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Alternative splicing of β-tropomyosin pre-mRNA: multiple cis-elements can contribute to the use of the 5′- and 3′-splice sites of the nonmuscle/smooth muscle exon 6

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ABSTRACT

We previously found that the splicing of exon 5 to exon 6 in the rat β-TM gene required that exon 6 first be joined to the downstream common exon 8 (Helfman *et al.*, Genes and Dev. 2, 1627–1638, 1988). Pre-mRNAs containing exon 5, intron 5 and exon 6 are not normally spliced *in vitro*. We have carried out a mutational analysis to determine which sequences in the pre-mRNA contribute to the inability of this precursor to be spliced *in vitro*. We found that mutations in two regions of the pre-mRNA led to activation of the 3′-splice site of exon 6, without first joining exon 6 to exon 8. First, introduction of a nine nucleotide poly U tract upstream of the 3′-splice site of exon 6 results in the splicing of exon 5 to exon 6 with as little as 35 nucleotides of exon 6. Second, introduction of a consensus 5′-splice site in exon 6 led to splicing of exon 5 to exon 6. Thus, three distinct elements can act independently to activate the use of the 3′-splice site of exon 6: (1) the sequences contained within exon 8 when joined to exon 6, (2) a poly U tract in intron 5, and (3) a consensus 5′-splice site in exon 6. Using biochemical assays, we have determined that these sequence elements interact with distinct cellular factors for 3′-splice site utilization. Although HeLa cell nuclear extracts were able to splice all three types of pre-mRNAs mentioned above, a cytoplasmic S100 fraction supplemented with SR proteins was unable to efficiently splice exon 5 to exon 6 using precursors in which exon 6 was joined to exon 8. We also studied how these elements contribute to alternative splice site selection using precursors containing the mutually exclusive, alternatively spliced cassette comprised of exons 5 through 8. Introduction of the poly U tract upstream of exon 6, and changing the 5′-splice site of exon 6 to a consensus sequence, either alone or in combination, facilitated the use of exon 6 *in vitro*, such that exon 6 was spliced more efficiently to exon 8. These data show that intron sequences upstream of an exon can contribute to the use of the downstream 5′-splice, and that sequences surrounding exon 6 can contribute to tissue-specific alternative splice site selection.

INTRODUCTION

A major unresolved question in pre-mRNA splicing is how exons are spliced together with such fidelity in genes that contain multiple exons. In addition, how alternatively spliced exons are chosen is largely unknown. Alternative RNA splicing is a fundamental process in eukaryotes, that contributes to tissue-specific and developmentally regulated patterns of gene expression (1,2). At present relatively little is known about the cellular factors and mechanisms that are responsible for the selection of alternative splice sites in complex transcription units and how the splicing signals in alternatively spliced exons differ from those in constitutively spliced exons (for reviews see 1–4). Significant progress has been made in Drosophila systems such as in the genes of the sex determination pathway, suppressor of white apricot and Drosophila P-transposase. In these systems alternative splicing is subject to regulation by factors that either inhibit or activate the use of alternative 5′ or 3′ splice sites (1,3–5). In contrast, in vertebrate systems, much less is known about the mechanisms and cellular factors involved in the regulation of alternative splicing, but a number of features in the pre-mRNA have been implicated in alternative splice site selection. These include the relative strengths of 5′ and 3′ splice sites (6–9), intron size (10), the pyrimidine content of a 3′ splice site (7,11,12), the location of branch points (13–18), multiple alternative branch points (14,16,19,20), branch point sequences (7,21–24), intron sequences between a 3′ splice site and upstream branch point (15,17,25), and exon sequences (11,13,25–39).

In addition to cis-acting elements in the pre-mRNA, at least one cellular factor isolated from mammalian cells termed ASF (alternative splicing factor) or SF2, has been found to affect the choice of alternative 5′ splice sites (40,41). *In vitro* splicing studies with pre-mRNA derived from the early region of simian virus (SV40) involving the alternative splicing of large T and small t splice sites, and with model pre-mRNA substrates derived from the human β-globin gene demonstrated that high concentrations of SF2/ASF promote the use of proximal 5′ splice sites, whereas low concentrations favor the use of distal 5′ splices (40,41). Although this factor is also required for general splicing (42), differences in the relative concentrations or activities of this factor in different cell types might play a role in regulated alternative splicing. In addition, hnRNP A1 can antagonize the effects of SF2/ASF (43), but this effect appears to be specific

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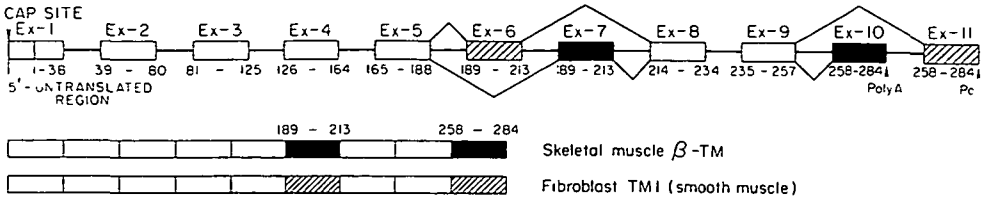

Figure 1. Organization of the rat β-tropomyosin gene. Open boxes represent constitutive exons, hatched and closed boxes represent tissue-specific exons as indicated. Horizontal lines represent introns; they are not drawn to scale. The amino acids encoded by each exon are indicated. The CAP site and polyadenylation sites are also indicated.

to only certain introns (44). SF2/ASF is part of a family of proteins termed SR proteins (45). Interestingly, different SR proteins have distinct functions in alternative pre-mRNA splicing *in vitro* and different members of the SR protein family are differentially expressed in various tissues and cell types (46). Collectively, these studies show that modulation of alternative splice site selection can, in principle, be achieved by changes in the levels of general splicing factors.

We have been using the rat β-tropomyosin (TM) gene as a model system to investigate the mechanisms responsible for developmental and tissue-specific alternative RNA splicing (16,17,25,30,32,47). This gene spans 10 kb of DNA with 11 exons and encodes two distinct isoforms, namely skeletal muscle β-TM and fibroblast TM-1 (Figure 1). Exons 1 through 5, 8 and 9 are common to all mRNAs expressed from this gene. Exons 6 and 11 are used in fibroblasts as well as smooth muscle, whereas exons 7 and 10 are used exclusively in skeletal muscle. We have found that use of the skeletal muscle exon 7 in nonmuscle cells is blocked in nonmuscle cells due to the interaction of factors with sequences upstream and within exon 7 (17,25,47). Our previous studies involving the use of exon 6 (nonmuscle-type splice) revealed an ordered pathway of splicing in which exon 6 must first be joined to the downstream common exon before it can be spliced to the upstream common exon (32). Pre-mRNAs containing exon 5, intron 5 and exon 6 are not normally spliced *in vitro*.

We have carried out a mutational analysis to determine which sequences in the pre-mRNA contribute to the inability of this precursor to be spliced *in vitro*. Our studies demonstrate that three distinct elements can act independently to activate the use of the 3'-splice site of exon 6. Using biochemical assays, we have determined that these sequence elements interact with distinct cellular factors for 3'-splice site utilization. We also find that introduction of a poly U tract in the intron upstream of exon 6 can contribute to the use of the downstream 5'-splice site. These results also demonstrate that, in addition to sequences in and around exon 7 (skeletal muscle-type splice), multiple cis-elements surrounding exon 6 contribute to tissue-specific alternative splice site selection.

MATERIALS AND METHODS

Plasmid constructions

The DNA templates for use in *in vitro* transcription with SP6 polymerase are derived from the rat β-TM gene (16,32). Mutations were introduced by the method of Kunkel (48), and the DNA sequences confirmed by the method of Sanger (49).

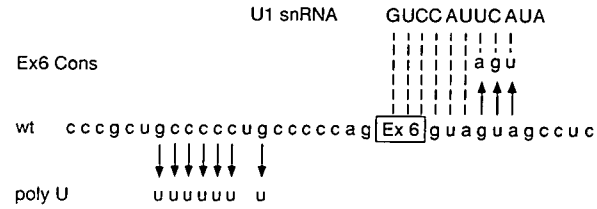

Figure 2. Nucleotide sequence of two mutations made in the 5' and 3' splice sites of exon 6. The wild-type (wt) nucleotide sequence at the 3' end of intron 5 and the 5'-splice site of exon 6 are shown. Also shown here is the U1snRNA sequence complementary to the 5'-splice site of exon 6.

Synthesis of RNA and *in vitro* splicing

The ^{32}P-labelled SP6/tropomyosin pre-mRNAs were synthesized *in vitro* primed with CAP analog as described (50). The pre-mRNAs contained a small amount of prematurely terminated transcripts, but their presence did not appear to affect the *in vitro* splicing reactions and the RNA substrates were not further purified.

HeLa cell nuclear extracts we prepared as described (51,52). *In vitro* splicing reactions were carried out at 30° for indicated times (0−240 min). Standard assay conditions used for these studies consisted of 15 μl of nuclear extract in a final reaction volume of 25 μl containing 2 $MgCl_2$, 500 μM ATP, 20 mM creatine phosphate, 2.6% (w/v) polyvinyl alcohol, 15−30 ng pre-mRNA, 12.8 mM HEPES (pH 8), 12% (v/v) glycerol, 60 mM KCl, 0.12 mM EDTA and 0.3 mM DTT. In some experiments the concentrations of $MgCl_2$ and KCl were varied as indicated.

The complementation assay was carried out in the presence of a HeLa cell cytoplasmic S100 fraction with or without the addition of SR proteins in in a 25 μl volume, containing 6 μl of HeLa cell S100 and 4 μl of buffer D or buffer D containing different dilutions of SR proteins. The SR proteins were prepared as described (46).

Analysis of processing products

After incubation for indicated time the reactions were stopped by addition of a solution containing SDS-proteinase K and the RNA was recovered as described (52). The products of the reaction were analyzed on 4% acrylamide/7M urea gels. Intermediates were distinguished from final products by their relative appearance and disappearance in time course experiments. Lariat RNAs were identified by their aberrant mobility on different percentage polyacrylamide gels (53). In addition, RNAs containing lariats were verified by treatment by a lariat debranching activity (54).

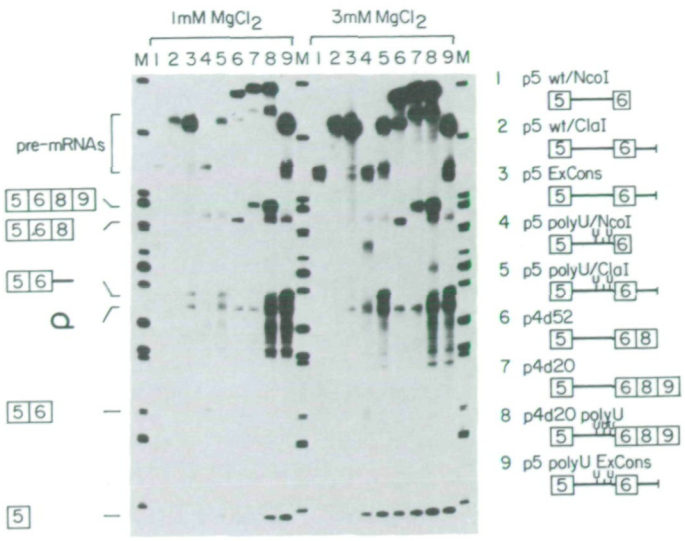

Figure 3. *In vitro* splicing of pre-mRNAs containing mutations in the 5′ and 3′ splice sites of exon 6. *In vitro* splicing reactions were carried out for 2 hours and the ^{32}P-RNA products were separated in a 4% denaturing polyacrylamide gel. The assay conditions used for these studies consisted of 15 μl of nuclear extract in a final reaction volume of 25 μl containing 1 mM or 3 mM $MgCl_2$, 500 μM ATP, 20 mM creatine phosphate, 2.6% (w/v) polyvinyl alcohol, 15–30 ng pre-mRNA, 12.8 mM HEPES (pH 8), 12% (v/v) glycerol, 60 mM KCl, 0.12 mM EDTA and 0.3 mM DTT. Schematic representation of the products and intermediates are indicated on the left and right of the panel; from top to bottom are: i) pre-mRNA, ii) lariat/exon intermediate, iii) free lariat, iv) free lariat but 3′ end is degraded up to the lariat branch points by 3′ exonucleolytic activity, v) splice product, vi) free exon 5. Lanes marked M are markers using pBR322 digested with MspI. Schematic diagrams of the pre-mRNAs used are shown on the right.

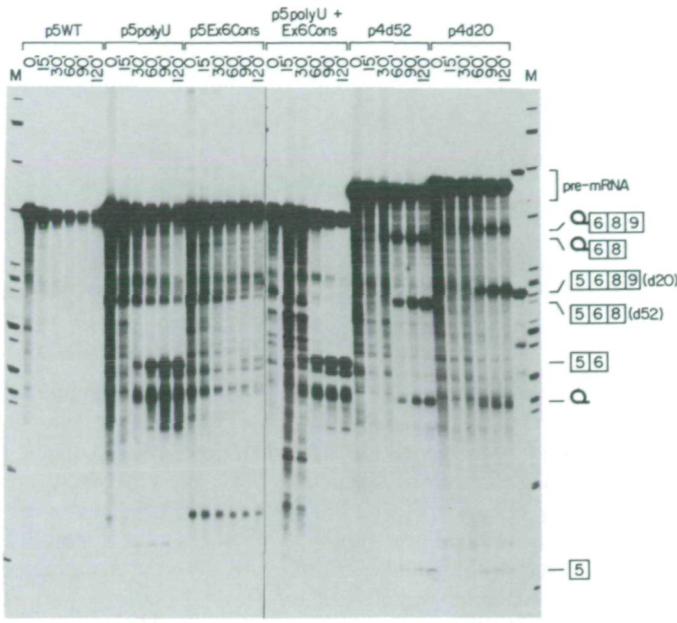

Figure 4. Analysis of *in vitro* splicing products of tropomyosin pre-mRNAs. Time course (in minutes) of formation of spliced products from different pre-mRNAs derived from the β-TM gene. The assay conditions were the same as described in Figure 3, except 1 mM $MgCl_2$ was used here. Schematic representation of products are indicated; the boxes represent exon sequences and the lines intron sequences.

RESULTS

Different elements can activate the 3′ splice site of exon 6

The splicing of exon 5 to exon 6 requires that exon 6 first be spliced to the downstream common exon 8 (32). Pre-mRNAs containing exon 5, intron 5 and exon 6 are not normally spliced *in vitro*. Therefore, the nucleotide sequences within exon 6 and the adjacent upstream intron does not seem to contain sufficient information to be recognized as a 3′-splice site by the splicing machinery. We have carried out a mutational analysis to determine which sequences in the pre-mRNA contribute to the inability of this precursor to be spliced *in vitro*. Two types of mutations were made in the 5′ and 3′ splice sites of exon 6 (Figure 2). The first one introduced a nine nucleotide stretch of poly U in the 3′-splice site of exon 6. The second mutation changed the 5′-splice site of exon 6 to a U1 consensus binding site. ^{32}P-labeled pre-mRNAs containing these mutations were synthesized and tested *in vitro* (Figure 3). In agreement with our previous studies, precursors containing only the 5′-half of exon 6 (generated from the template linearized at an NcoI site in the middle of exon 6) or those containing all of exon 6 and 17 nucleotides of downstream intron were not spliced under assay conditions using 1 mM or 3 mM $MgCl_2$ (Figure 3, lanes 1 and 2). In contrast, precursors containing a nine nucleotide poly U tract in the 3′-end of intron 5 were efficiently spliced (Figure 3, lanes 4 and 5) even with as little as 35 nucleotides of exon 6 (Figure 3, lane 4). Introduction of a consensus 5′-splice site in exon 6 led to splicing of exon 5 to exon 6 (Figure 3, lane 3). The results obtained with the consensus 5′-splice site in exon 6 are in agreement with the exon definition model (55). However, pre-mRNAs containing this mutation were spliced less efficiently (Figure 3, lane 3), compared to the mutants containing the poly U tract (Figure 3, lanes 4 and 5) or when exon 8 was joined to 6 (Figure 3, lanes 7 and 8). We also performed a time course study using the different pre-mRNAs described in Figure 3. The splicing reactions were carried out for 0, 15, 30, 60, 90 and 120 min, respectively (Figure 4). The wild-type pre-mRNA was not spliced at all . Interestingly, pre-mRNAs containing the poly U tract exhibited somewhat more rapid kinetics, as indicated by the appearance of splicing intermediates, i.e., free exon 5 and the lariat/exon intermediate, at as early as 30 min incubation. On the other hand, constructs containing exon 6 joined to exon 8 (p4d52 and p4d20) exhibited a somewhat slower rate of splicing kinetics. Again, pre-mRNAs containing the consensus sequence in the 5′-splice site of exon 6 was spliced less efficiently, and splice products were not detected until after 90 min. incubation. Thus these sequence elements exhibited different relative strengths with respect to their ability to activate the 3′-splice site of exon 6 presumably by interacting with distinct cellular factors (see below). Furthermore, these studies demonstrate that three distinct sequence elements can act independently to activate the use of the 3′-splice site of exon 6: (1) the sequences contained within exon 8 when joined to exon 6 (exon-recognition element), (2) a poly U tract in intron 5, and (3) the 5′-splice site of exon 6.

Effects of mutations in the 5′ and 3′ splice sites of exon 6 on alternative splice site selection

It was of interest to determine how these mutations in the 5′- and 3′-splice sites of exon 6 would affect alternative splice site

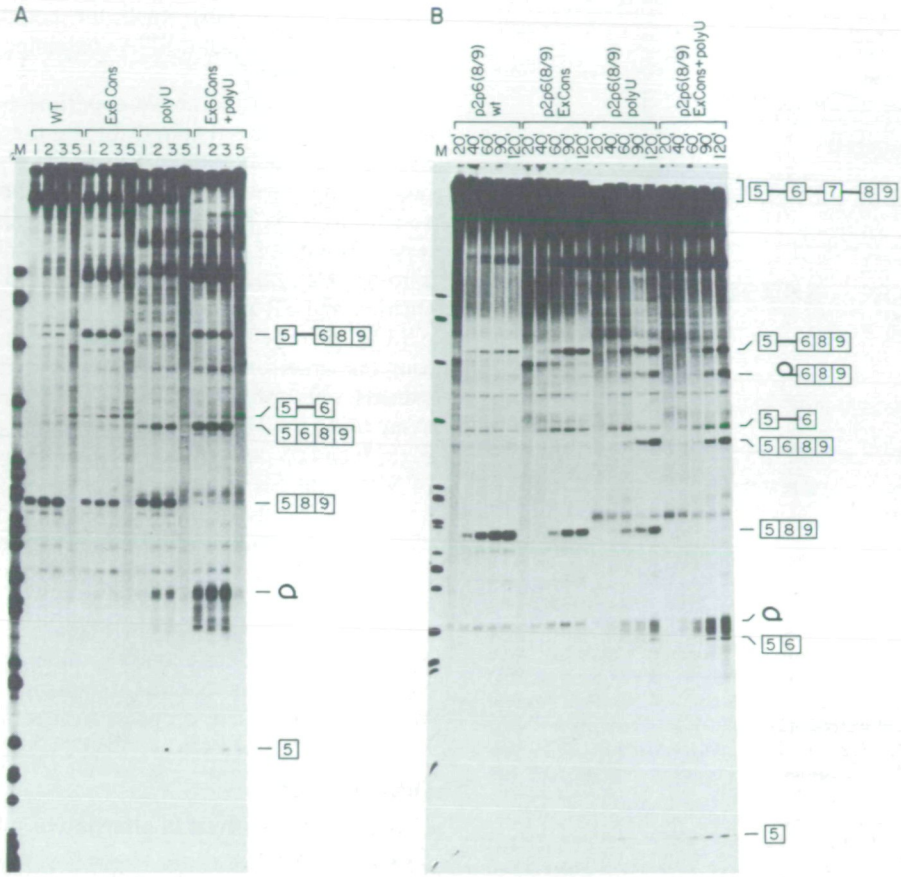

Figure 5. Effect of poly U and exon 6 consensus 5′-splice site on alternative splicing of β-tropomyosin pre-mRNA. **(Panel A)** Effect of magnesium concentration on alternative splicing *in vitro*. Tropomyosin pre-mRNAs derived from pSP64-p2p6 linearized with ClaI were processed for 2 h *in vitro* using the assay conditions described in Figure 3 except the magnesium concentration was varied as indicated at the top of the figure. The splicing products were analyzed on a 4% polyacrylamide denaturing gel. Schematic representation of the products are shown. The markers are ^{32}P-labelled Msp I fragments of pBR322 (M). **(Panel B)** Time course (in minutes) of formation of spliced products from transcripts terminating at the ClaI site of plasmids pSP64-p2p6. The ^{32}P-RNA products were separated in 4% denaturing polyacrylamide gels. The assay conditions used for were the same as described in Figure 3, except that 3 mM $MgCl_2$ was used here. Schematic representation of products and intermediates are shown.

selection using precursors containing the mutually exclusive, alternatively spliced cassette comprised of exons 5−8 (Figure 5). We analyzed the splicing of pre-mRNAs derived from pSP64-p2p6 linearized with ClaI (32). We previously found that alteration in magnesium concentration affect the choice of splice sites using the wild-type p2p6 pre-mRNA (32). Therefore, we examined the effects of changing the concentrations of $MgCl_2$ on splice site utilization in pre-mRNAs containing the mutations mentioned above. In agreement with our previous studies, at 1 mM $MgCl_2$ the wild-type pre-mRNA exhibited exon-skipping, i.e., joining exon 5 to exon 8 (Fig. 5). As the $MgCl_2$ concentration was increased above 1 mM, cleavage at the 5′ splice site of exon 6 as well as splicing of exon 6 to exon 8 were detected. Splicing for all pre-RNAs was found to be inhibited at 5 mM $MgCl_2$. In contrast, splicing of the precursor containing the consensus in the 5′-splice site of exon 6 was no longer sensitive to changes in the $MgCl_2$. Although this precursor still produced some exon skipping product, there was substantial amount of the product formed from splicing exon 6 to 8. In addition, there was only very low levels of fully-spliced RNA i.e., exons 5+6+8+9. *In vitro* splicing of the pre-mRNA containing the poly U tract at the 3′-end of intron 5 not only facilitated the splicing of exon 6 to 8, but also resulted in substantial amounts of fully-spliced pre-mRNA, i.e., exons 5+6+8+9. Finally, combining the poly U tract and consensus site in exon 6 had an additive effect. This double mutant precursor no longer exhibited any exon skipping, and produced significantly higher levels of fully spliced pre-mRNA.

Our previous studies demonstrated that there was an ordered pathway involved in utilization of exon 6, in which splicing of exon 6 to exon 8 preceded splicing of exon 5 to exon 6. We wished to determine if the poly U tract and consensus 5′-splice site would alter this ordered pathway. Therefore, we examined splicing pathways of the different tropomyosin pre-mRNAs in greater detail by carrying out a time course study (Figure 5, right panel). Both the wild-type precursor and the mutant precursor with the consensus 5′ splice site in exon 6 produced only two splice products, exons 5+8+9 (exon skipping) and exon 6 joined to exon 8. Neither substrate exhibited splicing of exon 5 to 6. Thus, the major effect of introducing a consensus 5′-splice site in exon 6 is promoting the splicing of exons 6 to 8. The time course of *in vitro* splicing of pre-mRNAs containing poly U tract

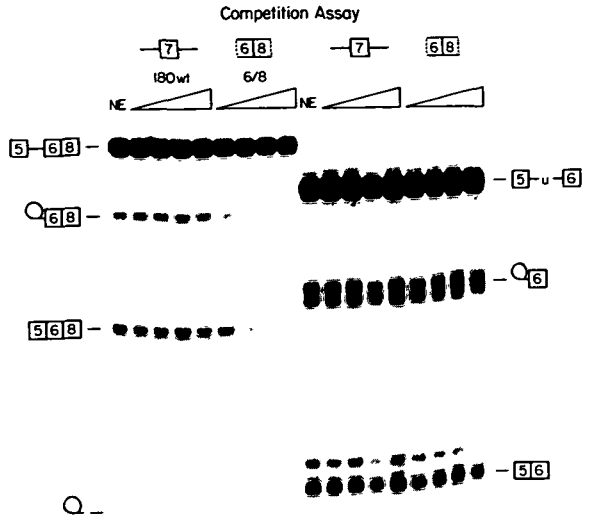

Figure 6. Effect of competitor RNAs on splicing *in vitro*. Tropomyosin pre-mRNAs derived from p4d52 and poly U tract-containing plasmids that were linearized with ClaI were processed for 2 h using the assay conditions described in Figure 3 except 10 μl of nuclear extract (NE) were used in a final reaction volume of 25 μl, in the presence of increasing concentrations of competitor RNAs. The splicing products were analyzed on a 4% polyacrylamide/UREA gel. Schematic representation of the precursors, intermediates, and products are indicated.

in intron 5, either alone or in combination with the consensus 5′-splice site in exon 6, revealed a preferred order of intron removal where exon 6 was first spliced to exon 8 before being spliced to the upstream exon 5. These data demonstrate that introduction of the poly U tract upstream of exon 6 and changing the 5′-splice site of exon 6 to a consensus sequence, either alone or in combination, facilitates the use of exon 6 *in vitro*, such that exon 6 is spliced more efficiently to exon 8, indicating that intron sequences upstream of an exon can contribute to the use of the downstream 5′-splice site. These results also demonstrate that, in addition to sequences in and around exon 7 (17, 25), multiple cis-elements surrounding exon 6 may also contribute to tissue-specific alternative splice site selection of β-TM pre-mRNA.

Distinct factors are required for different 3′-splice sites

The results presented above indicate that three distinct sequence elements can act independently to activate the 3′-splice site of exon 6. In order to determine if the activation by the poly U tract and by the sequence in exon 8 involved the same factors, we carried out an *in vitro* competition experiment using two kinds of competitor RNAs. One competitor had the sequence of the 3′-half of exon 6 joined to the 5′-half of exon 8, and the other contained exon 7 and the flanking intron sequences (Figure 6). *In vitro* splicing of the precursor containing exon 6 joined to exon 8 (p4d52 substrate) or containing the poly U tract in intron 5 was carried out in the absence or presence of increasing concentrations of competitor RNAs (Figure 6). Addition of the competitor RNA which contained sequences in and around exon 7 had virtually no effect on the splicing efficiency of either pre-mRNA. However, addition of the competitor which contained sequences in exons 6 and 8 inhibited the splicing of the p4d52 precursor. On the other hand, this competitor had no effect on the splicing of the pre-mRNA containing the poly U tract in the 3′-end of intron 5.

The data from the above experiments strongly suggest that distinct factors are required for the use of the 3′-splice site in exon 6 in the poly U tract containing and p4d52 substrates. In order to identify these factors we have begun to develop *in vitro* systems to assay for these 3′-splice site splicing factors. We first tested the ability of four different pre-mRNAs to be spliced in a restrictive splicing system comprised of a HeLa cell S100 fraction and SR proteins (Fig. 7). The pre-mRNAs used were p7(8/9), p5 poly U, p5 Ex6 consensus, and p4d52. Although all four precursors were able to be spliced in HeLa cell nuclear extracts, only two were spliced efficiently in the complementation assay using the HeLa cell S100 fraction plus SR proteins. Both p7(8/9) and p5 poly U pre-mRNAs were spliced in the presence of S100 plus SR proteins. By contrast, the p5 Ex6 consensus and the p4d52 pre-mRNAs were spliced relatively inefficiently. The difference between the ability of the p5 poly U and p4d52 pre-mRNAs to be spliced in the presence of S100 and SR proteins again suggests that use of the 3′-splice site in exon 6 in these two precursors require distinct factors. Thus it appears that the S100 fraction contains a limiting amount of a factor(s), other than SR proteins, that is required for splicing the p4d52 pre-mRNA. Work is currently in progress to identify this activity.

DISCUSSION

Cis-elements involved in alternative splice site selection in β-TM pre-mRNA

Our previous studies demonstrated that the nucleotide sequences contained in exon 6 itself do not contain sufficient information to interact with splicing factors to form a functional splicing complex with respect to utilization of its 3′-splice site (32). The present work extended these studies and showed that three distinct elements could act independently to activate the 3′-splice site of exon 6: (1) the sequences contained within exon 8 when joined to exon 6 (exon recognition element), (2) a poly U tract introduced in 3′-end of intron 5, and (3) a consensus 5′-splice site introduced in exon 6. We also investigated the effects these elements exerted on alternative splice site selection. We found that the poly U tract in intron 5 or the consensus 5′-splice site in exon 6, either alone or in combination, facilitated the splicing of exon 6 to exon 8. Thus, sequences in the 3′-splice site of exon 6 were found to facilitate the use of the downstream 5′-splice site of this exon (Figure 5). The mechanism by which the activation by these elements occurs is presently unknown. What factors the poly U tract recruits remains to be determined. It is possible that the poly tract simply serves as a better binding site for factors such as U2AF (56), and thereby promotes the binding of U2 snRNP which in turn facilitates the binding of U1 to the downstream 5′-splice site. In the exon definition model recently proposed by Berget and colleagues, exons are recognized and defined as units during early spliceosome assembly by factors that recognize the 3′ and 5′ splice sites bordering an exon (55). A number of laboratories have found that sequences at the 5′-splice site of an exon facilitates the use of the upstream 3′-splice site (6,8,55,57). The experiments presented here are in agreement with the exon definition, that is, sequences upstream of an exon can facilitate use of the downstream 5′-splice site. It is worth noting that both mutations in the 3′ or 5′ splice site

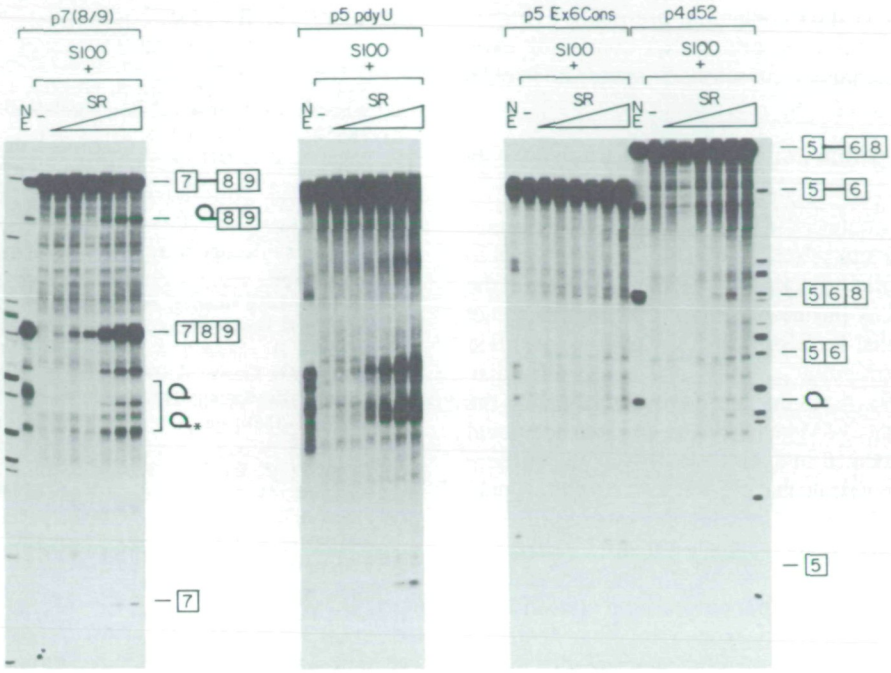

Figure 7. Effects of SR proteins on splicing of different β-tropomyosin pre-mRNAs *in vitro*. The names and structures of the RNAs are shown on the top and side of the panels. Each pre-mRNA was spliced in the presence of nuclear extract (NE) or S100 plus increasing amounts of SR proteins. The positions of the pre-mRNAs, products and intermediates are indicated.

of exon 6 facilitated the joining of exon 6 to exon 8 before the splicing of exon 5 to 6. Thus there was still a preferred order of intron removal. Interestingly, introduction of a 5′-splice site consensus in exon 6 promoted the joining of exon 5 to 6 in the absence of exon 8, but this effect was rather weak (Figure 3, lanes 3). However, the presence of both a consensus 5′-splice site in exon 6 and a polu U tract in intron 5 promoted the formation of fully spliced product. Collectively, these results suggest that distinct factors are likely interacting with these elements.

Previous studies from our laboratory have shown that use of the skeletal muscle exon 7 in nonmuscle cells is blocked by factors that interact with sequences within exon 7 and the adjacent upstream intron (17,25,30,47). The results reported here demonstrate that, in addition to sequences in and around exon 7, multiple cis-acting elements surrounding exon 6 (nonmuscle-type splice) also contribute to tissue-specific alternative splice site selection.

Distinct factors are likely required for certain 3′-splice sites

A number of sequence elements are known to play a role in the use of a 3′-splice site, including the branch point sequence and its adjacent polypyrimidine tract, as well as sequences contained within the adjacent downstream exon (reviewed in 58). The results in the present studies provide biochemical evidence that different factors are required for the use of certain 3′-splice sites. Using biochemical assays, we have determined that at least two of these sequence elements interact with distinct factors for 3′-splice site utilization. First, using a competition assay we were able to show the presence of a putative trans-acting factor specific for the p4d52 pre-mRNA. Although addition of a competitor

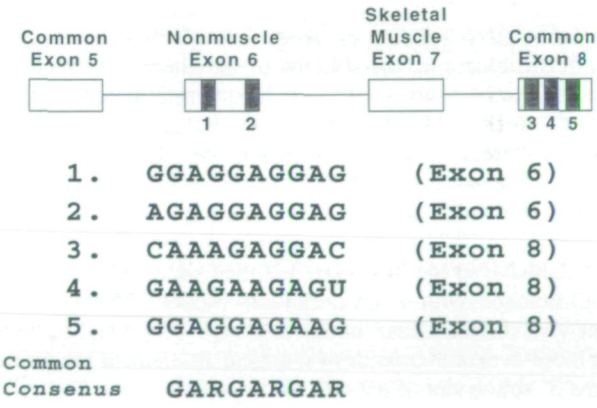

Figure 8. Position of purine-rich motifs in β-TM pre-mRNA. (top) The position of the purine-rich motifs are indicated by shaded regions in exons 6 and 8. Exon 6 contains two motifs and exon 8 contain three motifs. (lower) sequences of the five identified motifs and the sequence of a putative consensus motif as proposed by Xu *et al.*, 1993 (55).

RNA containing sequences from exon 6 and 8 inhibited the splicing of exon 5 to exon 6 with the p4d52 pre-mRNA, it had virtually no effect on the poly U tract-containing substrate (Figure 6). Second, HeLa cell nuclear extracts were able to splice three different pre-mRNAs used in the present studies, that contained the three sequence elements, respectively, but an S100 faction plus SR proteins was only able to support the splicing of exon

5 to exon 6 in the poly U tract containing pre-mRNA (Figure 7). In the latter assay conditions, pre-mRNA containing exon 6 joined to exon 8 or containing a consensus 5'-splice site in exon 6 was spliced poorly.

A purine-rich element within exon 8 likely serves as a splicing signal

Our data indicates that sequences in exon 8 contain an important determinant in 3'-splice site selection. The joining of exon 8 to exon 6 juxtaposes a splicing signal that thereby activates the 3'-splice site of exon 6. A purine-rich motif that appears to act as a general splicing signal for 3'-splice sites has been identified in the mouse immunoglobulin μ (IgM) and chicken cardiac troponin-T genes (38,39). A similar motif has been found at the 5'-end of exon 8 of the rat β-TM gene (Figure 8). Two additional purine-rich motifs are located in exon 8. However, our present and previous studies demonstrate that pre-mRNAs containing only 26 nucleotides of exon 8 are required for the splicing of exon 5 to exon 6, while pre-mRNAs containing less than 12 nucleotides of exon 8 are not processed (32, and present studies). Thus it appears that sequences within the first purine-rich motif in exon 8 are sufficient for activating the exon 5 to exon 6 splice. Additionally, exon 6 contains two copies of the purine-rich motif. Although these sequences alone are not sufficient for promoting the use of the 3'-splice site of exon 6 (Figure 3), it remains to be determined if these elements are required together with sequences contained within exon 8. In this regard, work on the mouse IgM gene demonstrated that multiple elements were required (38).

Studies of the rat preprotachykinin gene have also shown that alternative RNA splicing proceeds through an ordered pathway (8). In this example, the splicing of the alternative exon to the upstream exon cannot occur until the downstream common exon is first joined to the alternative exon. Interestingly, the critical cis-acting element for the use of the alternative exon in preprotachykinin pre-mRNA is located at the 5' splice site of the downstream common exon. Joining of the downstream common exon to the alternative exon promotes the binding of the U2snRNP to the upstream 3'-splice site of alternative exon in agreement with exon-definition model (55,57). This is somewhat different than in the case of β-TM, where a purine-rich motif in exon 8 provides the critical cis-element for activation of the 3' splice site of exon 6. Our results suggest that splicing factors cannot bind or recognize the purine-rich motif in exon 6, but requires the purine-rich motif in exon 8. Work on the IgM μ gene indicates that multiple elements are required for activation. Whether the sequence elements in both exons 6 and 8 are required for 3'-splice site activation remains to be determined.

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