Identification of two distinct intron elements involved in alternative splicing of β-tropomyosin pre-mRNA

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The rat β-tropomyosin gene encodes two isoforms, termed skeletal muscle β-tropomyosin and fibroblast last tropomyosin 1 (TM-1), via an alternative RNA processing mechanism. The gene contains 11 exons. Exons 1–5 and exons 8 and 9 are common to all mRNAs expressed from the gene. Exons 6 and 11 are used in fibroblasts, as well as smooth muscle, whereas exons 7 and 10 are used only in skeletal muscle. In the present studies we focused on the mutually exclusive internal alternative splice choice involving exon 6 (fibroblast-type splice) and exon 7 (skeletal muscle-type splice). We have identified two distinct elements in the intron, upstream of exon 7, involved in splice site selection. The first element is comprised of a polypyrimidine tract located 89–143 nucleotides upstream of the 3′ splice site, which specifies the location of the lariat branchpoints used, 144–153 nucleotides upstream of exon 7. The 3′ splice site AG dinucleotide has no role in selection of these branchpoints. The second element is comprised of intron sequences located between the polypyrimidine tract and the 3′ splice site of exon 7. It contains an important determinant in alternative splice site selection, because deletion of these sequences results in the use of the skeletal muscle-specific exon in nonmuscle cells. We propose that the use of lariat branchpoints located far upstream from a 3′ splice site may be a general feature of some alternatively excised introns, reflecting the presence of regulatory sequences located between the lariat branch site and the 3′ splice site. The data also indicate that alternative splicing of the rat β-tropomyosin gene is regulated by a somewhat different mechanism from that described for rat α-tropomyosin gene and the *transformer-2* gene of *Drosophila melanogaster*.

[*Key Words*: Alternative splicing; RNA processing; tropomyosin]

A growing number of cellular and viral genes have been characterized that encode multiple protein isoforms via the use of alternatively spliced exons (for reviews, see Ziff 1980; Leff et al. 1986; Breitbart et al. 1987). In many cases, alternative RNA splicing contributes to developmentally regulated and tissue-specific patterns of gene expression. At present, the molecular basis for alternative splice site selection is not known, although a great deal of information is available concerning the general splicing reaction of simple transcription units (for reviews, see Green 1986; Padgett et al. 1986; Krainer and Maniatis 1988).

In simple transcription units, splice site selection is achieved through the recognition of highly conserved sequences at the 5′ and 3′ splice junctions by different cellular factors (Breathnach and Chambon 1981; Mount 1982; Keller and Noon 1984; Green 1986; Padgett et al. 1986). Thus, the 5′ splice site consensus sequence AG/GTPuAGT, has been shown to interact with the 5′ end of the U1 small nuclear RNA (Mount et al. 1983; Black et al. 1985; Zhuang et al. 1987; Seraphin et al. 1988), whereas the 3′ splice site consensus (Py)$_n$NPyAG/G (Breathnach and Chambon 1981; Mount 1982) interacts

with several cellular factors whose precise nature has not been completely established. One of these, however, is a 70- to 100-kD protein that is probably associated with the U5 small nuclear ribonucleoprotein (snRNP) (Gerke and Steitz 1986; Tazi et al. 1986). In addition, analysis of the branchpoints in a number of cellular and viral genes identified a loosely conserved sequence PyN-PyUPuAPy, with the adenosine residue usually serving as the site of branchpoint formation 18–40 nucleotides upstream of a 3′ splice site (Keller and Noon 1984; Reed and Maniatis 1985; Ruskin et al. 1985). U2 snRNP is thought to interact with the pre-mRNA at the branch-point region, most likely through direct base-pairing with the pre-mRNA (Black et al. 1985; Konarska and Sharp 1986; Parker et al. 1987; Bindereif and Green 1987; Hartmuth and Barta 1988; Wu and Manley 1989; Zhuang and Weiner 1989). In addition, a factor termed U2AF (U2 snRNP auxiliary factor) has been reported to be necessary for the binding of U2 snRNP to the branch-point and for splicing complex assembly (Ruskin et al. 1988).

At present little is known about the 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. Sequence comparisons between splice junctions of alternative and constitutive exons have failed to reveal any significant differences, suggesting that the regulation of splice site selection in transcripts containing alternative 5' or 3' splice sites will involve other cis-acting elements (Breitbart et al. 1987). A number of features in the pre-mRNA may be involved in alternative splice site selection, including intron size (Fu and Manely 1987), the relative strengths of 5' splice sites (Zhuang et al. 1987), the pyrimidine content of a 3' splice site (Fu et al. 1988), location of branchpoints (Gattoni et al. 1988; Helfman and Ricci 1989; Smith and Nadal-Ginard 1989), multiple alternative branchpoints (Noble et al. 1987 1988; Gattoni et al. 1988; Helfman and Ricci 1989), branchpoint sequences (Reed and Maniatis 1988; Zhuang et al. 1989), and exon sequences (Somasekhar and Mertz 1985; Reed and Maniatis 1986; Mardon et al. 1987; Helfman et al. 1988; Cooper and Ordahl 1989; Hampson et al. 1989; Streuli and Saito 1989). In addition, exon and intron sequences could play a role in alternative splicing by regulating the accessibility of different exons to the splicing machinery by the formation of RNA secondary structures (Khoury et al. 1979; Munroe 1984; Solnick 1985; Eperon et al. 1986, 1988; Edlind et al. 1987; Leff et al. 1987).

We have used the rat β-tropomyosin gene as a model to investigate developmental and tissue-specific alternative splicing (Helfman et al. 1986, 1988; Helfman and Ricci 1989). This gene spans 10 kb with 11 exons and encodes two distinct isoforms, namely skeletal muscle β-tropomyosin and fibroblast TM-1 (see Fig. 1). Exons 1–5, 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. Our previous studies of tropomyosin pre-mRNA splicing in vitro, using HeLa cell systems, revealed an ordered pathway of splicing in which either of the internal alternatively spliced exons (exon 6 or 7) must first be joined to the downstream common exon before they can be spliced to the upstream common exon (Helfman et al. 1988). We characterized the branchpoints formed during use of these alternatively spliced exons. The splicing of exon 5 to exon 7 (skeletal muscle-type splice) is accompanied by the selection of multiple branchpoints, which are located an

unusually long distance (144, 147, and 153 nucleotides) from the 3' splice site of exon 7 (Helfman and Ricci 1989). These results are different from most branchpoints mapped, in which a single adenosine residue located 18–40 nucleotides from a 3' splice site is used during lariat formation.

We have now investigated the functional role of the intron sequences between the unusual branchpoints and the 3' splice site of the skeletal muscle exon (exon 7) in splice site selection of the rat β-tropomyosin pre-mRNA. Our results indicate that two distinct elements are present in this region. The first element is a polypyrimidine tract that specifies the use of the branchpoints located far upstream from the 3' splice site; the second element is located immediately upstream of exon 7 and inhibits the use of the skeletal muscle exon (exon 7) in nonmuscle cells. The unusual location of these branchpoints located far upstream from the 3' splice site in this and perhaps other alternatively excised introns thus reflects the presence of new cis-acting elements located just upstream of the 3' splice site, which are involved in alternative splice site selection.

Results

The splicing of exon 5 to exon 7 (skeletal muscle-type splice) is associated with the use of multiple branchpoints located 144–153 nucleotides upstream of the 3' splice site of exon 7 (Helfman and Ricci 1989). To determine what sequences in intron 6 contributed to the use of these branchpoints, first we constructed a series of deletion mutants in which 53–167 nucleotides were removed upstream of the 3' splice site. All of these deletions from the 3' end of the intron maintained the last 7 nucleotides of intron 6 at the 3' splice site of exon 7. The sequence of intron 6 and the position of the deletions are indicated in Figure 2. To determine whether sequences upstream of the branchpoint sequences affect the efficiency of splicing or the position of branch formation, a set of deletion mutants was constructed in which 14–180 nucleotides were removed downstream of the 5' splice site of exon 6. These deletions maintained the first 17 nucleotides of intron 6 at the 5' splice site of exon 6. The deletions were introduced into the plasmid pSP64-p2(7/8) for in vitro experiments and into plasmids pSV40-p2(7/8) and pSV40-p2 for in vivo analyses (Helfman et al. 1988).
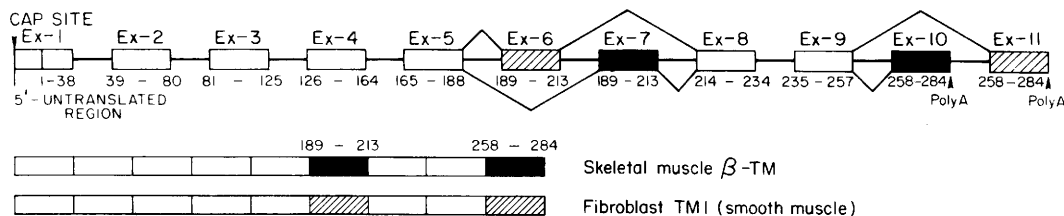

Figure 1. Schematic diagram of the rat β-tropomyosin gene. Open boxes represent constitutive exons; hatched and solid boxes represent tissue-specific exons, as indicated. Horizontal lines represent introns (not drawn to scale). The amino acids encoded by each exon are indicated. The cap site and polyadenylation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

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                              cd21
                                |
     -291 guaguagccucucuaggccuuucuaggcaauggcaccuuuucucacuuca

                         cd3      cd14
                           |        |
     -241 cgucccuccccagcucagcaucucaggcuacugucucucuccugcgguag

          cd30           cd27 d85        d15   d84
            |               |   |          |  * |  *    *
     -191 gaggggcgggggcggagcuuccaaacagccugcugucacucucaucacu

             d81              d92                  cd22
               |                |                    |
     -141 uugcucuucucuuuucuccuccucccuccuccacugugccaccccuccc

              d25          d29            d9
                |            |             |
      -91 ccuaaccccaccccucaccccgucgucgcgccacccccacugucucaccu

                                                    -1
      -41 cacugugcccucacgcuccauccugccacacgcccugcag:EXON 7
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Figure 2. Nucleotide sequence of deletion mutants in intron 6. The nucleotide sequence of intron 6 is shown, as well as the locations of the lariat branch sites used in the splicing of exon 5 to exon 7 (*) and are located 144, 147, and 153 nucleotides from the 3' splice site of exon 7. The 55-nucleotide long polypyrimidine tract located 89–143 nucleotides upstream from the 3' splice site of exon 7 is underlined. Deletions beginning from the 3' end of the intron are indicated: d9, d29, d25, d92, d81, d84, d15, and d85. These deletions maintained the last 7 nucleotides of intron 6. Deletions from the 5' end of the intron are also indicated: cd21, cd3, cd14, cd30, cd27, and cd22. These deletions maintained the first 17 nucleotides of intron 6.

Intron sequences immediately upstream of the skeletal muscle specific exon are not required for splice site utilization in vitro and in vivo

We demonstrated previously that exon 5 is spliced accurately and efficiently to exon 7 in HeLa cell nuclear extracts, using pre-mRNAs derived from plasmid pSP64-p2(7/8) (Helfman et al. 1988). The products of the in vitro splicing reactions with the wild-type and mutant pre-mRNAs containing deletions immediately upstream of exon 7 are shown in Figure 3. In this experiment, RNA transcripts were incubated in the in vitro splicing reaction for 120 min. Following the splicing reactions, the RNA was fractionated on a 4% polyacrylamide/urea gel (Fig. 3, left). As shown in Figure 3, removal of up to 116 nucleotides upstream of the 3' splice site of exon 7 had little affect on splicing efficiency in vitro. On the other hand, pre-mRNAs containing deletions d15 and d85, which removed sequences upstream of the functional branched nucleotides, were not spliced at all, as indicated by the lack of a free 5' exon. In no case did we detect splicing of exon 5 to exon 6 with any of these precursors. These results are in agreement with our previous observations that splicing of exon 5 to exon 6 (fibroblast-type splice) absolutely requires that exon 6 first be joined to exon 8 (Helfman et al. 1988). Thus, splicing of exon 5 to exon 7 requires some, but not all, sequences between the branchpoint sequences and 3' splice site.

Next we determined whether the splicing of exon 5 to exon 7 in the various deletion mutants involves the use of the same branch sites. Precursors derived from the wild-type template and four competent splicing mutants (d9, d29, d25, and d92) were spliced in vitro and the products corresponding to the free lariat were isolated. Half of the RNA was analyzed directly, and the other half was incubated in a HeLa cell S-100 fraction that contained a lariat debranching activity prior to primer extension analysis (Ruskin and Green 1985). Primer extension was performed with a 15-nucleotide ^{32}P-labeled
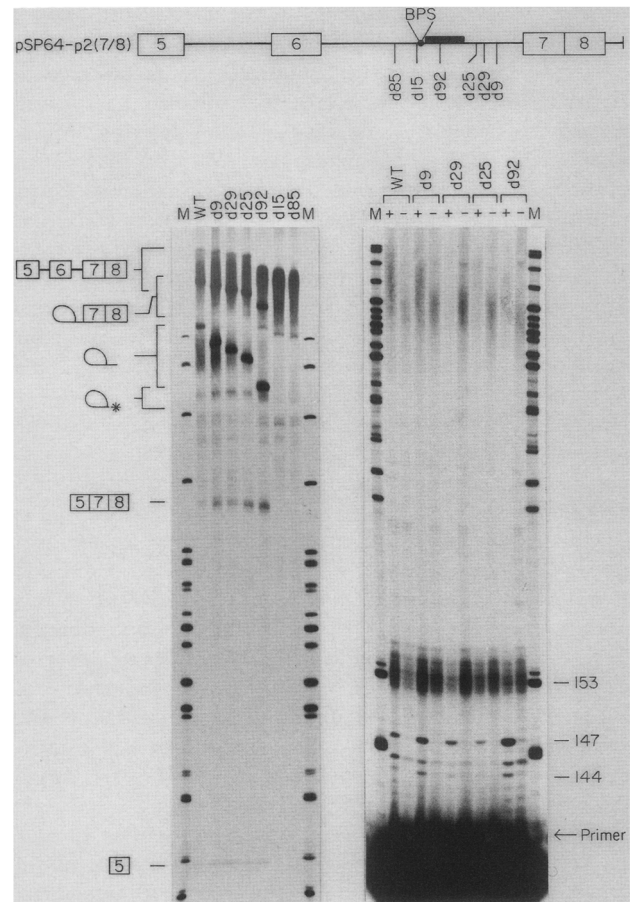

Figure 3. In vitro splicing of pre-mRNAs containing deletions of intron sequences upstream of exon 7. The diagram at top represents the DNA templates derived from pSP64-p2(7/8) linearized with EcoRI. The position of the branchpoint sequences (BPS) and adjacent polypyrimidine tract (shaded box) in intron 6 are also indicated. In vitro splicing reactions were carried out for 2 hr, and the ^{32}P-labeled RNA products were separated in a 4% denaturing polyacrylamide gel. Schematic representation of the products and intermediates are indicated to the left of the autoradiographs and are (from top to bottom) pre-mRNA, lariat/exon intermediate, free lariat, free lariat but 3' end is degraded up until the lariat branchpoints by 3' exonucleolytic activity, splice product, and free exon 5. (Lanes M) Markers using pBR322 digested with MspI. The isolated free lariat was analyzed by primer extension using a ^{32}P-labeled oligonucleotide complementary to sequences in intron 6 (right). Part of the RNA was treated with debranching activity from an S-100 fraction prior to reverse transcription. Analyses of branched RNA (+) and debranched RNA (−) for each pre-mRNA are indicated. The positions of the lariat branch nucleotides, located 144, 147, and 153 nucleotides upstream of the 3' splice site of the wild-type precursor, are indicated. At this level of exposure, the expected band of 396 nucleotides corresponding to the 5' end of the linearized intron following treatment with debranching activity is not visible in this autoradiograph.

deoxyoligonucleotide complementary to sequences within intron 6. Reverse transcriptase has been shown to stop at branched nucleotides (Ruskin et al. 1985). As indicated in Figure 3 (right), splicing of exon 5 to exon 7 with all five substrates involves the use of the same lariat branchpoints. Identical results were also obtained using the isolated lariat/exon intermediates (data not shown). Thus, the position of the lariat branchpoints used was specified independently of the distance between the 3' splice site AG dinucleotide and the branchpoint sequences.

The effects of these intron deletions were also assayed in vivo. The different deletions were introduced into plasmid pSV40-2(7/8), which carries a minigene consisting of the SV40 early promoter and a functional poly(A) site flanking genomic tropomyosin sequences. In previous studies we demonstrated that in HeLa cells, the 3' splice site of exon 7 was used efficiently in this construct (Helfman et al. 1988). HeLa cells were transfected with these seven plasmids, and the resulting transiently expressed cytoplasmic RNAs were isolated. The RNA protection analyses of these RNAs, as well as RNA isolated from mock-infected cells, were carried out with probes derived from cDNA clones encoding skeletal muscle β-tropomyosin (Fig. 4). Transient expression of wild-type plasmid pSV40-p2(7/8) and deletions d9, d29, d25, and d92 in HeLa cells results in spliced RNA that contains exons 5 + 7 + 8 + 9. In contrast, deletions d15 and d85 did not yield spliced products corresponding to the splicing of exons 5 + 7 + 8 + 9. Thus, in agreement with the in vitro data presented above, the splicing of exon 5 to exon 7 in vivo requires some, but not all, sequences between the branchpoint sequences and the 3' splice site.

Most sequences in the 5' end of intron 6 are not required for use of the skeletal muscle-specific exon in vitro

We also introduced a series of deletions in plasmid pSP64-p2(7/8), starting near the 5' end of intron 6 (see Fig. 2). Pre-mRNAs derived from these 5'-intron deletion mutants were processed in vitro (Fig. 5). All but one deletion mutant were accurately and efficiently spliced in vitro (Fig. 5, left). In addition, splicing of these pre-mRNAs is associated with the use of the same branchpoints (Fig. 5, right). Pre-mRNAs derived from pSP64-p2(7/8)cd22 were not spliced at all. This deletion removed the functional branchpoints, including the adjacent downstream polypyrimidine tract. However, this deletion leaves intact a number of potential branch site sequences that are located closer to the 3' splice site of exon 7. For example, good branchpoint consensus sequences are found at positions 29, 74, and 87 nucleotides upstream of the 3' splice site (see Fig. 2). Interestingly, these branchpoints were not selected, even in the absence of the branch nucleotides usually used upstream of these sites. Because the intron sequences within the first 90 nucleotides upstream of exon 7 contain purines distributed every 2–7 nucleotides, the purines might
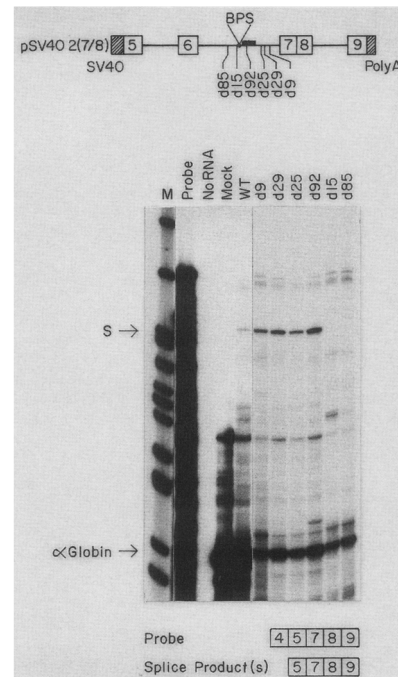

Figure 4. RNA analysis of HeLa cells transfected with tropomyosin minigenes. (*Top*) Schematic diagram of the tropomyosin minigenes used in the HeLa cell transfections. The position of the branchpoint sequences (BPS) and adjacent polypyrimidine tract (shaded box) in intron 6 are also indicated. (*Middle*) Autoradiograph of RNA protection analyses, using probes derived from cDNA clone encoding rat skeletal muscle β-tropomyosin of cytoplasmic RNAs from HeLa cells transfected with the minigenes, as indicated. The band corresponding to the reference α-globin RNAs is labeled (α-globin). (*Bottom*) Schematic diagram of the structure of the RNA probe and protected fragments (labeled S in *middle*) corresponding to spliced RNA from the seven minigenes.

disrupt the interaction of specific splicing factors with these sequences and thereby prevent their use. This hypothesis is supported by recent studies indicating that substitution of purines in the polypyrimidine tract located at the 3' end of intron 1 of the adenovirus major late transcript affects the efficiency of the in vitro splicing reaction (Freyer et al. 1989).

The branch site upstream of the skeletal muscle exon is specified independent of the 3' splice site AG dinucleotide

The above results showed that deletion of most sequences between the 3' splice site of exon 7 and the branchpoints did not affect the splicing of exon 5 to exon 7. To further investigate the nature of the cis-acting sequences that specify the location of the lariat branch points used in intron 6, a series of pre-mRNAs were transcribed from the deletions derived from pSP64-p2(7/8) linearized with PstI. The PstI site (CTGCAG) is located at the 3' end on intron 6. These pre-mRNAs terminate 5 bases short of the 3' end of the intron and have no
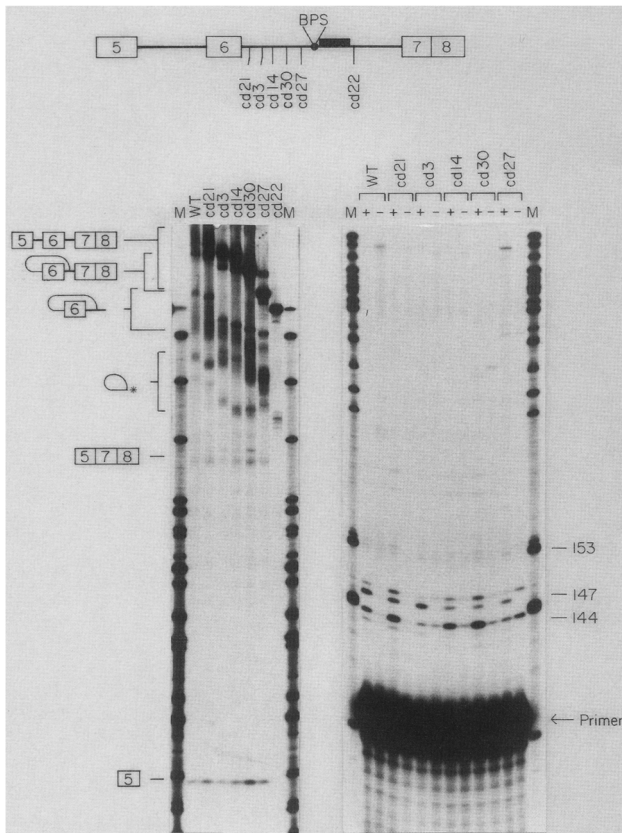
Figure 5. In vitro splicing of pre-mRNAs containing deletions of intron sequences downstream of exon 6. (*Top*) DNA templates derived from pSP64-p2(7/8) linearized with *Eco*RI. The position of the branch point sequences (BPS) and adjacent polypyrimidine tract (shaded box) in intron 6 are also indicated. In vitro splicing reactions were carried out for 2 hr, and the ^{32}P-labeled RNA products were separated in a 4% denaturing polyacrylamide gel. Schematic representation of the products and intermediates are indicated to the left of the autoradiographs and are (from *top* to *bottom*) pre-mRNA, lariat/exon intermediate, free lariat, free lariat with the 3′ end degraded up until the lariat branchpoints by 3′ exonucleolytic activity, splice product, and free exon 5. (Lanes *M*) Markers using pBR322 digested with *Msp*I. One-half of the reaction products were analyzed by primer extension using a ^{32}P-labeled oligonucleotide complementary to sequences in intron 6 (*right*). Part of the RNA was treated with debranching activity from an S-100 fraction prior to reverse transcription. Analyses of branched RNA (+) and debranched RNA (−) for each pre-mRNA are indicated. The positions of the lariat branch nucleotides, located 144, 147, and 153 nucleotides upstream of the 3′ splice site of the wild-type precursor, are indicated. At this level of exposure, the expected band of 396 nucleotides corresponding to the 5′ end of the linearized intron following treatment with debranching activity is not visible in this autoradiograph.

downstream exon sequences. The products of the in vitro splicing reactions with pre-mRNA transcripts from wild-type and mutant pSP64-p2(7/8) linearized with *Pst*I are shown in Figure 6. Interestingly, the wild-type pre-mRNAs and deletions d9, d29, d25, and d92 went through the first step in splicing efficiently, resulting in

cleavage at the 5′ splice site of exon 5 and lariat formation (Fig. 6). On the other hand, deletions d15 and d85 did not exhibit any splicing, as indicated by no detectable cleavage at the 5′ splice site of exon 5. In addition,
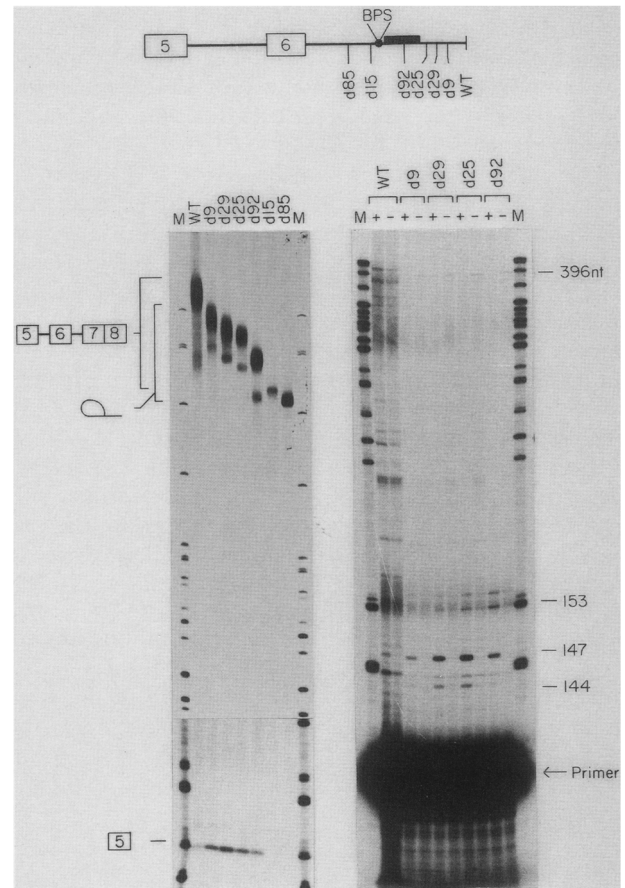

Figure 6. The branch site upstream of the skeletal muscle exon is specified independent of the 3′ splice site AG dinucleotide. (*Top*) DNA templates derived from pSP64-p2(7/8) linearized with *Pst*I. The position of the branchpoint sequences (BPS) and adjacent polypyrimidine tract (shaded box) in intron 6 are also indicated. In vitro splicing reactions were carried out for 2 hr and the ^{32}P-labeled RNA products were separated in a 4% denaturing polyacrylamide gel. Schematic representation of the products and intermediates are indicated to the left of the autoradiographs and are (from *top* to *bottom*) pre-mRNA, free lariat, and free exon 5. (Lanes *M*) Markers using pBR322 digested with *Msp*I. To see free exon 5, it was necessary to photograph the autoradiograph at two exposures; hence, the break in the film above free exon 5. The free lariat formed using the wild-type precursor does not resolve from the unspliced precursor in this gel. The isolated free lariat was analyzed by primer extension using a ^{32}P-labeled oligonucleotide complementary to sequences in intron 6 (*right*). Part of the RNA was treated with debranching activity from an S-100 fraction prior to reverse transcription. Analyses of branched RNA (+) and debranched RNA (−) for each pre-mRNA are indicated. The positions of the lariat branch nucleotides, located 144, 147, and 153 nucleotides upstream of the 3′ splice site of the wild-type precursor, are indicated. The band of 396 nucleotides corresponding to the 5′ end of the linearized intron following treatment with debranching activity is also indicated.

precursors derived from deletions d81 and d84 (see Fig. 2) linearized with *Pst*I also did not undergo the first step in splicing (data not shown). Figure 6 (right) shows that the first step in splicing with all five substrates involves the use of the same branchpoints. Thus, the use of the branchpoints located upstream of exon 7 is specified independently of the AG dincleotide of this 3' splice site.

These results suggested that the branchpoints were specified by the polypyrimidine tract located immediately downstream of the branch sites and that this tract is an important element for splice site utilization. To address this question further, we constructed a series of pre-mRNAs in which the polypyrimidine tract adjacent to the branch nucleotides was deleted from pSP64-p2(7/8). The deletion mutants pSP64-p2(7/8)d92/cd22, pSP64-p2(7/8)d81/cd22, pSP64-p2(7/8)d84/cd22, and pSP64-p2(7/8)d15/cd22 were obtained (Fig. 7). These deletions begin 94 nucleotides upstream of the 3' splice site of exon 7 and remove sequences toward the 5' end of intron 6. The products of the in vitro splicing reactions with the wild-type and mutant pre-mRNAs are shown in Figure 7. Precursors derived from wild-type and pSP64-p2(7/8)d92/cd22, which contains 27 nucleotides of the polypyrimidine tract downstream of the branched nucleotides were spliced accurately and efficiently. In contrast, the three precursors derived from pSP64-p2(7/8)d81/cd22, d84/cd22, and d15/cd22, in which 8 nucleotides or less of the downstream polypyrimidine tract were present, were not spliced at all. These results indicate that the polypyrimidine tract located 89–143 nucleotides upstream of exon 7 represents an important *cis*-acting element.

Removal of intron sequences upstream of exon 7 results in use of the skeletal muscle-specific exon in nonmuscle cells

The data described above demonstrate that splicing of exon 5 to 7 does not require most sequences between the 3' splice site of exon 7 and the upstream branchpoints. However, it is possible that these sequences are involved in alternative splice site selection. The experiments described above used precursors in which there was no *cis*-competition between alternative splice sites (i.e., exons 6 and 7) for upstream and downstream common exons. Accordingly, a series of deletions were introduced into plasmid pSV40-p2, which carries a minigene consisting of the SV40 early promoter and a functional poly(A) site flanking genomic tropomyosin sequences. We demonstrated previously that transient expression of plasmid pSV40-p2 in HeLa cells resulted in spliced RNA that contains exons 5 + 6 + 8 + 9 (Helfman et al. 1988). No other products were observed, and no RNAs were detected that contained exon 7. These results were expected because HeLa cells express the same isoform as rat fibroblasts, namely TM-1 and, therefore, would not be expected to use exon 7.

HeLa cells were transfected with the wild-type and six deletion mutant plasmids (d9, d29, d25, d92, d15, and d85), and the resulting transiently expressed cyto-
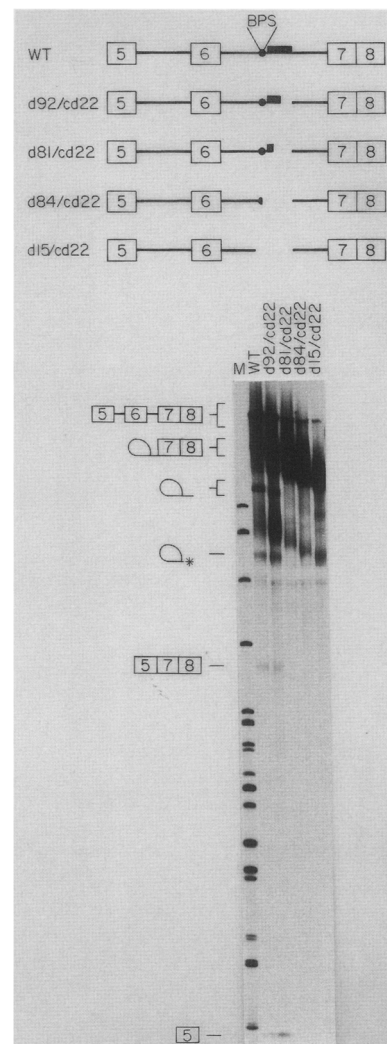

Figure 7. Deletion of the polypyrimidine tract affects pre-mRNA splicing. (*Top*) DNA templates derived from pSP64-p2(7/8) linearized with *Eco*RI. The position of the branchpoint sequences (BPS) and adjacent polypyrimidine tract (shaded box) in intron 6 are also indicated. The nucleotides removed in these deletions correspond to sequences in intron 6 and are shown in Fig. 2 from, −95 to −116 (d92/cd22); −95 to −135 (d81/cd22); −95 to −149 (d84/cd22); and −95 to −155 (cd15/cd22). In vitro splicing reactions were carried out for 2 hr and the ^{32}P-labeled RNA products were separated in a 4% denaturing polyacrylamide gel. Schematic representation of the products and intermediates are indicated at the left and are (from *top* to *bottom*) pre-mRNA, lariat/exon intermediate, free lariat, free lariat but 3' end is degraded up until the lariat branchpoints by 3' exonucleolytic activity, splice product, and free exon 5. (Lanes *M*) Markers using pBR322 digested with *Msp*I.

plasmic RNAs were isolated. The RNA protection analyses of these RNAs, as well as RNA isolated from mock-infected cells, were carried out using RNA probes derived from cDNA clones encoding rat fibroblast TM-1 and skeletal muscle β-tropomyosin (Fig. 8). Transient expression of wild-type plasmid pSV40-p2 in HeLa cells resulted in spliced RNA that contains exons
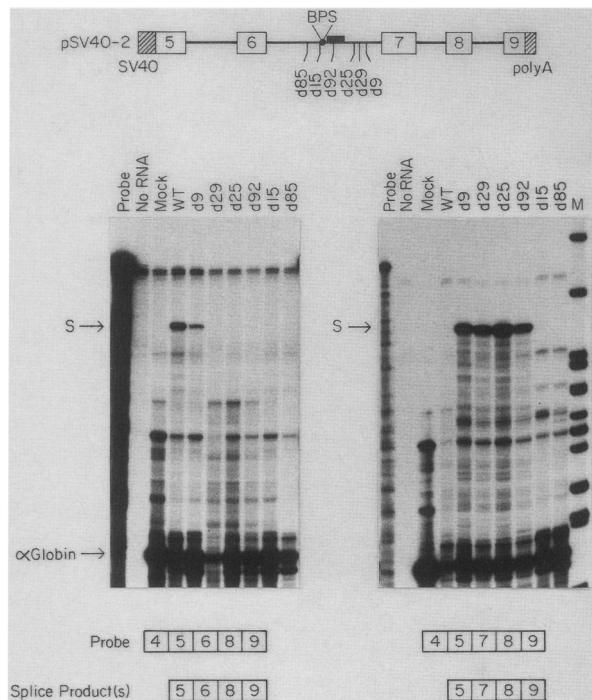
Figure 8. RNA analysis of HeLa cells transfected with tropomyosin minigenes. (*Top*) Schematic diagram of the tropomyosin minigenes used in the HeLa cell transfections. The position of the branchpoint sequences (BPS) and adjacent polypyrimidine tract (shaded box) in intron 6 are also indicated. (*Middle*) Autoradiographs of RNA protection analyses using probes derived from cDNA clones encoding rat fibroblast TM-1 (*left*) and skeletal muscle β-tropomyosin (*right*) of cytoplasmic RNAs from HeLa cells transfected with minigenes, as indicated. The band corresponding to the reference α-globin RNAs is labeled (α-globin). (*Bottom*) Schematic diagram of the structure of the RNA probe and protected fragments (labeled S in *middle*) corresponding to spliced RNA from the seven minigenes.

5 + 6 + 8 + 9 (Fig. 8). No other products were observed, and no RNAs were detected that contained exon 7. Surprisingly, expression of pSV40-p2d9 in HeLa cells resulted in expression of two forms of spliced RNA: one containing exons 5 + 6 + 8 + 9 (fibroblast-type splice), and the other containing exons 5 + 7 + 8 + 9 (skeletal muscle-type splice). Expression of mutants d29, d25, and d92 exhibited only the skeletal muscle-type splice products, with no detectable use of the fibroblast-type splice products. On the other hand, transient expression of deletions d15 and d85 did not result in spliced RNA containing either the fibroblast or the skeletal muscle-type products. At present, the reason for a lack of inclusion of the fibroblast-type exon using these two deletions is unclear. The results presented in Figure 8, together with the results presented earlier in this paper, indicate that intron sequences immediately upstream from the 3' splice site of exon 7 are not required for utilization of this splice site but function by influencing the choice of alternative splice sites by inhibiting the

use of the skeletal muscle-specific exon in nonmuscle cells.

Discussion

The results presented in this paper identify two distinct elements in the intron upstream of the internal skeletal muscle-specific exon (exon 7) of the rat β-tropomyosin gene, which play a role in pre-mRNA splicing. The first element is a polypyrimidine tract located 89–143 nucleotides upstream of the 3' splice site. This polypyrimidine tract is required for the formation of the lariat branchpoints located 144–153 nucleotides upstream of the 3' splice site. The second element consists of intron sequences immediately upstream of the 3' splice site of exon 7, which play an important role in the selection of alternative splice sites. The identification of these two *cis*-acting elements raises some important questions concerning the general mechanism of branchpoint selection and the role of the elements in the regulation of alternative splicing.

Cis-*acting elements involved in branchpoint location*

Most branch sites that have been mapped are located within 18–40 nucleotides of the 3' splice site (Grabowski et al. 1984; Padgett et al. 1984; Ruskin et al. 1984; Zeitlin and Efstratiadis 1984; Konarska et al. 1985; Reed and Maniatis 1985; Ruskin et al. 1985). In addition, mutation of branch nucleotides was found to result in the use of cryptic branchpoints that were also always located within the 18–40 nucleotides upstream from the 3' splice site (Padgett et al. 1985; Ruskin et al. 1985). These data have led to the conclusion that there is a distance constraint responsible for positioning the branchpoint no more than 40 nucleotides from the AG dinucleotide of a 3' splice site (for review, see Green 1986). However, a number of recent studies have demonstrated the use of branchpoints located a relatively long distance from a 3' splice site of alternatively spliced viral and cellular genes. Splicing of adenovirus E1A RNA was found to use multiple branchpoints located 51–59 nucleotides from the 3' splice site (Gattoni et al. 1988). Splicing of α-tropomyosin pre-mRNAs was found to be associated with the use of a single branch site located 177 nucleotides from the 3' sites (Smith and Nadal-Ginard 1989), whereas alternative splicing of β-tropomyosin pre-mRNAs was found to involve the use of multiple branchpoints located 144–153 nucleotides from a 3' splice site (Helfman and Ricci 1989). These studies raise the possibility that the use of branchpoints located a relatively long distance from a 3' splice site may be an essential feature of some alternatively spliced exons. Whether such distant branch sites are found in introns that are not associated with alternative splice site selections is unknown.

The use of branchpoint sequences located far upstream from a 3' splice raises a number of questions regarding the *cis*-acting elements and cellular factors involved in the use of these unusual branchpoints. Pre-

vious studies have demonstrated that formation of the branch nucleotide involves the interaction of U2 snRNP with the pre-mRNA branchpoint sequences (Black et al. 1985; Konarska and Sharp 1986; Bindereif and Green 1987; Parker et al. 1987). Mutational analyses demonstrate that the sequence most important for the U2 snRNP branch point interaction is not the nucleotides in the branch point region itself but, rather, the 3' splice site consensus including the polypyrimidine tract and AG dinucleotide (Ruskin and Green 1985; Chabot and Steitz 1987). Deletions or mutations in the 3' splice site decrease or abolish U2 snRNP binding and splicing complex formation (Frendewey and Keller 1985; Ruskin and Green 1985; Bindereif and Green 1986; Chabot and Steitz 1987). On the other hand, mutations in the branchpoint sequences generally are not associated with the loss of U2 snRNP binding but, instead, result in activation of cryptic branchpoints (Padgett et al. 1985; Ruskin et al. 1985). In addition, the interaction of U2AF with the 3' splice site of a pre-mRNA requires the AG dinucleotide for efficient binding (Ruskin et al. 1988). These results demonstrate that the sequences near the 3' splice site are important for the interaction of U2 snRNP at a given branchpoint.

The present studies address the nature of some of the cis-acting elements involved in the use of the branchpoint sequences located 144–153 nucleotides upstream of the 3' splice site of exon 7 in the rat β-tropomyosin gene. We demonstrated that a polypyrimidine tract located immediately downstream of a branch site functions as an important cis-acting element in branchpoint formation. Deletion of sequences between the 3' splice site and polypyrimidine tract had no effect on branchpoint selection. In addition, we found that the positions of these branchpoints were specified independent of downstream exon sequences and the AG dinucleotide of the 3' splice site. Even in the absence of the AG dinucleotide we were able to obtain the first step in splicing, that is, cleavage at the 5' splice site and formation of the lariat intermediate, as long as there was a polypyrimidine tract adjacent to the branchpoint sequences (Fig. 6). These data indicate that a polypyrimidine tract downstream of a branch sequence can function independently of a 3' splice site in branch site selection. A similar polypyrimidine tract has been found adjacent to the branchpoint, which is located 177 nucleotides upstream of the 3' splice site of exon 3 of the rat α-tropomyosin gene (Smith and Nadal-Ginard 1989). Furthermore, the use of this branchpoint was found to be specified by the adjacent polypyrimidine tract, independent of the 3' splice site (C.W.J. Smith and B. Nadal-Ginard, pers. comm.). In a number of tropomyosin genes, we have observed relatively long polypyrimidine tracts located in the intron upstream of an alternatively spliced exon (Ruiz-Opazo and Nadal-Ginard 1987; Clayton et al. 1988; Wieczorek et al. 1988; Helfman and Ricci 1989; Libri et al. 1989). These include exons 3, 8, 10, and 11 of the rat α-tropomyosin gene (Ruiz-Opazo and Nadal-Ginard 1987; Wieczorek et al. 1988), exons 8 of the human slow α-tropomyosin gene (Clayton et al. 1988),

exon 7 and 10 of the rat β-tropomyosin gene (Helfman and Ricci 1989; S. Erster and D. Helfman, unpubl.) and exons 6b and 10 of the chick β-tropomyosin gene (Libri et al. 1989). In the future, it will be of interest to determine what role, if any, these sequences play in the use of these alternatively spliced exons.

Our current model is that the polypyrimidine tract adjacent to the branch sites specifies the position of the lariat branchpoint. Following formation of the lariat/exon intermediate during the first step in splicing, the splicing machinery selects the first downstream AG dinucleotide, which defines the 3' splice site. In this respect it is worth noting that there are no AG dinucleotides between the branchpoints and the authentic AG dinucleotide of the 3' splice site of exon 7. Whether the same splicing factors are responsible for the use of those branchpoints located near (<40 nucleotides) versus those located far (>40 nucleotides) from a 3' splice site is not known. For example, although U2AF has been reported to require the AG dinucleotide at the 3' splice site, the presence of a long polypyrimidine tract may, itself, function as a binding site for U2AF. Alternatively, other factors may interact with the pre-mRNA in this region.

Function of distant branch sites in splicing

The functional significance of branch sites situated farther than 40 nucleotides from a 3' splice site is not fully understood. In one case the use of a branch site located a long distance from a 3' splice site was found to play an important role in alternative splice site selection. Alternative splicing of the rat α-tropomyosin gene involves mutually exclusive use of exons 2 and 3. These two exons are never spliced together in any cell type. The intron between exons 2 and 3 is 218 nucleotides in length. Interestingly, the branchpoint used upstream of exon 3 was found to be located 177 nucleotides from the 3' splice site of this exon (Smith and Nadal-Ginard 1989). This branch site is positioned only 42 nucleotides from the 5' splice site of exon 2. As a result of the relatively short distance between the 5' splice site of exon 2 and this branch site, exon 2 is unable to splice to exon 3, presumably because the proximity of the 5' splice site and branchpoint results in steric hindrance, thereby preventing splicing factors from interacting with these splice sites. In contrast, this mechanism cannot explain the mutually exclusive use of exons 6 and 7 in alternative splicing of the rat β-tropomyosin gene. The lariat branchpoints located upstream of exon 7 are 136–147 nucleotides from the 5' splice site of exon 6. Thus, a model based simply on steric hindrance cannot explain the lack of splicing of exon 6 to exon 7. Therefore, the locations of these branch sites are likely to play a different role in alternative splicing of the β-tropomyosin gene. The results presented in this paper indicate that the primary role of sequences immediately upstream of the 3' splice site of exon 7 (skeletal muscle-type splice) is to influence the selection of alternative exons. Removal of these sequences resulted in the use of the skel-

etal muscle-specific exon in nonmuscle cells (Fig. 8). It should also be noted that the presence of regulatory sequences between a branch site and a 3′ splice site involved in alternative splicing is not restricted to those cases associated with the use of distant branchpoints. For example, studies of alternative splicing of SV40 large-T and small-t pre-mRNAs demonstrated that mutations in the sequences immediately upstream of the shared 3′ splice site had substantial effects on the use of alternative 5′ splice sites (Fu et al. 1988). In this case, splicing involves the use of multiple branch sites located within 18–32 nucleotides of the 3′ splice site (Noble et al. 1987). Thus, the use of branchpoints located far upstream from a 3′ splice site may be a feature of some introns to provide space for regulatory sequences between a lariat branch site and a 3′ splice site.

Role of intron sequences in regulation of alternative splicing

The mechanism by which the intron sequences immediately upstream of exon 7 play a role in alternative splice site selection is not known. One way in which these sequences could regulate splice site selection is by binding to factor(s) in nonmuscle cells that inhibit the use of this 3′ splice site. Thus, deleting those sequences that bind to a negative-acting factor would consequently lead to activation of the splice site. Such a mechanism, or so-called blockage model, has been proposed for the regulation of a number of alternatively spliced genes, including the *transformer* gene and the *suppressor of white apricot* gene of *Drosophila melanogaster* (Chou et al. 1987; Zachar et al. 1987; Sosnowski et al. 1989). However, the blockage model cannot explain the regulation of the rat β-tropomyosin gene described in this study. For example, by using precursors without deletions in intron 6, we were able to detect splicing of exon 5 to exon 7 both in vitro and in vivo (present studies and Helfman et al. 1988). Therefore, it is unlikely that a negative-acting factor simply binds only to the 3′ splice site of exon 7 and prevents its use in nonmuscle cells. On the other hand, a factor bound to sequences at the 3′ splice site of exon 7 might function by preventing the use of the 5′ splice site of this exon. Such a mechanism would be consistent with the observation that the splicing of exon 5 to exon 7 in nonmuscle cells requires that exon 7 first be spliced to exon 8 (Helfman et al. 1988).

A second way in which these sequences may regulate splice site selection is by participating in the formation of RNA secondary structures that sequester this exon

and prevent its utilization in nonmuscle cells. RNA secondary structure has been suggested to play a role in alternative RNA splicing by regulating the accessibility of different splice sites to the splicing machinery (Khoury et al. 1979; Munroe 1984; Solnick 1985; Eperon et al. 1986, 1988; Edlind et al. 1987). Deleting sequences immediately upstream of exon 7 might prevent the formation of RNA structures that normally block the use of this exon in nonmuscle cells. We have analyzed the possible secondary structures that can form in this region of the pre-mRNA by using a computer program (Zucker and Stiegler 1981). Computer analysis of possible RNA secondary structure involving intron sequences upstream and downstream of the skeletal muscle exon reveals that this exon may be sequestered in a stable hairpin structure (G = −93.2 kcal/mole) (Fig. 9). The sequences analyzed in Figure 9 include 70 nucleotides upstream of exon 7 and 39 nucleotides downstream. We also analyzed sequences including 160 nucleotides upstream of exon 7 (which comprise the polypyrimidine tract and branchpoint sequences) through 126 nucleotides downstream and have found a predicted structure of −196.5 kcal (data not shown). The sequences analyzed in Figure 9 correspond to those sequences upstream of exon 7 which, when deleted, resulted in use of the skeletal muscle exon in nonmuscle cells. Although the use of computer algorithms are not without limitations, it is worth noting that analysis of intron sequences of the same isogene from chick reveals a similar secondary structure that could sequester this exon (Libri et al. 1989). Thus, on the basis of computer analysis, there is phylogenetic conservation of these structures. Additional data consistent with the hypothesis that these intron sequences may function via RNA secondary structures comes from our previous studies demonstrating that splicing of exon 5 to exon 7 in nonmuscle cells required that exon 7 first be joined to the downstream common exon (Helfman et al. 1988). Joining exons 7 and 8 and thereby removing the flanking intron may have prevented the formation of RNA secondary structures that interfered with the interactions of splicing factors with the splice sites of exon 7.

Possible mechanisms for alternative splice site selection

The data in the present studies demonstrate that intron sequences upstream of exon 7 act as a negative regulatory element preventing the use of this exon in nonmuscle cells, possibly by participating in the formation of an RNA structure that sequesters this exon. Non-
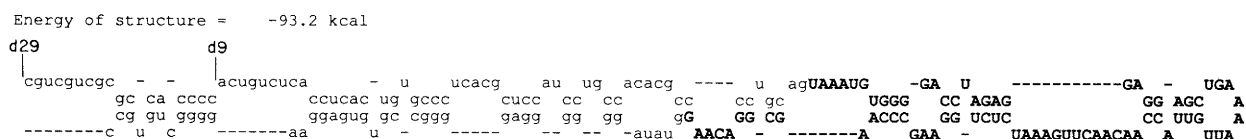

Figure 9. Computer-predicted structure that could sequester the skeletal muscle specific exon. Sequences of exon 7 are shown in uppercase letters. Intron sequences upstream and downstream of this exon are indicated in lowercase letters. The positions of deletions from the 3′ end of intron 6 are indicated (d9, d29).

muscle cells could express factors that interact with the RNA and inhibit the use of exon 7. Alternatively, skeletal muscle could express factors that destabilize an RNA structure and thereby lead to utilization of this exon. Such factors could be RNA-binding proteins that act stoichiometrically by binding to the RNA or they could act catalytically, such as an RNA helicase. For example, a protein that has homology to a family of presumed RNA helicases was found to be required for splicing of the mitochondrial cytochrome *b* (*cob*) and cytochrome *c* oxidase subunit (*cox 1*) genes (Seraphin et al. 1989). Interestingly, this protein was not required for splicing of all introns in these genes but only specific introns. Such an intron-specific RNA helicase could, in principle, lead to tissue-specific alternative splice site selection. In addition, an activity that destabilizes RNA helices has been found in a number of heterogeneous nuclear RNP (hnRNP) preparations (Razziuddin et al. 1982; Thomas et al. 1983; Valentini et al. 1985; Dreyfuss 1986). On the other hand, a factor may simply bind to the pre-mRNA and prevent the formation of a secondary structure. For example, a number of hnRNP proteins have been found to bind to the 3' end of introns (Swanson et al. 1988). Factors may bind to the 3' end of intron 6 of the rat β-tropomyosin gene and inhibit the formation of an RNA structure that would sequester this exon. It is also worth noting that if RNA secondary structure does play a role in alternative splicing, mechanisms of regulation do not have to be limited to factors interacting solely with the pre-mRNA. Work by Eperon et al. (1988) has suggested that there is a critical period in which an RNA can fold after transcription, which could contribute to the regulation of tissue-specific splicing if the rate of transcription along a gene was subject to regulatory control. For example, if, in skeletal muscle, the RNA polymerase were to pause during the transcription of sequences upstream and downstream of exon 7, this could provide time for cellular factors to interact with the pre-mRNA and thereby prevent the RNA from forming a structure that prevents its use in nonmuscle cells.

In conclusion, having identified an important *cis*-acting element in the regulation of alternative splice site selection, we are now in a position to ask how these sequences contribute mechanistically to tissue-specific splicing. Further work will be required to precisely define the sequences involved in splice site selection. We also hope to determine whether muscle and nonmuscle cells express cell-type-specific factors that interact with sequences surrounding exon 7.

Experimental procedures

Plasmid constructions

The DNA templates for use in in vitro transcription are derived from the rat β-tropomyosin gene (Helfman et al. 1986). pSP64-p2(7/8) was described in detail elsewhere (Helfman et al. 1988). Plasmids containing deletions from the 3' end of intron 6 toward the 5' end of the intron were obtained from pSP64-p2(7/8) by first digesting pSP64-p2(7/8) with *Pst*I. The DNA was then

treated with BAL-31 nuclease, and the Klenow fragment of DNA polymerase I to generate blunt ends, followed by ligation to *Pst*I linkers. Plasmids pSp64-p2(7/8) d9, d29, d25, d92, d84, d81, d15, and d85 were obtained by ligating the *Nco*I–*Pst*I fragments from the various deletions to the *Nco*I–*Pst*I sites of pSP64-p2(7/8). The *Nco*I site is located in the middle of exon 6. All deletions from the 3' end of the intron maintained the last 7 nucleotides of intron 6. Plasmids containing deletions from the 5' end of intron 6 toward the 3' end of the intron were obtained from pSP64-p2(7/8) by first digesting pSP64-p2(7/8) with *Stu*I. The *Stu*I site in intron 6 is located 17 bp downstream of the 5' splice site of exon 6. Therefore, these deletions from the 5' end of the intron maintain the first 17 nucleotides of intron 6. Then the DNA was treated with BAL-31 nuclease and the Klenow fragment of DNA polymerase I to generate blunt ends, followed by ligation to *Cla*I linkers. Plasmids pSP64-p2(7/8)cdwt (wild type), cd21, cd3, cd14, cd30, cd27, and cd22 were obtained by ligating the *Cla*I–*Pst*I fragments from the various deletions to the *Cla*I–*Pst*I sites of pSP64-p2(7/8)cdwt. The sequences of all deletions were determined by DNA sequence analysis (Sanger et al. 1977) and are shown in Figure 2.

For studies designed to analyze the internal alternatively spliced region of the rat β-tropomyosin gene, in vivo plasmids pSV40-p2(7/8) and pSV40-p2 were used (Helfman et al. 1988). The deletions prepared from the 5' and 3' ends of intron 6 described above were subcloned into plasmids pSV40-p2(7/8) and pSV40-p2. In all transfection studies HeLa cells were cotransfected with an α-globin test gene (Treisman et al. 1983).

Synthesis of RNA and in vitro splicing

The ^{32}P-labeled SP6/tropomyosin transcripts were synthesized in vitro, primed with Cap analog, as described (Konarska et al. 1984). The ^{32}P-labeled 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 purified further.

HeLa cell nuclear extracts were prepared as described (Dignam et al. 1983; Krainer et al. 1984). In vitro splicing reactions were carried out at 30°C 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 1 mM MgCl$_2$, 500 μM ATP, 20 mM creatine phosphate, 2.7% (wt/vol) polyvinyl alcohol, 15–30 ng pre-mRNA, 12.8 mM HEPES (pH 8), 14% (vol/vol) glycerol, 60 mM KCl, 0.12 mM EDTA, and 0.7 mM dithiothreitol (DTT).

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 (Krainer et al. 1984). The products of the reaction were analyzed on denaturing urea-polyacrylamide 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 (Grabowski et al. 1984; Ruskin et al. 1984). In addition, RNAs containing lariats were verified by treatment by a lariat de-branching activity (Ruskin and Green 1985). Processing products were also analyzed by primer extension analysis. Primer extension analysis was carried out essentially as described (Ghosh et al. 1978; Erster et al. 1988; Helfman and Ricci 1989). An oligodeoxynucleotide (5'-AGGAGGAGAAAA-GAGAA-3'), complementary to sequences in intron 6 located 119–135 nucleotides from the 3' splice site of exon 7, was used

as a primer. The primer was end-labeled with $[\gamma\text{-}^{32}\text{P}]$ATP, using T4 polynucleotide kinase. The primer was annealed to the RNA by incubation for 3 min at 55°C, followed by 30 min at 37°C in 30 µl of primer extension buffer [50 mM Tris-HCl (pH 8.3), 140 mM KCl, 10 mM $MgCl_2$]. For primer extension, reactions were transferred to 42°C and supplemented with DTT (final concentration of 5 mM), deoxynucleotide triphosphates (final concentration of 0.5 mM) and 10 units of avian myeloblastosis virus reverse transcriptase. After 30 min, the reactions were terminated by addition of EDTA to a final concentration of 25 mM, and the radiolabeled pre-mRNA was degraded by treatment with RNase A prior to gel electrophoresis.

Transfections and RNA analysis

HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HeLa cells were transfected with 5 µg of tropomyosin plasmid DNA and 1 µg of α-globin test plasmid (Treisman et al. 1983) per 100-mm plate, using a calcium phosphate coprecipitation procedure, and the cells were harvested 48 hr later. The α-globin plasmid served as an internal control for transformation efficiency. For preparation of cytoplasmic RNA, each 100-mm plate of HeLa cells was washed three times with ice-cold PBS, and the cells were scraped from the plates with a rubber policeman in 1 ml of PBS and transferred to a microfuge tube. The cells were centrifuged for 15 sec and resuspended in 375 µl of ice-cold buffer containing 150 mM NaCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl (pH 8.0), 10% NP-40 was added to a final concentration of 0.5% to lyse the cells. The nuclei were pelleted by 2 min of centrifugation in the cold room, and the supernatant was transferred to a clean tube containing 4 µl of 10% SDS and 5 µl of proteinase K (10 mg/ml). The mixture was incubated at 37°C for 15 min and extracted with phenol, and the nucleic acids were precipitated with ethanol. The RNA preparations were then treated with DNase in the presence of RNasin and reprecipitated.

RNase protection assays were carried out as described (Zinn et al. 1983). The plasmids used for synthesis of ^{32}P-labeled antisense RNA were derived from cDNA clones encoding rat fibroblast TM-1 and skeletal muscle β-tropomyosin (Yamawaki-Kataoka and Helfman 1985; Helfman et al. 1986). The plasmids were prepared by inserting the PstI–ClaI fragments of the fibroblast and skeletal muscle cDNA clones into pSP64. The PstI site, corresponding to amino acids 144 in exon 4, was modified with EcoRI linkers, and the ClaI site, corresponding to amino acids 252 in exon 9, was modified with HindIII linkers. The resulting EcoRI–HindIII fragments were ligated into an EcoRI–HindIII double-cut pSP64 vector. Plasmid was linearized with EcoRI and transcribed with SP6 polymerase to yield a transcript of ~330 nucleotides. Because the transfected genes do not contain sequences in exon 4, it was possible to distinguish RNA derived from the transfected genes from endogenous mRNA. Hybridizations contained 10 µg of total cytoplasmic RNA, 500,000 cpm of tropomyosin probe, and 500,000 cpm of α-globin anti-sense probes (Herr and Clarke 1986) in a total volume of 30 µl. All subsequent steps were carried out as described previously (Zinn et al. 1983), except the RNase digestions were carried out at 30°C for 60 min.

Acknowledgments

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