceosome (Brody and Abelson 1985; Frendewey and Keller 1985; Grabowski et al. 1985; Grabowski and Sharp 1986; Bindereif and Green 1987; Konarska and Sharp 1987). Within the structure of the spliceosome, the 5' splice site is cleaved and the 5' end of the IVS becomes linked to the branch site adenosine residue in a 2', 5' phosphodiester bond. The 3' splice site is then cleaved to release the IVS in a lariat form, and the exons are ligated to form the spliced mRNA.

The procedure of spliceosome assembly is believed to facilitate the folding of pre-mRNA into a specific structure in which proximal 5' and 3' splice sites are poised

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for reaction. Yet, the way in which particular sites are selected for splicing is a central question that is not well understood, even for simple systems. For systems involving alternative splicing, this question is made more complex because certain splice sites have variable activities. In the case of exon skipping, the splice sites immediately flanking the optional exon are recognized, in one instance, to produce the constitutively spliced mRNA, containing the optional exon. In another instance, the same splice sites are ignored to produce the alternatively spliced mRNA, lacking the optional exon. This inherent flexibility in the choice of splice sites is unique to alternative splicing and must have a structural explanation. Thus, it is important to consider the contribution of pre-mRNA structure and the role of RNA-binding components in the recognition of alternative splice sites. Each of these features may influence the relative "strength" and, hence, utilization of a given splice site.

We are interested in determining the role of RNA secondary structure and splice site strength in the alternative splicing of pre-mRNA molecules. In this paper we describe the analysis of the splicing of pre-mRNAs containing an optional exon, E4, from the preprotachykinin gene. The preprotachykinin I gene encodes substance P, neurokinin A, and related tachykinin peptides by alternative splicing of a common pre-mRNA. Three mRNAs are produced and can be distinguished by the splicing of all seven exons to form the β mRNA or by the exclusion of an internal exon, E4 or E6, to form the γ or α mRNAs, respectively (Nawa et al. 1984; Kawaguchi et al. 1986; Krause et al. 1987). The alternative splicing event in which E4 is excluded gives rise to the predominant mRNA of the three found in rat brain, the γ mRNA form. Each mRNA encodes a distinct set of peptides that are generated by post-translational processing events (MacDonald et al. 1988). In addition, a previous report has indicated the involvement of tissue-specific splicing in the generation of the α and β mRNA forms in bovine brain, intestine, and thyroid (Nawa et al. 1984).

In this study, we present a molecular analysis of alternative splicing by use of preprotachykinin pre-mRNA as a model system. The predominant splicing mechanism *in vitro* and *in vivo* is exon skipping, which removes IVS3, E4, and IVS4 in unison. The competing mechanism, constitutive splicing, which incorporates E4 into the final spliced product, removes the two IVSs in a strict sequential fashion, that is, IVS4 is spliced out before IVS3. Sequential splicing is due to an initial block or defect in the splicing of IVS3. We have used mutational analysis to ask what sequence changes restore splicing activity to substrates that are defective for the splicing of IVS3. Results are presented that demonstrate the importance of repositioning a downstream consensus 5' splice site in the constitutive splicing mechanism.

Results

To investigate the structural basis of exon skipping, a model substrate containing a central, optional exon was subjected to splicing in a HeLa nuclear extract. The pre-

mRNA substrate for *in vitro* splicing, E3–E4–E5 (dashes signify IVSs), contained E3, IVS3, E4, IVS4, E5, and short regions of flanking IVSs derived from the rat preprotachykinin gene. Previous work involving analysis of cDNAs from rat brain has shown that E4, an optional exon, is excluded to produce the γ mRNA, which is the most abundant of the three mRNAs expressed from this gene (Kawaguchi et al. 1986; Krause et al. 1987).

Detection of spliced product RNAs in vitro and in vivo

An RNase protection assay was used to detect the spliced product RNAs, E3E5 and E3E4E5, from alternative and constitutive splicing, respectively. Synthetic oligonucleotides containing sequences diagnostic for each spliced product RNA were hybridized to the ³²P-labeled products of splicing, followed by RNase T1 digestion (Fig. 1A). Synthetic oligonucleotides were complementary to the splice junction in the E3E5 spliced product (oligonucleotide 35), or to E4 and adjacent splice junctions in the E3E4E5 spliced product (oligonucleotide 345). To detect the first splicing event of the constitutive splicing pathway, which involves two complete rounds of splicing, two additional oligonucleotides were employed (IVS45 and 34IVS). These oligonucleotides were characterized by hybridization to RNAs of known sequence, synthetic mRNA as well as E3–E4–E5 pre-mRNA, and related deletion mutants lacking single exons (data not shown).

The product of alternative splicing, E3E5 RNA, appeared in a time and ATP-dependent fashion (Fig. 1B). This protected product migrated as a characteristic doublet at ~65 nucleotides, in agreement with its expected size. The E3E5 junction sequence was also detected with oligonucleotide 345 because RNase T1 does not cleave the RNA strand opposite the loop in the RNA–DNA hybrid (Fig. 1C). From the same reactions, the product of constitutive splicing, E3E4E5 RNA, was shown by protection of a 108-nucleotide fragment containing both splice junctions (Fig. 1C). Thus, alternative and constitutive splicing occur accurately for the E3–E4–E5 model substrate. Alternative splicing is clearly the more efficient pathway *in vitro* by approximately 5 : 1, under these conditions. This is in agreement with the preference observed *in vivo* (see below).

To determine whether the splicing pattern observed *in vitro* in HeLa nuclear extracts was comparable to the pattern observed in intact cells, we used a minigene segment containing sequences E2–E3–E4–E5–E6–E7 from the preprotachykinin gene. This minigene was transiently expressed in several mammalian cell types, followed by S1 nuclease protection analysis with a 5'-end-labeled cDNA probe containing all exons. As shown in Figure 2, HeLa cells produced the three spliced mRNA forms expected. Similarly, rat (F111) and mouse (BALB/c) fibroblast and rat hippocampal (HT4) cells gave rise to all three species. Hippocampal and other neuronal cells in the animal are known to express substance P. The mock-transfected controls demonstrated no endogenous expression of this gene in the cultured cells;

Figure 1. Kinetics of splicing of preprotachykinin pre-mRNA. (A) Scheme for detection of spliced product RNAs by RNase protection analysis. Four synthetic oligonucleotides (thick black lines) distinguish among four possible spliced RNAs. The tripartite pre-mRNA substrate E3-E4-E5 is indicated (top). Sizes of protected products observed are indicated in nucleotides. (ND) Not detected. (B-E). Products (32 P-labeled) from splicing reactions were hybridized to oligonucleotide 35 (B), oligonucleotide 345 (C), IVS45 (D), or 34IVS (E) and treated with RNase T1 to remove single-stranded RNA by using the method of Padgett et al. (1983). Protected RNA products were resolved by electrophoresis on 12% polyacrylamide/7 M urea gels. Schematic representation of protected products is indicated (right); dots signify ATP-dependent products. Incubation times of each reaction are indicated at top. (Control) A 270-min reaction incubated in the absence of ATP and creatine phosphate. Molecular weight markers (M) from pBR322 *Ban*I DNA: 114, 89, and 43 nucleotides (top to bottom). An important limitation of the RNase protection assay is the protection of several background bands in the vicinity of the E4E5 fragment (C). These products are not ATP dependent and are not due to partial digestion (data not shown). It is likely that these bands represent protected products due to the folding of unspliced RNA in the presence of the oligonucleotide. A further limitation of this assay is the inability to resolve ATP-dependent products that migrate at the same or faster rates than fragments of unspliced pre-mRNA.

the appearance of mRNA species was dependent on the presence of the transfected minigene. In all four cell types examined, the γ mRNA form, in which E4 is excluded, was the most abundant species. The γ and β mRNA forms were also detected when a shortened minigene, containing E2-E5 sequences, was expressed in these cells (H. Hoffmann and P. Grabowski, unpubl.). Densitometry of the autoradiograph of Figure 2 demonstrated that the γ , β , and α mRNA species are expressed in an approximate ratio of 72 : 24 : 5, respectively. Sim-

ilar results were obtained when the RNA samples of Figure 2 were analyzed with a 3'-end-labeled cDNA probe (data not shown). Thus, the model substrate used for in vitro studies reflects the in vivo splicing preference for E4 skipping.

Sequential splicing results in the ordered removal of IVS4 before IVS3 for the constitutive pathway

An ordered series of splicing events leading to the for-

Figure 2. E4 skipping predominates during *in vivo* splicing. (Top) S1 nuclease analysis of RNA produced by transfection of mammalian cell lines with a minigene containing E2–E7 of the rat preprotachykinin gene. Alternative splicing results in production of α (E6 excluded), β (constitutive), and γ (E4 excluded) mRNA forms, which correspond to protected fragments of 234, 586, and 312 nucleotides, respectively (indicated by arrowheads). Samples were transfected with the minigene (+) or mock-transfected with vector backbone alone (–). Cell lines tested include BALB/c-3T3 (mouse embryonal), HT4 (rat neuronal; gift of Ron McKay, MIT), HeLa (human fibroblast), and F111 (rat fibroblast).

mation of the E3E4E5 spliced RNA was indicated first by RNase protection analysis. Given that the removal of either IVS3 or IVS4 could, in principle, occur first (Fig. 3A, pathways I and II), it was surprising to find protection of only one partially spliced RNA, E3–E4E5, in which IVS4 was spliced out before IVS3. This product was protected by oligonucleotide IVS45 and appeared as an ATP-dependent product of expected size, 110 nucleotides (Fig. 1D, lanes 90' and 180'). Evidence for protection of a shorter region of the E3–E4E5 molecule, the E4E5 junction sequence, was also observed with a different oligonucleotide, 345 (Fig. 1C). In contrast, no ATP-dependent species that would indicate production of the E3E4–E5 molecule was observed either for oligonucleotide 34IVS (Fig. 1E) or 345 (Fig. 1C). These data indicated, although were not sufficient to prove, sequential splicing.

We were intrigued by the possibility of sequential splicing because a structural change brought about by the first splicing event (removal of IVS4) might be required to drive the second splicing event (removal of IVS3). To examine this question more rigorously, Northern blot analysis was used as a direct test for the partially spliced RNA forms, E3–E4E5 and E3E4–E5. This is a highly sensitive method and allows a comprehensive display of splicing intermediates and products. Detection of only the E3–E4E5 RNA species would provide evidence for sequential splicing in agreement with the RNase protection results, whereas detection of both

RNA forms would provide evidence for random splicing (see Fig. 3A). RNAs from splicing reactions containing the RP23 substrate (a shortened version of E3–E4–E5 RNA), were separated on 5 and 7% polyacrylamide/7 M urea gels and transferred to nylon membranes. Individual RNA molecules were then identified by hybridization with radioactive DNA probes specific for IVS3, IVS4, and exon sequences. The scheme shown in Figure 3A serves as a reference for the RNA structures (numbered) discussed below.

Key evidence for sequential splicing can be observed in the Northern blot of the 5% gel (Fig. 3B). RNA5 is a linear product diagnostic for pathway II and corresponds to the RNA structure E3–E4E5, in which IVS4 but not IVS3, has been spliced out. RNA5 migrates at its expected molecular weight, 700 nucleotides, and is detected by IVS3- and exon-specific probes but not by an IVS4-specific probe (Fig. 3B, lanes 2a, 2b, 2c, and 2d). The intermediates that directly precede production of RNA5 (RNA4 and RNA6), can also be observed on the same Northern blot (Fig. 3B, lanes 2a, 2b, and 2c). Cleavage of RNA5 at the 5' splice site of E3 and ligation of the 5' end of IVS3 to the branch site upstream of E4 generates RNA3. RNA3 migrates very closely to RNA4 on a 5% gel but is characteristically present in low amounts and can be distinguished by hybridization to IVS3-, E4-, and E5-specific probes. RNA4 is present at a higher level and hybridizes only to IVS4- and E5-specific probes. Thus, Northern blot analysis demonstrates the RNA species diagnostic for pathway II.

No RNA species unique to pathway I could be identified on the same Northern blots. The partially spliced RNA, E3E4–E5, is a diagnostic for pathway I and would be expected to hybridize to IVS4- and exon-specific probes, but not IVS3. The E3E4–E5 RNA was not detected above background, even though its size (726 nucleotides) is similar to RNA5 (700 nucleotides), which is clearly resolved on the 5% gel. In case splicing was blocked at the step prior to formation of the E3E4–E5 RNA, we also attempted to identify the lariat RNA species containing IVS3 in a lariat configuration linked to E4, IVS4, and E5 sequences. This lariat intermediate would be expected to migrate above precursor RNA on the 5% gel; however, no evidence for this RNA species could be detected by IVS4- or exon-specific probes. Thus, Northern analysis provides strong evidence for sequential splicing in which IVS4 is excised prior to IVS3 (pathway II, Fig. 3A).

The lariat RNA molecules produced by alternative splicing can also be effectively separated on the 5% denaturing polyacrylamide gel. RNA2 is the IVS product of alternative splicing, which contains the sequences IVS3, IVS4, and E4 in a large loop (Fig. 3B, lanes 2a, 2b, and 2d). The related intermediate from the same pathway, RNA1, contains all of these sequences plus E5 (Fig. 3B, lanes 2a, 2b, 2c, and 2d). The spliced product RNAs from alternative and constitutive splicing pathways contain only exon sequences and migrate at their expected molecular weights, E3E5 at 217 nucleotides and E3E4E5 at 262 nucleotides, respectively (Fig. 3B, lanes 2a, 2b, and

Figure 3. Northern analysis demonstrates sequential splicing of IVS4 prior to IVS3. (A) Possible products and intermediates for constitutive splicing (I and II) and alternative splicing (III). Underscored numbers indicate those RNA species identified in the Northern blots that follow in descending order from the top of the gel. The box indicates the observed partially spliced form, E3-E4E5 RNA. (B) Splicing reactions of tripartite substrate RP23 (bottom) were separated on a 5% polyacrylamide/7 M urea gel. The blot was probed first with IVS3 (lanes 1a and 2a), and stripped and reprobed with IVS4 (lanes 1b and 2b), E5 (lanes 1c and 2c), and E4 (lanes 1d and 2d). DNA probes are indicated below as thick bars (drawn to scale). Underscored numbers at right indicate the RNA species corresponding to schematic of A. The RNA species below RNA6 in lane 2a is a degradation product containing IVS3 sequences. The inability to detect the RNA6 and E3E4E5 spliced product using the E4-specific probe is due to the poor hybridization efficiency and lower specific activity of this oligonucleotide probe compared to the longer DNA fragments (see Experimental methods). Results obtained using the E4-specific oligonucleotide probe were verified by using an internally labeled probe containing E3 and E4 sequences generated from the β -preprotachykinin mRNA (data not shown). (C) Splicing reactions of indicated substrates (diagrammed below) were separated on a 7% polyacrylamide/7 M urea gel. The blot was probed first with IVS3 and stripped and reprobed with IVS4. Some reactions were preincubated at 37°C for 30 min to decrease the second step of splicing (+) or not treated (-). Times of incubation under splicing conditions are indicated at top.

2c). The predominance of the E3E5, compared to E3E4E5 spliced product, is in close agreement with that observed using RNase protection analysis (Figs. 1C and 3B). This product ratio was confirmed when the Northern blot of Figure 3B was hybridized to a probe containing E3 and E4 sequences (P. Spears and P. Grabowski, unpubl.).

IVS3 splicing is rate limiting for the generation of the constitutively spliced product E3E4E5. This is consistent with the sequential splicing pattern observed in which IVS4 is spliced out before IVS3. IVS3 and IVS4 can be clearly distinguished above the pre-mRNA by hybridization to the IVS-specific probes on a 7% gel (Fig. 3C, lanes 2 and 3). Mutant substrate RNAs containing either IVS3 or IVS4 demonstrate the specificity of the IVS probes used in the experiments of Figure 3. As expected for final reaction products, IVS3 and IVS4 accumulate to higher levels in those reactions not subjected to preincubation at 37°C (Fig. 3C, lanes 3, 6, 9, and 12). This heat treatment diminishes an activity necessary for the second step of splicing (Reed et al. 1988). Because the IVS-specific probes were labeled at the same specific activity, these results demonstrate an overall decreased rate of IVS3 compared to IVS4 splicing in the tripartite substrates E3–E4–E5 and RP23.

An intrinsic defect in the splicing of IVS3

To determine whether the splicing of IVS3 was intrinsically inefficient, we constructed deletion mutants that contained only a single pair of splice sites (Fig. 4, bottom); that is, the splicing of E3 to E4 was tested in the absence of the competing downstream exon. Splicing activity of each mutant was tested by RNase protection analysis, as described above. A characteristic ATP-dependent product of expected size was detected for both the E4–E5 and E3–E5 mutant substrates (Fig. 4, lanes 3–6). For the IVS3-containing E3–E4 mutant substrate, however, only a miniscule level of product was detected (Fig. 4, lanes 1 and 2). This immediately indicated a defect in IVS3 splicing. Defective splicing of IVS3 persisted over a wide range of reaction conditions, as determined by titration of salts, cofactors, and nuclear extract (data not shown). In addition, when the 5' half of this substrate containing E3 was substituted by a different 5' exon, the L1 exon of adenovirus and flanking IVS sequences, splicing to E4 remained defective (data not shown). Thus, the inactivity of E4 splicing is intrinsic to the sequences of IVS3 containing the E4 3' splice site. It is worth noting that the splicing efficiency of all of the preprotachykinin substrates shown in Figure 4 reproducibly falls short of that exhibited by the prototype adenovirus substrate L1-L2 (data not shown); however, IVS3 splicing is particularly defective. This defect in IVS3 splicing is likely to be due to the sequence or structure of the 3' splice site region of E4 (see Discussion).

Sequential splicing relieves an initial block to the splicing of IVS3

Up to this point, two results appeared to be closely con-

nected: (1) IVS3 splicing was defective in the absence of sequences downstream of IVS4, and (2) sequential splicing occurred to excise IVS4 prior to IVS3. Therefore, we reasoned that the defect in IVS3 splicing might be overcome by the splicing of E4 to E5 (IVS4 splicing). Specifically, we hypothesized that the RNA structural rearrangement brought about by the splicing of IVS4 directly promotes IVS3 splicing. To test this hypothesis we constructed a synthetic version of the partially spliced RNA, E3–E4E5, the RP18 substrate (see RNA5, Fig. 3A). That is, we wished to compare directly the splicing activity of the RP18 substrate to the IVS3 splicing-defective mutants, RP23 Bgl and RP16 Bgl, (see Fig. 5). These latter two substrates are identical to the splicing-defective mutant, E3–E4 RNA, shown in the experiment of Figure 4, except for the shortening of sequences upstream of E3; all three of these substrates were found to be splicing defective. The level of IVS3 produced by these mutant substrates was detected by Northern analysis, using an IVS3-specific probe. This experiment shows that whereas the splicing of IVS3 is barely detectable ($\leq 2\%$ splicing efficiency) for the RP23 Bgl and RP16 Bgl substrates, splicing is strongly enhanced in RP18 (Fig. 5, lanes 1–4, 9, and 10). In addition to detec-

Figure 4. RNase protection analysis of mutant substrates indicates a block in the splicing of IVS3 in the absence of the competing downstream exon E5. Pre-mRNAs E3–E4, E4–E5, and E3–E5, labeled with ^{32}P , were incubated under splicing conditions with (+) or without (–) ATP and creatine phosphate. (Bottom) Schematic of mutant substrates. Reactions were then tested for accurate splicing by the RNase protection assay by using oligonucleotide 345 (lanes 1–4), or oligonucleotide 35 (lanes 5–6).

Figure 5. Northern analysis confirms that IVS3 splicing is activated by the RNA rearrangement resulting from the splicing of IVS4. Reactions were separated on a 7% polyacrylamide/7 M urea gel, and the Northern blot was probed with the IVS3-specific probe as shown in Fig. 3B. Diagrams of substrates used for splicing reactions 0 and 180 min are shown (below).

tion of IVS3, accurate spliced product formation was demonstrated by RNase protection analysis for the RP18 substrate (data not shown). In agreement with the results observed for the tripartite model substrate, IVS3 excision is extremely inefficient unless IVS4 sequences downstream of E4 have been replaced with E5 and a portion of IVS5; that is, the structural change resulting from the splicing of E4 to E5 strongly promotes IVS3 excision. In the same experiment, runoff transcripts ending in the middle of E5 show that the junction sequence, E4 (45 nucleotides)/E5 (14 nucleotides), by itself, is not sufficient to promote IVS3 splicing (Fig. 5, lanes 5–8). Additional information/structure downstream of the middle of E5 is required.

Thus, sequential splicing relieves the initial block, or defect, in IVS3 splicing by producing an important structural change in the pre-mRNA. To determine how the block to IVS3 splicing was relieved by this structural change, the following models were tested. Model 1: E5, or downstream sequences may contain a factor-binding site(s) that enhances recognition of the E4 3' splice site. Splicing would then be promoted by the repositioning of the binding site(s). Model 2: The juxtaposition of E4 and

E5 may prevent the formation of an inhibitory secondary structure or interaction that initially blocks the splicing of IVS3. That is, splicing would be promoted by the removal of the inhibitory structure.

Exon-specific mutants

To test the importance of E5 sequences in promoting IVS3 splicing, mutants were constructed containing one or three copies of E5 but no downstream IVS5 sequences. The first of these, mutant RP26, contained E3, IVS3, and E4 joined to one exact copy of E5. The RP26 substrate is analogous to the RP18 substrate but lacks IVS5 sequences. The RP26 substrate was inactive in splicing in contrast to the splicing-competent RP18 substrate (Fig. 3C, lanes 11 and 12; Fig. 5, lane 10; Fig. 6, lanes 8–11). Control reactions containing the highly active substrate representative of alternative splicing, RP29, and the splicing-defective substrate, RP16 *Bgl*II, are also shown (Fig. 6, lanes 1–7). Thus, a single E5, when juxtaposed to E4, is not sufficient to promote IVS3 splicing. Downstream IVS5 sequences must also be present. In case a single E5 was not sufficient to promote IVS3 splicing, three tandem copies of E5 were cloned immediately downstream of E4, in the RP27 substrate. In addition to multimerizing E5, this served to increase the length of exon sequences downstream of E4 from 24 to 72 nucleotides; however, no splicing activity could be detected for the RP27 substrate (Fig. 6, lanes 12–15). We are currently determining the effect of downstream E6 and E7 sequences on the splicing of IVS3; however, it is worth noting that E6 is an optional exon and cannot be an obligatory requirement for IVS3 splicing.

A very different result was obtained in an exon-swap experiment, where the three tandem E5 sequences of RP27 (72 nucleotides) were exactly replaced by the adenovirus L2 exon (72 nucleotides) but with no downstream IVS sequences (RP28). The L2 exon was found to activate splicing of IVS3, regardless of the identity of the 5' exon (RP28 and RP21; Fig. 6, lanes 16–23). The RP21 substrate, containing the adenovirus L1 exon and 101 nucleotides of adjacent IVS, 260 nucleotides of IVS3, and E4 joined to L2, was found to have particularly high activity. Its activity is comparable to that of the splicing-competent substrate RP29 (Fig. 6, lanes 2–3 and 21–22).

These results demonstrate that E5 sequences alone do not promote IVS3 splicing when they are present in one or three tandem copies; however, when E5 is substituted by the adenovirus L2 exon, splicing activity is restored. It is interesting to note that no IVS sequences are present downstream of the L2 exon in either of the splicing-competent substrates, RP28 or RP21. These results suggest that the presence of L2 sequences may cause a different folding of the pre-mRNA substrate into an active conformation for the splicing of IVS3.

Site-specific mutagenesis of the 5' splice site of E4

The idea that the splicing of IVS3 might be promoted by a factor(s) binding downstream of E4 (model 1) led us to

Figure 6. Sequences downstream of E4 govern splicing of IVS3. Splicing reactions containing ^{32}P -labeled pre-mRNA were separated as in Fig. 5 and autoradiographed. Structures of substrates are shown (below). Times of incubation under splicing conditions are indicated (top). (●) Lariat intron–3' exon intermediate RNA; (○) lariat product RNA; (arrowheads) spliced product RNA.

test the involvement of the 5' splice site of E4. The 5' splice site of E4, GUAAAC, deviates in two positions (underlined) from the consensus 5' splice site, $\text{GU}^{\text{A}}/\text{CAGU}$. In contrast, the 5' splice site of E5, GUAAGU, is an exact match. As a consequence of sequential splicing, the 5' splice site of E4 is removed and the 5' splice site of E5 is repositioned just downstream [45(E4) + 24 (E5) = 69 nucleotides] of the alternative 3' splice site of E4. The following experiments were designed to test the hypothesis that the strength of the downstream 5' splice site has an important influence on the splicing of IVS3; that is, the efficiency of binding of U1 snRNP to the 5' splice site of the small exon E4 may either promote or inhibit the upstream splicing event.

Site-specific mutagenesis was performed to alter the E4 5' splice site to a perfect match with the consensus sequence, substrate RP32 (2-nucleotide change), or to an increased mismatch, substrate RP33 (3-nucleotide change; Fig. 7, bottom). These mutants showed opposite

effects and thereby demonstrate a dramatic influence of the E4 5' splice site. The "up" mutant RP32, which has a perfect match to the consensus site, splices much more efficiently than the wild-type substrate RP23 Bg/II (Fig. 7, lanes 6–8, and 3–5). The wild-type substrate shows its characteristic activity (barely detectable), compared to the active, control substrate RP18 (Fig. 7, lanes 5 and 2). It is also interesting to note that the degree of splicing activity in the "up" mutant is comparable to that of the control substrate in spite of the structural differences between these two substrates (Fig. 7, lanes 2 and 8). The "down" mutant RP33 has no detectable splicing activity (Fig. 7, lanes 9–11). Thus, the strength of the downstream 5' splice site has a strong influence on the upstream splicing event. When that site has an increased match to the consensus U1 small nuclear RNA (snRNA)-binding site, IVS3 splicing is clearly enhanced; when the site has a decreased match, splicing is abolished.

Figure 7. Site-specific mutagenesis reveals a significant effect of the 5' splice site of E4 on IVS3 splicing. Substrate RNAs labeled with ^{32}P (bottom) were incubated under splicing conditions for the time points indicated at top. RP23 *Bgl*II is the wild-type substrate containing the unchanged 5' splice site of E4. RP32 and RP33 substrates contain nucleotide changes in the 5' splice site as indicated (underlined nucleotides). (Control) The RP18 substrate incubated under splicing conditions at the 120-min time point. The control reaction serves as a marker for the IVS3 lariat product. The lariat intermediate RNA generated by the splicing of RP18 does not enter the 7% gel. (Consensus) 5' splice site consensus sequence beginning at the first nucleotide of the intervening sequence [Mount et al. 1983].

A 15-nucleotide sequence containing a consensus 5' splice site is sufficient to activate splicing of IVS3

To this point, our results demonstrated that E5 sequences alone did not promote IVS3 splicing but that splicing was strongly enhanced by increasing the match

of the E4 5' splice site to the consensus sequence. Thus, the observation of sequential splicing could be explained by the removal of the comparatively weak 5' splice site of E4 and the repositioning of E5 with its adjacent strong 5' splice site. That is, of the 557 nucleotides of IVS5 found in the partially spliced RNA, E3–E4E5 RNA (RNA5 and the synthetic version RP18), only the 5' splice site might be necessary to activate IVS3 splicing. To test this hypothesis, we constructed IVS5 deletion mutants to ask what IVS5 sequences were important for IVS3 splicing.

Compared to the splicing-competent substrate RP18 RI, which contained 557 nucleotides, the substrate RP35 RI, which contained only the first 96 nucleotides of IVS5, was equally active (Fig. 8, lanes 1–6). Similar activity was observed when only the first 190 nucleotides of IVS5 were present (data not shown). When all IVS5

Figure 8. The first 15 nucleotide of IVS5, containing a consensus 5' splice site, are sufficient to activate splicing of IVS3. Substrates labeled with ^{32}P were incubated under splicing conditions at the indicated times. Structures of substrates containing varying portions of IVS5 are diagrammed below.

sequences were removed, splicing was abolished, as shown above for the splicing-defective substrate RP26 RI (Fig. 8, lanes 7–9). However, when the first 15 nucleotides of IVS5 were inserted just downstream of the exon structure E4E5, RP26.rsc RI, significant splicing activity was restored (Fig. 8, lanes 10–12); that is, the splicing-defective mutant RP26 could be “rescued” simply by the insertion of a 15-nucleotide segment containing a consensus 5' splice site. These results strongly indicate that U1 snRNP binding downstream of the alternative 3' splice site of E4 strongly activates that site.

Discussion

An RNA structural rearrangement activates splicing

To investigate the structural basis of alternative splicing we analyzed the splicing of model substrates containing a central, optional exon, E4, from the preprotachykinin gene. This work describes the strategy of sequential splicing in the mechanism that incorporates the optional E4 into the final spliced product RNA. This strategy involves an initial block or defect in the splicing of the immediate upstream IVS3, which is removed by the splicing of the immediate downstream IVS4. The first splicing event in the series results in the repositioning of the small downstream exon (E5, 24 nucleotides) with its adjacent IVS (IVS5) sequences. By using a synthetic version of the partially spliced RNA, E3–E4E5 (IVS3-containing substrate, RP18), we tested the hypothesis that the RNA rearrangement resulting from the splicing of IVS4 was directly responsible for the increase in IVS3 splicing. This RNA rearrangement was found to activate IVS3 splicing significantly compared with splicing-defective mutants, which contained only IVS4 sequences downstream of E4 (Fig. 5). Thus, sequential splicing removes an initial block to IVS3 splicing by altering the structure of the RNA substrate.

The importance of a downstream consensus 5' splice site in the activation of the upstream IVS3 splicing event was determined by asking what sequences in RP18 were responsible for the activation of IVS3 splicing. E5 and the majority of IVS5 sequences were found to have no effect on IVS3 splicing; however, when the consensus 5' splice site of E5, GUAAGU, was deleted from the RP18 substrate, IVS3 splicing activity was lost.

The effect of an immediate downstream consensus 5' splice site was tested in two ways. Site-specific mutagenesis was used to alter the wild-type, 5' splice site of E4, GUAAAC, to a perfect consensus match, GUAAGU. This 2-nucleotide change (underlined) dramatically improved IVS3 splicing, compared to the wild-type substrate. In contrast, decreasing the match of this site to the consensus abolished splicing. In a second experiment, we attempted to rescue the splicing-defective mutant RP26, which contains E3, IVS3, and E4 joined to E5 but no IVS5 sequences. By simply inserting the first 15 nucleotides of IVS5, beginning with the sequence GUAAGU, IVS3 splicing (substrate RP26.rsc) was significantly improved.

These results strongly indicate that increased binding

of U1 snRNP just downstream of the E4 3' splice site is responsible for promoting splicing to that site. Sequential splicing, in removing IVS4 prior to IVS3, serves to remove the relatively poor 5' splice site of E4 and reposition the consensus 5' splice site of E5. When repositioned after IVS4 splicing, the 5' splice site of E5 is located only 69 nucleotides away from the 3' splice site of E4 [E4(45 nucleotides), plus E5 (24 nucleotides)]. Perhaps the consensus 5' splice site of E5 drives the upstream splicing event by providing a more efficient binding site for U1 snRNP. It has been shown previously that by increasing the base pairing of an SV40 5' splice site to the 5' end of U1 snRNA, splicing of this site to a downstream 3' splice site is significantly improved (Zhuang et al. 1987). In the case of RP18, increased binding of U1 snRNP would have to exert its effect immediately upstream, rather than far downstream, of that site.

It is curious that a downstream consensus 5' splice site would exert such a dramatic influence on a splicing event upstream. In a recent study utilizing adenovirus substrates, U1 snRNP binding to a 5' splice site was found to enhance U2 complex formation at the upstream 3' splice site of the same exon (Robberson et al. 1990). These investigators proposed that the selection, or “definition” of an exon could be significantly enhanced by interactions of U1 and U2 snRNPs bound to adjacent splice sites. One important difference between the Robberson et al. (1990) study and our results is the difference in the types of 3' splice sites activated. The former study utilized the constitutive and highly efficient 3' splice site of the adenovirus L2 exon. In contrast, the 3' splice site of preprotachykinin E4 is an alternative splice site, which we have shown to be intrinsically defective in splicing. One obvious difference in these sites is that the E4 3' splice site has a short polypyrimidine stretch, 6 uninterrupted nucleotides, compared to a stretch of 14 nucleotides for the adenovirus L2 exon. Future experiments, however, will be needed to determine what structural features of the E4 3' splice site are responsible for the defect in splicing.

A strict requirement for a consensus 5' splice site just downstream of E4 need not always be met. Mutants in which IVS4 sequences were substituted with the 72-nucleotide L2 exon of adenovirus (substrates RP21 and RP28) also were found to activate IVS3 splicing significantly compared with the wild-type substrate. It is important to note that no 5' splice site exists downstream of E4 in these splicing-competent substrates. A search for the sequence GU^A/CAGU in L2 revealed only a three of six match to this consensus. Thus, IVS3 splicing can be activated by a hybrid exon, E4L2, in the absence of a downstream 5' splice site.

How can these apparently different activation mechanisms be explained? In other systems, exon-specific sequences have been shown to have an important influence on upstream splicing events. In the case of the alternatively spliced β -tropomyosin pre-mRNA, incorporation of two mutually exclusive exons is dependent on a prior splicing event that joins downstream exon sequences (Helfman et al. 1988). In this case, the required

exon sequences are tropomyosin specific. In the case of α -tropomyosin pre-mRNA, the proximity of the 5' splice site and branch site prevents the joining of E2 and E3 [Smith and Nadal-Ginard 1989]. The α -tropomyosin mechanism, however, cannot explain the mutually exclusive splicing of E6 and E7 in the case of β -tropomyosin [Helfman and Ricci 1989]. In addition, by using model β -globin substrates, Reed and Maniatis (1986) have shown that exon sequences can play a significant role in the selection of genuine splice site sequences of constitutive exons.

Mechanisms for activation

The activation of IVS3 splicing reported here is consistent with two simple models. Both of these models take into account the fact that the E4 3' splice site is intrinsically defective. One model would invoke the role of a factor-binding site that enhances recognition of the E4 3' splice site. This model is consistent with the RP18 substrate, in which enhanced IVS3 splicing is due to the repositioning of the consensus 5' splice site of the next exon, E5. Simultaneous binding of (most likely) U1 snRNP to the newly positioned 5' splice site and to a 3' splice site-binding factor would account for enhanced splicing by increasing the local concentration of the 3' splice site factor near the defective 3' splice site. These interactions would be expected to drive the binding equilibrium in the direction of increased complex formation at the defective 3' splice site of E4. This model is intriguing because it has been shown that the same U1 snRNP-rich fraction that binds to 5' splice site sequences also binds efficiently to 3' splice site sequences [Tatei et al. 1987]. In this same study it was shown that further fractionation of the original U1-rich fraction effectively separated the 5' and 3' splice site-binding activities. This suggests that U1 snRNP associates loosely with a factor that recognizes 3' splice sites and lends credence to the proposed model. This model is consistent with the important role of proteins in recognizing sequences from the branch site to the AG dinucleotide [Gerke and Steitz 1986; Tazi et al. 1986; Ruskin et al. 1988; Swanson and Dreyfus 1988; Garcia-Blanco et al. 1989].

In an alternative model, local RNA secondary structure may have a pronounced effect on the activity of the 3' splice site of E4. Specifically, refolding of the RNA substrate due to a newly introduced sequence would be proposed to activate splicing, by relieving a putative inhibitory structure involving the 3' splice site of E4. This model is consistent with substrates RP21 and RP28, in which a hybrid E4L2 exon activates splicing of the E4 3' splice site. We have determined the hypothetical secondary structure of the wild-type splicing-defective substrate, RP23 *Bg*/II by using the program of Zuker and Stiegler (1981). This hypothetical model indicates that multiple interactions involving E4 and IVS3 sequences sequester the E4 3' splice site region in a double-stranded form. All of the proposed E4 and IVS3 interactions, including a perfect 10-bp complementarity, occur

within a distance of <100 nucleotides. Models of the splicing-competent substrates containing the hybrid E4L2 exon fold differently due to proposed base-pairing interactions between E4 and L2 utilizing sequences at the 5' end of L2. This refolding results in a putative single-stranded E4 3' splice site. What is most interesting about these structural models is that they provide a simple explanation of differences in splicing activity based on differences in RNA structure. Because these structural models are hypothetical, we are currently challenging them experimentally.

Secondary structure effects on splicing have previously been introduced artificially by the insertion of inverted repeats that sequester an exon or splice site [Solnick 1985; Solnick and Lee 1987]. In some cases, this has resulted in exon skipping *in vitro* and *in vivo*. The effect of secondary structure on splicing has been met with some skepticism because of the generally accepted idea that heterogeneous nuclear ribonucleoproteins (hnRNPs) bind to the pre-mRNA substrate during transcription and prevent extensive secondary structure from forming. Some of these proteins are important in splicing and may have RNA unwinding activity [Choi et al. 1986]. Nonetheless, a study by Eperon et al. (1988) has shown that a significant effect on splicing can occur by the formation of secondary structure within a short stretch of RNA. In particular, these investigators observed a significant effect of secondary structure for stem-loop structures having a loop size of ~50 nucleotides or less. The model we propose for the initial structural inhibition of IVS3 splicing involves proposed interactions that fall well within this distance.

Potential for regulation

We have shown that alternative splicing of optional E4 of the preprotachykinin gene proceeds predominantly by an exon-skipping mechanism *in vitro* and *in vivo*. Interpretation of the results presented here indicates that the predominance of exon skipping is due to the combined effect of a defect in the 3' splice site region of E4 and also to the less than ideal 5' splice site of the same exon. When this 5' splice site is artificially altered to a perfect consensus match, the defect in the E4 3' splice site is substantially overcome. These results indicate that interactions at the 5' splice site of an optional exon are capable of regulating splicing of the same exon and establishing the functional characteristics of an optional exon. Consequently, alteration of the level or specificity of factors in the cell that bind to 5' splice sites could, in principle, provide a simple strategy to regulate alternative splicing of many different genes. U1 snRNP is one candidate for such a regulatory role. It is of interest that variations in sequence and levels of U1 snRNA have been shown to occur during *Xenopus* development [Lund and Dahlberg 1987; Lund et al. 1987]. Our results indicate that considerations about the regulatory potential of U1 snRNP should be extended to effects on 3' splice site activity.

Experimental methods

Construction of plasmids

The plasmid pBSE3-E4-E5 contains 2000 bp of the rat prepro-tachykinin I gene from the *Xho*I site 332 bp upstream of exon 3 to the *Bam*HI site 557 bp downstream of exon 5, cloned into the *Hind*III and *Bam*HI sites of the pBS (–) backbone (Stratagene). The related deletion mutants follow: pBSE4-E5 is the *Bam*HI fragment of pBSE3-E4-E5 cloned into the *Bam*HI site of pBS(–); pBSE3-E5 is a deletion of the central *Sph*I fragment of pBSE3-E4-E5; the pRP23 substrate, used to prepare the smaller tripartite substrate, was constructed from pBSE3-E4-E5 by using the polymerase chain reaction (PCR) technique with oligonucleotides hybridizing 96 bp downstream of exon 5 (bottom strand) and at the 5' edge of exon 3 (top strand). PCR was performed in a Coy temperature cycler with *Taq* DNA polymerase (Perkin-Elmer) by use of conditions as specified by the manufacturer. PCR was also used to generate the pRP29 mutant by use of the deletion mutant pBSE3-E5 and the oligonucleotides indicated above. The remaining plasmids, pRP18, pRP21, pRP26, pRP27, pRP28, pRP32, pRP33, and pRP26.rsc, were generated by PCR with oligonucleotides containing the desired changes and the T3-specific (opposite strand) oligonucleotide located just upstream of E3. Oligonucleotides were synthesized on a Biosearch 8600 DNA synthesizer, followed by gel purification of the full-length molecules. Each plasmid was sequenced by the Sanger chain-termination method to ensure that the desired changes had been made. Further details about the construction of these plasmids will be made available upon request.

Transfection and RNA analysis

Cells were grown in Dulbecco's modified medium supplemented with 10% calf serum or, for HT4 cells, 10% fetal bovine serum. Cells were transfected with 15 µg of plasmid DNA/10-cm plate by use of the calcium phosphate coprecipitation procedure (Graham and van der Eb 1973). RNA was prepared after 48 hr by use of guanidinium thiocyanate (Chirgwin et al. 1979). Nuclease S1 analysis was performed by the method of Favaloro et al. (1980) with 20 µg of total RNA and a 5' end-labeled (β) cDNA probe. The 844-bp *Pvu*I–*Bst*EII cDNA probe contained 258 nucleotides of vector sequence and E1 to the *Bst*EII site within E7 of the prepro-tachykinin gene (Krause et al. 1987). The minigene used for in vivo splicing assays contained gene sequences E2–E7 inserted between an SV40 promoter and polyadenylation site in a pBS (–) backbone.

Pre-mRNA preparation and in vitro splicing reactions

Capped pre-mRNA was prepared by transcription of 40 µg/ml linearized template DNA in the presence of 0.5 mM diguano-sine triphosphate and T3 polymerase, as specified (Stratagene). Template DNA was removed by addition of 5 units of DNase I (RQ1, Promega), followed by incubation for 10 min at room temperature. Reactions were eluted through G50-150 Sephadex spin columns, followed by phenol/chloroform extraction and ethanol precipitation. Splicing reactions contained 100,000 cpm, or less, of freshly prepared pre-mRNA, 44% by volume HeLa nuclear extract (Dignam et al. 1983), 1.5 mM MgCl₂, 2 mM ATP, and 5 mM creatine phosphate, in volumes of 12.5 or 25 µl. Reactions were incubated at 30°C, followed by proteinase K digestion (100 U/ml for 10 min at 30°C), phenol/chloroform extraction, and ethanol precipitation. RNA samples were resolved on 7% polyacrylamide/7 M urea gels, unless otherwise indicated.

Northern analysis

Splicing reactions containing unlabeled RNA were separated by denaturing gel electrophoresis (5 or 7% polyacrylamide/7 M urea) and transferred to GeneScreen membrane (DuPont NEN) by electroblotting or by capillary flow from top (gel) to bottom (membrane). Northern analysis was performed essentially by the method of Konarska and Sharp (1987). DNA probes were labeled with ³²P by use of Klenow polymerase and random hexanucleotide primers (Boehringer–Mannheim). The E4-specific DNA probe was a synthetic 19-mer labeled at its 5' end with [γ-³²P]ATP and polynucleotide kinase (New England Biolabs). Membranes were stripped by boiling in 0.1× SSC, 0.1% SDS, and checked by autoradiography before reprobing.

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