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# Donor site competition is involved in the regulation of alternative splicing of the rat $\beta$ -tropomyosin pre-mRNA

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## ABSTRACT

The rat  $\beta$ -tropomyosin ( $\beta$ -TM) gene encodes both skeletal muscle  $\beta$ -TM mRNA and nonmuscle TM-1 mRNA via alternative RNA splicing. This gene contains eleven exons: exons 1–5, 8, and 9 are common to both mRNAs; exons 6 and 11 are used in fibroblasts as well as in smooth muscle, whereas exons 7 and 10 are used in skeletal muscle. Previously we demonstrated that utilization of the 3' splice site of exon 7 is blocked in nonmuscle cells. In this study, we use both in vitro and in vivo methods to investigate the regulation of the 5' splice site of exon 7 in nonmuscle cells. The 5' splice site of exon 7 is used efficiently in the absence of flanking sequences, but its utilization is suppressed almost completely when the upstream exon 6 and intron 6 are present. The suppression of the 5' splice site of exon 7 does not result from the sequences at the 3' end of intron 6 that block the use of the 3' splice site of exon 7. However, mutating two conserved nucleotides GU at the 5' splice site of exon 6 results in the efficient use of the 5' splice site of exon 7. In addition, a mutation that changes the 5' splice site of exon 7 to the consensus U1 snRNA binding site strongly stimulates the splicing of exon 7 to the downstream common exon 8. Collectively, these studies demonstrate that 5' splice site competition is responsible, in part, for the suppression of exon 7 usage in nonmuscle cells.

**Keywords:** *cis*-competition; RNA processing; splice site selection

## INTRODUCTION

Alternative RNA splicing is a common mechanism regulating gene expression in eukaryotic cells (for reviews, see Adams et al., 1996; Wang et al., 1997). By this mechanism, a single gene can produce different mRNAs that are translated into distinct protein isoforms often with different functions. This process requires precise splice site recognition and pairing in a tissue- and developmental stage-specific manner (Reed, 1996; Wang et al., 1997), and the mechanisms responsible for splice site selection are just beginning to be elucidated.

RNA splicing reactions take place in an RNA–protein complex called the spliceosome. The first step during formation of the spliceosome is the assembly of the commitment complex (for review, see Reed & Palandjian, 1997). The formation of the commitment complex involves (1) direct base pairing between the sequences

in 5' splice sites and the 5' end of U1 snRNA, the RNA component of U1 small nuclear ribonucleoprotein (U1 snRNP), which results in recognition of 5' splice site by U1 snRNP, and (2) the association of polypyrimidine tracts (PPT) immediately downstream of branchpoint sequences (BPS) with U2 snRNP accessory factor (U2AF). Because a commitment complex defines the boundary of the intron that will be spliced, its formation represents a primary step for the regulation of alternative splice site selection (Chabot, 1996). One determinant for 5' splice site recognition is the degree of the complementarity of the 5' splice site to the 5' end of U1 snRNA, with the consensus sequence AG:GUAA/GUA being the most effective one (Eperon et al., 1986; Zhuang et al., 1987; Lear et al., 1990). Interestingly, alternatively spliced exons often have suboptimal 5' splice sites (reviewed by Horowitz & Krainer, 1994). The association of U1 snRNP and 5' splice sites can be regulated by other *cis*-acting elements and cellular factors. A variety of *cis*-acting sequences in introns and exons have been demonstrated to affect the recognition of 5' splice sites either through activation (Black, 1992; Dominski & Kole, 1992; Huh & Hynes, 1993;

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Humphrey et al., 1995; Sirand-Pugnet et al., 1995; Elrick et al., 1998) or by inhibition (Siebel et al., 1992; Elrick et al., 1998). Both constitutive splicing factors such as SR proteins and heterogeneous nuclear ribonucleoprotein (hnRNP) proteins, and regulated splicing factors such as KH-type splicing regulatory protein (KSRP) and P element somatic inhibitor (PSI), have been shown to affect the recognition of alternative 5' splice sites (Harper & Manley, 1991; Cáceres et al., 1994; Kohtz et al., 1994; Staknis & Reed, 1994; Yang et al., 1994; Zuo & Manley, 1994; Jamison et al., 1995; Min et al., 1995, 1997; Sreaton et al., 1995; Siebel et al., 1995; Tarn & Steitz, 1995; Jumaa & Nielsen, 1997). These proteins exert their effects on splice site selection presumably by binding to their cognate *cis*-acting elements, stabilizing or unstabilizing the association of U1 snRNP with 5' splice sites through direct protein-protein interactions. RNA secondary structures have also been demonstrated to participate in the regulation of splice site selection (D'Orval et al., 1991; Libri et al., 1991). The association of U2AF with the PPT in 3' splice site was found to be dependent upon the length and pyrimidine content of the PPT (Mullen et al., 1991; Noble et al., 1988), as well as on a number of other *cis*-acting elements and *trans*-acting factors (Valcarcel et al., 1993; Wu & Maniatis, 1993; Lin & Patton, 1995; Lynch & Maniatis 1996; Zhang & Wu, 1996; Zuo & Maniatis, 1996; Buvoli et al., 1997; Gooding et al., 1998).

We have been using the rat  $\beta$ -TM gene as a model system to study the regulation of alternative RNA splicing (Helfman et al., 1986, 1988, 1990; Helfman & Ricci, 1989; Guo et al., 1991; Mulligan et al., 1992; Guo & Helfman, 1993; Tsukahara et al., 1994; Grossman et al., 1998). This gene consists of eleven exons; two exon pairs, exons 6 and 7 and exons 10 and 11, are alternatively spliced. Exons 6 and 11 are used in TM-1 mRNA in nonmuscle cells, and exons 7 and 10 are used in  $\beta$ -TM mRNA in skeletal muscle cells (Fig. 1). Previous studies from our laboratory indicated that utilization of the 3' splice site of exon 6 in nonmuscle cells requires exon 8 sequences that contain three putative exonic splicing enhancers (Helfman et al., 1988; Tsukahara et al., 1994; Selvakumar & Helfman, 1999). This led us to propose a model in which the splicing of exon 6 in nonmuscle cells followed an ordered pathway in which exon 6 must first join the downstream common exon 8 before it can be spliced to the upstream common exon 5 (Helfman et al., 1988; Guo & Helfman, 1993). This model raises at least two questions. First, how is the 3' splice site of exon 7 suppressed in nonmuscle cells? This suppression is established through *cis*-acting elements located in intron sequence upstream of exon 7, designated as the intron regulatory element (IRE), and probably also in exon sequence at the 5' end of exon 7 (Helfman et al., 1990; Guo et al., 1991; Guo & Helfman, 1993). Further studies indicated that a constitutive splicing factor, poly-

pyrimidine tract binding protein, as well as other proteins such as FUSE binding protein and a homolog of human Sam 68 tyrosine phosphoprotein, form a complex on IRE and are possibly involved in blocking the recognition of the 3' splice site of exon 7 in nonmuscle cells (Mulligan et al., 1992; Grossman et al., 1998). Second, how is the 5' splice site of exon 7 suppressed in nonmuscle cells? Suppression of the 5' splice site of exon 7 is critical because it spares the 3' splice site of exon 8 for exon 6, thus not only enabling exon 8 to be spliced to exon 6 so that the 3' splice site of exon 6 can be activated, but also excluding exon 7 from mRNA in nonmuscle cells. However, the mechanism underlying this regulation remains to be determined.

In the present studies, we carried out a series of *in vitro* and *in vivo* experiments to examine the roles of intron, exon, and splice site sequences on the suppression of the 5' splice site of exon 7 in nonmuscle cells. Our data demonstrate that utilization of the 5' splice site of skeletal muscle-specific exon 7 in nonmuscle cells is suppressed by competition of the alternative 5' splice site of nonmuscle-specific exon 6. Thus, our data extend our previously proposed model that utilization of exon 6 in nonmuscle cells follows an ordered pathway, and demonstrate that in addition to the blockage of the 3' splice site, 5' splice site competition plays a role in the exclusion of the skeletal muscle-specific exon 7 in nonmuscle cells.

## RESULTS

### Sequences upstream of exon 7 are responsible for suppressing the recognition of the 5' splice site of exon 7

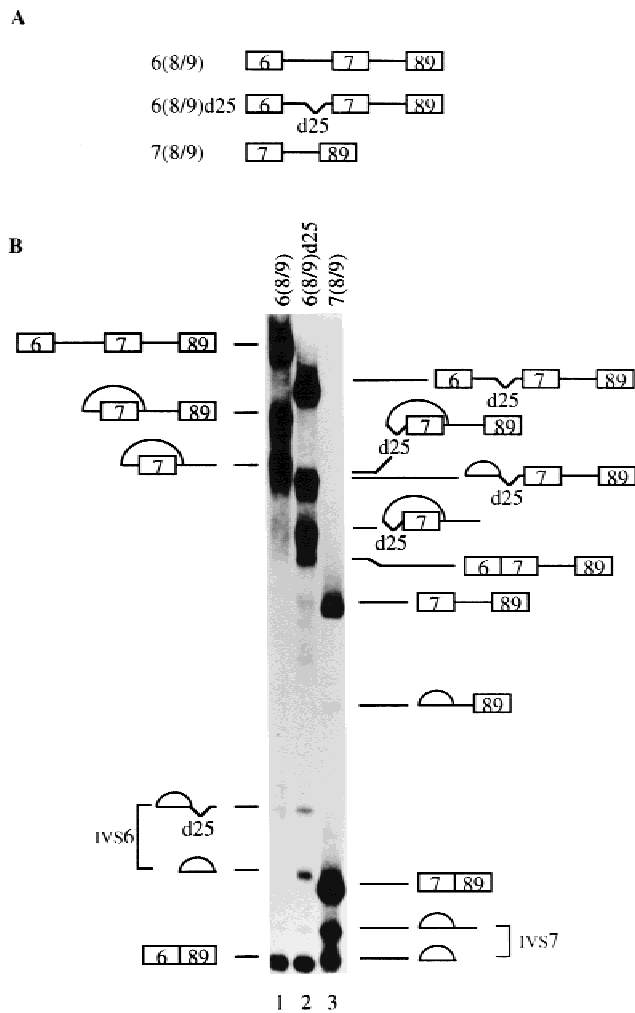
Our previous *in vitro* and *in vivo* data shows that a minigene containing sequences from exon 5 to exon 8 contains enough *cis*-information to maintain the correct exon choice in nonmuscle cells, in which exon 6 is included and exon 7 is excluded (Helfman et al., 1988). This data suggests that suppression of the 5' splice site of exon 7 in nonmuscle cells is either an intrinsic property, by which exon 7 cannot be spliced to exon 8, even in the absence of its flanking sequences, or an extrinsic effect, by which other sequences are required. Exon 7 can be spliced efficiently to exon 8 *in vitro* using a simple pre-mRNA that contains exon 7, intron 7, and exon 8 in a HeLa cell system, which suggests that the inability of exon 7 to join exon 8 in nonmuscle cells is not an intrinsic property (Helfman & Ricci, 1989; Tsukahara et al., 1994). However, when the upstream exon 6 and intron 6 were present, splicing of exon 7 to exon 8 was undetectable (Helfman & Ricci, 1989). This result suggests that sequences in intron 6, in exon 6, or in both are responsible for suppression of the 5' splice site of exon 7. To extend these studies, we examined the splicing of two substrates, 7(8/9) and 6(8/9), in



**FIGURE 1. A:** A schematic diagram of the rat  $\beta$ -tropomyosin pre-mRNA. Open boxes represent constitutive exons; shaded boxes represent tissue-specific exons as indicated. Horizontal lines represent introns (not drawn to scale). **B:** Nucleotide sequence from exon 6 to exon 8 of the rat  $\beta$ -tropomyosin pre-mRNA. The upper cases are exon sequences and the lower cases are intron sequences. d25 deletes the sequence within the two sites marked d25. cd, cd27, and cd22 contain sequences from the 3' end of intron 6 to their respectively marked locations (Helfman et al., 1990). In ex-1, UGUGGG is mutated to GGAUCC (Guo et al., 1991). Primers Int6 and Ex8 used in the PCR reactions are underlined, and the restriction enzyme sites for *Nco*I and *Pst*I that were used to confirm the identities of in vivo products are also indicated.

HeLa cell nuclear extracts. Substrate 7(8/9) consists of exon 7, intron 7, and the joined exons 8 and 9; substrate 6(8/9) includes the 3' half of exon 6 and the entire intron 6 in the upstream of 7(8/9). As shown in Figure 2, substrate 7(8/9) was spliced efficiently to form the product exons 7 + 8/9 (Fig. 2, lane 3), but splicing of exon 7 to exon 8 was suppressed almost completely when the upstream intron 6 and exon 6 were present (Fig. 2, lane 1); splicing of substrate 6(8/9) gave rise only to product exons 6 + 8/9 (Fig. 2, lane 1). The intermediate and final products were identified accord-

ing to published data (Helfman & Ricci, 1989; Tsukahara et al., 1994), and further confirmed by the spliced intermediate and final products of a deletion substrate 6(8/9)d25 that has a deletion of 76 nt in the IRE (Fig. 1). The splicing of 6(8/9)d25 gave rise to the same product exons 6 + 8/9 as the wild-type substrate, but the intermediate and final lariats from the splicing of 6(8/9)d25 were 76 nt shorter than those from the wild-type substrate (Fig. 2, lane 2). Interestingly, splicing of 6(8/9)d25 also gave rise to another spliced product exon 6 + exon 7 + intron 7 + exons 8/9, and the lariat

Splice site competition in  $\beta$ -TM

**FIGURE 2.** *Cis*-acting elements required for the suppression of the 5' splice site of exon 7 are located in sequences upstream of exon 7. **A:** Schematic diagrams of the splicing substrates. **B:** The pre-mRNAs were transcribed in a standard run-off transcription reaction, subjected to *in vitro* splicing reactions for 2 h, and the products were separated in a 4% denaturing polyacrylamide gel. Schematic representations of the precursors and products are indicated. The cleaved exon 6 ran out of the gel because of its small size. IVS: intervening sequences.

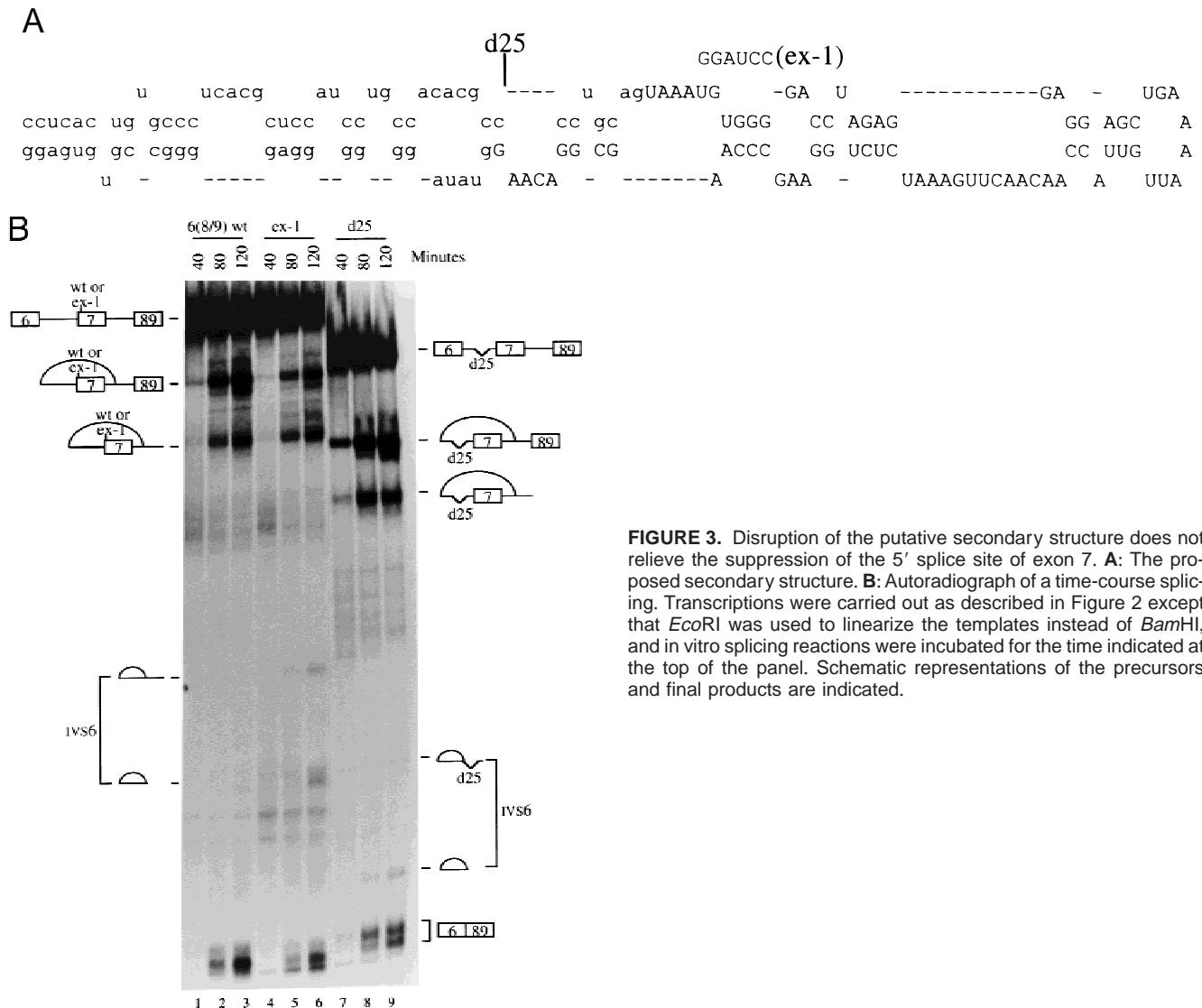
derived from the deleted intron 6. This suggests that sequences in the IRE do not play a role in preventing the use of the 5' splice site of exon 7. These results confirm that suppression of the 5' splice site of exon 7 requires the sequences in intron 6, exon 6, or both.

### The proposed secondary structure is not responsible for suppression of the 5' splice site of exon 7

Studies of the chicken  $\beta$ -TM pre-mRNA have revealed that a secondary structure formed by the sequences in and around the skeletal muscle-specific exon is responsible for the suppression of its 5' splice site usage in

nonmuscle cells (D'Orval et al., 1991; Libri et al., 1991). A similar secondary structure was also proposed to be present in the rat  $\beta$ -TM gene (Helfman et al., 1990). In addition, two types of mutations, one deleting or mutating the IRE sequence and the other mutating the exon sequence UGUGGG at the 5' end of exon 7 to GGAUCC(ex-1) (Fig. 1B), have been shown to activate the utilization of skeletal muscle-specific exon 7 in HeLa cells *in vitro* and *in vivo* (Helfman et al., 1990; Guo et al., 1991); these two types of mutations alter sequences that are important for the formation of the putative secondary structure. From these results, we speculated that the putative secondary structure in the rat  $\beta$ -TM gene may also be responsible for the suppression of the 5' splice site of exon 7. In order to test this hypothesis, we examined the effects of the d25 and ex-1 mutations on the utilization of the 5' splice site of exon 7 in the context of 6(8/9). Instead of using *Bam*HI to linearize the templates for synthesizing the substrates as in Figure 2, we used *Eco*RI because the sequence of the ex-1 mutation produces a *Bam*HI site. Substrates synthesized using templates linearized by *Eco*RI are 18 nt longer than those linearized with *Bam*HI.

To determine the kinetics of splicing, a time course splicing reaction was carried out. Splicing of the wild-type substrate generated only one product: exons 6 + 8/9 (Fig. 3, lanes 1–3). Splicing of the deletion mutant d25 also gave rise to product exons 6 + 8/9, with the same kinetics as the wild-type substrate had (Fig. 3, lanes 7–9). However, this deletion did not activate the 5' splice site of exon 7, as we did not detect any of the cleaved exon 6 + intron 6 + exon 7, the intermediate lariat intron 7 + exons 8/9, the final lariat intron 7, or the final product exon 6 + intron 6 + exon 7 + exons 8/9, as these intermediates or products would be generated if the 5' splice site of exon 7 was activated. Instead, the final lariat derived from the deleted intron 6 was detected (Fig. 3, lanes 7–9), which indicates that the deletion of the IRE sequence does not activate the 5' splice site of exon 7 but, instead, the 3' splice site of exon 7. Besides the product exons 6 + 8/9, splicing of the ex-1 mutation also gave rise to the lariat derived from intron 6 (Fig. 3, lanes 4–6). Splicing of the ex-1 mutation produced less spliced product exons 6 + 8/9 than the wild-type substrate because exon 6 was also spliced to exon 7 to form exon 6 + exon 7 + intron 7 + exons 8/9. This result suggests that the ex-1 mutation has the same effect as d25, that is, activation of the 3' splice site of exon 7. We could not detect the product exon 6 + exon 7 + intron 7 + exons 8/9 when d25 was spliced (compared to Fig. 2, lane 2) because products derived from the substrates linearized by *Eco*RI were not stable in this batch of HeLa cell nuclear extract, as can be seen from the fuzzy final product exons 6 + 8/9. These data demonstrate that the putative secondary structure is not responsible for the suppression of the 5' splice site of exon 7.



**FIGURE 3.** Disruption of the putative secondary structure does not relieve the suppression of the 5' splice site of exon 7. **A:** The proposed secondary structure. **B:** Autoradiograph of a time-course splicing. Transcriptions were carried out as described in Figure 2 except that *EcoRI* was used to linearize the templates instead of *Bam*HI, and in vitro splicing reactions were incubated for the time indicated at the top of the panel. Schematic representations of the precursors and final products are indicated.

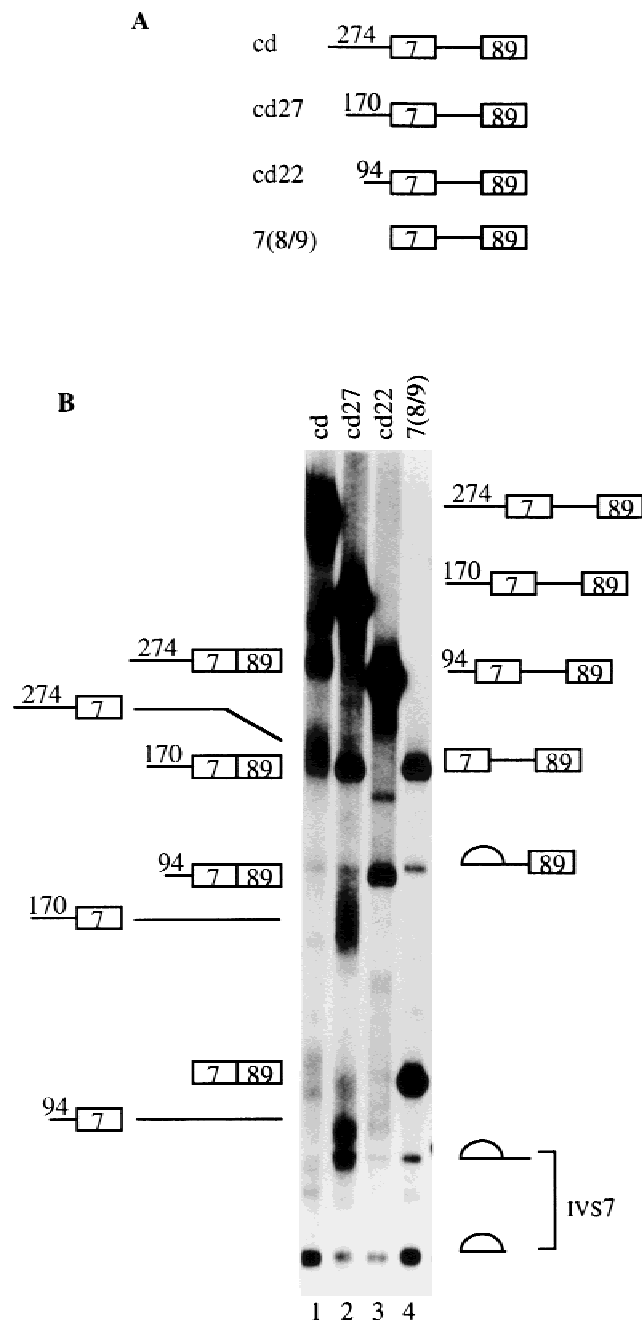
### The upstream intron sequences do not affect the efficient utilization of the 5' splice site of exon 7

To determine whether the sequence in intron 6 is responsible for the suppression of the 5' splice site of exon 7, we generated three substrates that contain different lengths of intron 6 starting from the 3' end. These substrates contain 274 nt (cd), 170 nt (cd27), or 94 nt (cd22) of intron 6 sequences upstream of 7(8/9) (Fig. 1B, Fig. 4A). The substrates were subjected to the in vitro splicing assay. As shown in Figure 4B, the 5' splice sites of exon 7 were used to splice to exon 8 efficiently with all substrates. Because different substrates contain different lengths of the intron 6 sequences upstream of exon 7, the spliced product from each substrate migrated to a different position. However, the intermediates and final lariats are the same and their intensities were comparable for different substrates. Although in this experiment the intensities of

both intermediate and final lariats from the splicing of cd22 were weaker than those of others, cd22 was spliced with the same efficiency in other experiments. These results demonstrate that the sequences in intron 6 are not responsible for suppressing the 5' splice site of exon 7. We also found that this suppression was not due to the sequences in exon 6 because substituting exon 6 sequence with exon 1 of the human  $\beta$ -globin gene or exon 5 of the rat  $\beta$ -TM gene in the context of 6(8/9) also suppressed the splicing of exon 7 to exon 8 (data not shown).

### Donor site competition is responsible for the suppression of the 5' splice site of exon 7 in vitro

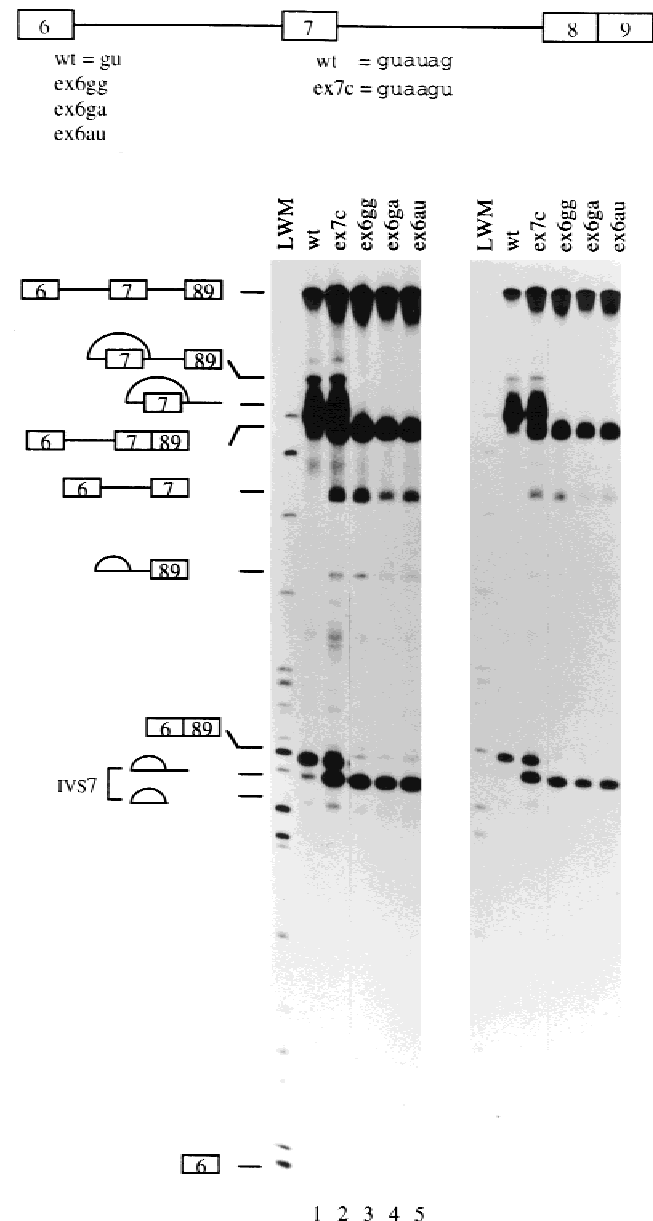
The above-mentioned results demonstrated that specific sequences in either intron 6 or exon 6 were not required for the suppression of the 5' splice site of

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**FIGURE 4.** Sequences in intron 6 are not involved in the suppression of the 5' splice site of exon 7. **A:** A schematic diagram of the substrates. Intron 6 sequences retained in the substrates are indicated as numbers above the intron upstream of exon 7. **B:** Autoradiograph of the in vitro splicing. The precursors, intermediates, and final products are indicated.

exon 7. However, we had not studied the role of the first 17 nt in intron 6. Because this sequence contains the 5' splice site of exon 6, it is possible that the 5' splice site of exon 6 is responsible for the regulation of the 5' splice site of exon 7. Thus, we hypothesized that the 5' splice site of exon 6 competes with that of exon 7 to splice to exon 8, thereby suppressing the 5' splice site of exon 7. To test this hypothesis, we examined the

splicing of three substrates in which two conserved nucleotides GU at the 5' splice site of exon 6 were mutated to either GG, GA, or AU. When these mutated substrates were tested for splicing, the formation of the product exons 6 + 8/9, which the wild type produced (Fig. 5, lane 1), was abolished. Instead, splicing of these mutant substrates gave rise to a product containing exon 6 + intron 6 + exon 7 + exons 8/9 (Fig. 5, lanes 3–



**FIGURE 5.** *Cis*-competition is responsible for the suppression of the 5' splice site of exon 7 in vitro. Transcription and in vitro splicing reactions were carried out as described in Figure 2 with the exception that the RNA molecules were separated in denaturing 6% PAGE gel. A schematic diagram of the different mutated substrates is shown at the top; the precursors, intermediates, and final products are indicated on the left. A lighter exposure is also shown on the right. In ex7c, GUAUAG is mutated to GUAAGU. ex6gg, ex6ga, and ex6at are mutations of the 5' splice site of exon 6.

5). These results suggest that the 5' splice site of exon 6 is preferred to that of exon 7 in HeLa cell nuclear extract *in vitro*.

Another way to examine *cis*-competition between two 5' splice sites is to strengthen the weak one to determine if this change leads to an increasing utilization of the improved splice site. Therefore, we examined splicing of a substrate *ex7c* in which sequence of the 5' splice site of exon 7 (AG:GUAUAG) was mutated to the consensus sequence (AG:GUAAGU) of the U1 snRNP binding site. Strengthening the 5' splice site of exon 7 resulted in its efficient usage, as evidenced by the formation of spliced product exons 7 + 8/9 (Fig. 5, lane 2). It is not surprising that the level of the spliced product exons 6 + 8/9 that uses the 5' splice site of exon 6 was maintained, because the splicing efficiency of exon 6 to exon 8 is high, and the standard splicing reaction uses an excess amount of HeLa cell nuclear extract. These data confirm our conclusion that utilization of the 5' splice site of exon 7 is suppressed by competition from the 5' splice site of the alternative exon 6 in nonmuscle cells.

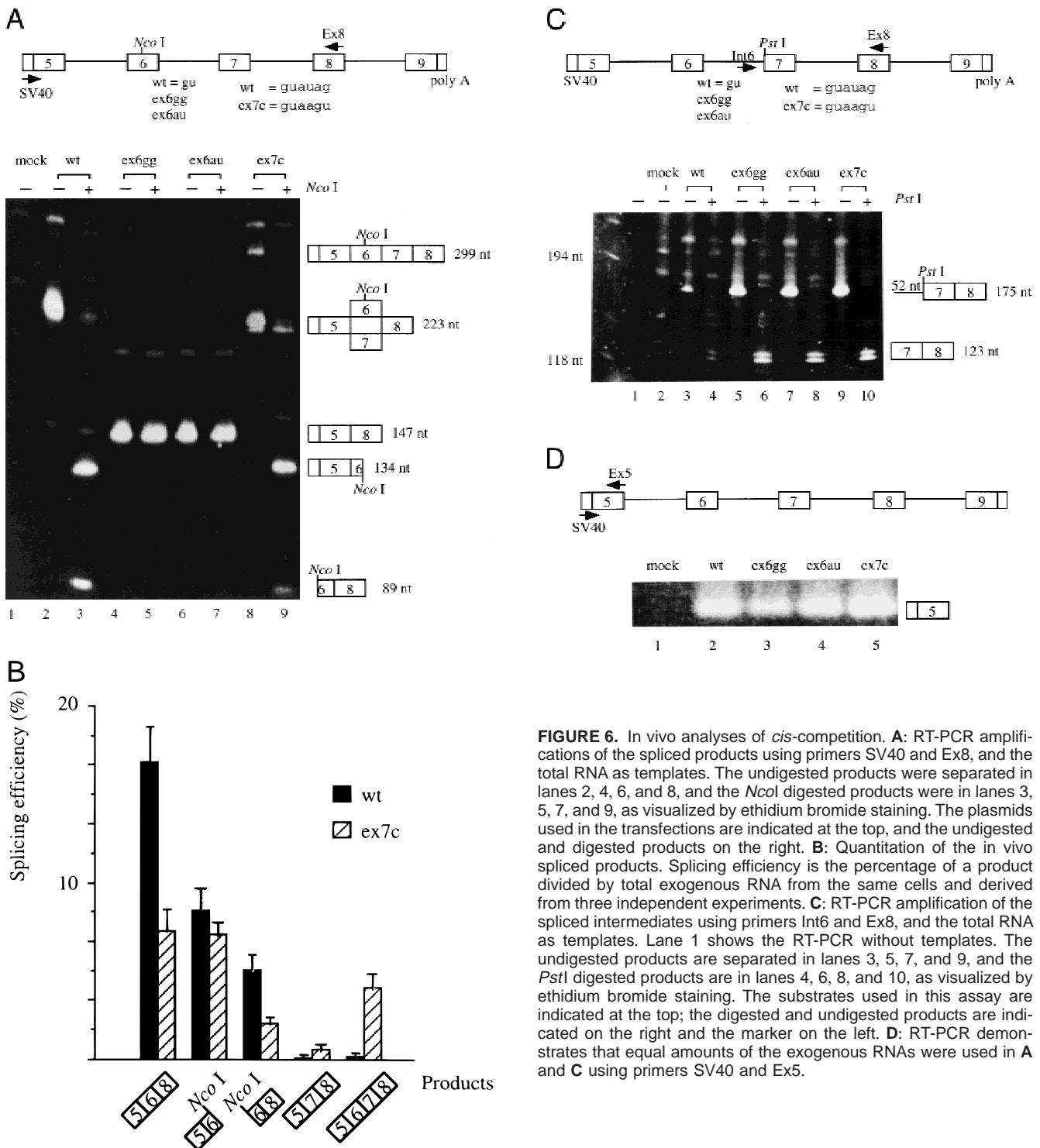
#### In vivo study of donor site competition

We previously reported that the mutations or deletions of the 5' splice site of exon 6 did not result in increasing use of exon 7 in nonmuscle cells *in vivo* (Guo et al., 1991), which is inconsistent with the *in vitro* results described above. However, in the previous experiments, cytoplasmic mRNA was used in an RNA protection assay, by which only the fully spliced products would have been detected. Thus, it is possible that the effects of the deletions or mutations of the 5' splice site of exon 6 on the utilization of the 5' splice site of exon 7 might not have been observed because the 3' splice site of exon 7 is still blocked. To examine this possibility and to determine whether donor site competition occurs *in vivo*, we performed transient transfection assays in HeLa cells using the same series of p2 substrates as in the previous experiments. p2 consists of the rat  $\beta$ -TM gene fragment from exon 5 to exon 9, and it has been demonstrated to contain enough information for the regulation of mutually exclusive splicing of exons 6 and 7 in HeLa cells (Helfman et al., 1988). Instead of isolating cytoplasmic mRNA and using an RNA protection assay, whole-cell RNA was isolated 48 h after the transfection and analyzed using reverse transcription-polymerase chain reaction (RT-PCR). This assay facilitates the detection of intermediate products. When we used a sense primer (SV40) homologous to the SV40 promoter and an antisense primer (Ex8) complementary to exon 8, we detected a major PCR product corresponding to spliced products exons 5 + 6 + 8 or exons 5 + 7 + 8 (both are at the same size), and a skipping product containing exons 5 + 8 from cells transfected with the wild-type substrate (Fig. 6A, lane 2).

To determine if the major PCR product contained exon 6 or exon 7, the products were digested with *NcoI* because exon 6 contains a *NcoI* site. The major product was composed of exons 5 + 6 + 8, as it was completely digested by *NcoI* (Figs. 1B and 6A, lane 3). These results are consistent with our previous data (Guo et al., 1991; Guo & Helfman, 1993). In contrast, when the 5' splice site of exon 6 was mutated from GU to GG or AU, a significantly increasing amount of the skipping product containing exons 5 + 8 was detected (Fig. 6A, lanes 4–7). Furthermore, the mutation converting the 5' splice site of exon 7 to the consensus sequence of U1 snRNP binding site reduced the level of the product exons 5 + 6 + 8 about half-fold (Fig. 6A, lane 8; Fig. 6B). This mutation also gave rise to a detectable amount of the product containing exons 5 + 7 + 8, as *NcoI* could not completely digest the band corresponding to the product exons 5 + 6 + 8 or exons 5 + 7 + 8 (Fig. 6A, lane 9; Fig. 6B). It is worth noting that a significant amount of the product exons 5 + 6 + 7 + 8 was also detected from cells transfected with the *ex7c* mutant (Fig. 6A, lane 8; Fig. 6B). This result supports our hypothesis that the regulation of the 5' splice site of exon 7 is important for the mutually exclusive splicing of exons 6 and 7; deregulation of the 5' splice site of exon 7 leads to the inclusion of skeletal muscle-specific exon 7 in nonmuscle cells. The product exons 5 + 7 + 8 was not detected when the 5' splice site of exon 6 was mutated (Fig. 6A, lanes 4–7), which is consistent with our model that suppression of the 3' splice site of exon 7 is important to block the utilization of exon 7 in nonmuscle cells (Guo & Helfman, 1993). Because digestion of the product exons 5 + 6 + 7 + 8 by *NcoI* also gave rise to exon 5 + half of exon 6, the intensities of this digested product exon 5 + half of exon 6 were comparable between the wild-type and mutant *ex7c*. We could not detect the intermediate product exon 5 + intron 5 + exon 6 + intron 6 + exons 7 + exon 8 using this pair of primers, which is probably because of the competition with the short PCR products for amplification.

To determine whether any intermediate products were present in the transfected cells, we carried out PCR reactions using another sense primer, Int6, homologous to the intron 6 sequence, together with the antisense primer Ex8. This pair of primers allowed us to detect the intermediate in which exon 7 is spliced to exon 8 and the product also contains intron 6. The expected PCR product is 175 nt long and can be digested by restriction enzyme *PstI* to generate a product containing exons 7 + 8 (123 nt) because a *PstI* site is at the boundary between intron 6 and exon 7 (Fig. 1B). A low level of splicing intermediate intron 6 + exons 7 + 8 was detected from cells transfected with the wild-type substrate (Fig. 6C, lanes 3 and 4), which is consistent with our previous result (Guo et al., 1991). When the 5' splice site of exon 6 was mutated from GU



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**FIGURE 6.** In vivo analyses of *cis*-competition. **A:** RT-PCR amplifications of the spliced products using primers SV40 and Ex8, and the total RNA as templates. The undigested products were separated in lanes 2, 4, 6, and 8, and the *Nco*I digested products were in lanes 3, 5, 7, and 9, as visualized by ethidium bromide staining. The plasmids used in the transfections are indicated at the top, and the undigested and digested products on the right. **B:** Quantitation of the in vivo spliced products. Splicing efficiency is the percentage of a product divided by total exogenous RNA from the same cells and derived from three independent experiments. **C:** RT-PCR amplification of the spliced intermediates using primers Int6 and Ex8, and the total RNA as templates. Lane 1 shows the RT-PCR without templates. The undigested products are separated in lanes 3, 5, 7, and 9, and the *Pst*I digested products are in lanes 4, 6, 8, and 10, as visualized by ethidium bromide staining. The substrates used in this assay are indicated at the top; the digested and undigested products are indicated on the right and the marker on the left. **D:** RT-PCR demonstrates that equal amounts of the exogenous RNAs were used in **A** and **C** using primers SV40 and Ex5.

to GG or AU, splicing of exon 7 to exon 8 was significantly increased (Fig. 6C, lanes 5–8). The ex7c mutation also increased the splicing of exon 7 to exon 8 (Fig. 6C, lanes 10 and 11). The digested product exons 7 + 8 was a doublet because the two strands differ by 4 nt and were separated in denatured gel. An equal amount of exogenous RNA from different transfected cells was used in the RT-PCR reactions (Fig. 6D). Collectively, these results indicate that donor site compe-

titition is responsible for suppressing the 5' splice site of exon 7 in nonmuscle cells in vivo.

## DISCUSSION

In the present paper we demonstrate that the 5' splice site of the skeletal muscle-specific exon 7 is regulated in nonmuscle cells. Our studies show that donor site competition is responsible for the suppression of the 5'

splice site of exon 7, and furthers our understanding of the regulation of alternative splicing of the rat  $\beta$ -TM gene.

Until now, it was not known how the 5' splice site of exon 7 is regulated. One simple mechanism we considered is that a *trans*-acting factor(s) selectively represses the 5' splice site of exon 7 in nonmuscle cells. This mechanism has been demonstrated to affect 5' splice site selection in other pre-mRNAs. The PSI, a somatic cell-specific splicing factor in *Drosophila*, binds to an exon inhibitory sequence and blocks the binding of U1 snRNP to the authentic 5' splice site. This leads to inhibition of splicing of the P element third intron in somatic cells (Siebel et al., 1995). SF2/ASF has been suggested to inhibit the recognition of splice donor site in alternatively spliced SRp20 gene (Jumaa & Nielsen, 1997). However, this type of *trans*-regulation seems unlikely to be responsible for the suppression of the 5' splice site of exon 7 in the  $\beta$ -TM pre-mRNA because sequences of intron 6 and exon 6 outside of the 5' splice site of exon 6 play no role on this regulation (Fig. 4). Another possibility we considered is that sequences in and flanking exon 7 are involved in the formation of an inhibitory secondary structure, which prevents the recognition of the 5' splice site of exon 7. The involvement of the upstream intron sequence in the regulation of the downstream exon splicing has been demonstrated in the chicken  $\beta$ -TM pre-mRNA (D'Orval et al., 1991; Libri et al., 1991). In the chicken pre-mRNA, sequences in and around the skeletal muscle-specific exon form a secondary structure, which masks the recognition of the 5' splicing site of the skeletal muscle-specific exon in nonmuscle cells. We previously proposed a similar secondary structure that may be involved in the regulation of the rat  $\beta$ -TM gene (Helfman et al., 1990). However, we found no evidence for such regulation because deletions or mutations of the sequences involved in the formation of this putative secondary structure did not activate its downstream 5' splice site (Fig. 3).

Our data demonstrate that splice site competition is responsible for the suppression of the 5' splice site of exon 7: exon 7 was used when two conserved nucleotides GU at the 5' splice site of exon 6 were mutated or when the 5' splice site of exon 7 was improved (Figs. 5 and 6). The sequences of 5' splice sites have been demonstrated to be critical for their recognition in both constitutive and alternative splicing (Eperon et al., 1986; Aebi et al., 1987; Zhuang et al., 1987; Lear et al., 1990). Although the sequences of 5' splice sites are more or less deviated from the consensus (reviewed in Horowitz & Krainer, 1994), it is a general phenomenon that the consensus sequence is preferably recognized by the splicing machinery (Eperon et al., 1986), and there is a sequence hierarchy for 5' splice site preference (Gallego & Nadal-Ginard, 1990; Lear et al., 1990). Our data indicate that the AG:GUAGUA sequence of the 5' splice site of exon 6 (a purine insertion between

AG in positions 3 and 4 would make it a perfect match to the consensus), matches better the consensus AG:GURAGU than the AG:GUAUAG sequence of the 5' splice site of exon 7 does. As a consequence, the association of U1 snRNP with the 5' splice site of exon 6 is stronger than with that of exon 7. It is possible that a factor(s) associated with U1 snRNP is limited so that the commitment complex can only be formed between the donor site of exon 6 and the acceptor site of exon 8, which results in the exclusion of exon 7 in nonmuscle cells. In contrast, in skeletal muscle cells, the 5' splice site of exon 7 may be activated by an activator such as SF2/ASF. This protein was shown to activate the donor site of exon 7 in nonmuscle cells in vitro (Meyeda et al., 1993), but not in vivo (Cáceres et al., 1994).

Splice site competition has been found to be responsible for splice site selection in several alternatively spliced exons. In the chicken  $\beta$ -TM gene, the donor site of exon 6b (GA:GUAUGA) is preferred to that of exon 6a (GA:GUAUGA); this preference results in utilization of exon 6b in myotubes (Libri et al., 1992). In the rat fibronectin gene, exon EIIIB is not used because its 5' splice site is outcompeted by a stronger one in exon III-8b (Huh & Hynes, 1993). Furthermore, the production of E1A 13S mRNA at early times after adenovirus infection results from a bona fide *cis*-competition between two 5' splice sites for 13S and 12S (Harper & Manley, 1991). Similarly, 3' splice site competition has also been demonstrated to be involved in splice site selection. Because binding of U2AF to the PPT immediately downstream of the BPS is a prerequisite for the association of U2 snRNP with the branchpoint (Wang et al., 1995; Valcarcel et al., 1996; Rudner et al., 1998), the contents of PPT significantly affects the recognition of 3' splice sites. A long PPT located downstream of the branchpoint in the IVS 3 of  $\alpha$ -TM pre-mRNA makes the branchpoint of IVS 3 stronger than that of IVS 2; this difference results in the default utilization of exon 3 in most cell types except for the smooth muscle cells (Mullen et al., 1991). 3' splice site competition was also found to be involved in the regulation of the chicken  $\beta$ -TM pre-mRNA (Libri et al., 1992). The strength difference of the BPSs was also shown to control alternative splice-site selection in SV40 early pre-mRNA splicing (Noble et al., 1988).

The regulation of alternative splicing of the rat  $\beta$ -TM gene is similar to that of the chicken gene in that both intron sequences immediately upstream of skeletal muscle-specific exon and exon sequences at the 5' end of that exon are involved in the suppression of its 3' splice sites in nonmuscle cells (Helfman et al., 1990; Guo et al., 1991; Guo & Helfman, 1993; Gallego et al., 1996). In addition, each of the genes contains an unusually long distant BPS for skeletal muscle-specific exon 7 (exon 6b in the chicken gene) (Helfman & Ricci, 1989; Goux-Pelletan et al., 1990). However, the results presented here suggest that the regulation of the 5'

splice sites of the skeletal muscle-specific exon is different in mammalian and avian systems. We show that in the rat  $\beta$ -TM gene, the 5' splice site of exon 7 is suppressed due to competition of the 5' splice site of exon 6 in nonmuscle cells. In contrast, a secondary structure formed by the sequences in and around the skeletal muscle-specific exon masks the recognition of its 5' splice site in the chicken  $\beta$ -TM gene in nonmuscle cells (D'Orval et al., 1991; Libri et al., 1991). Our present data argue that a similar secondary structure is not involved in the 5' splice site suppression of the skeletal muscle-specific exon in the rat  $\beta$ -TM gene because disruptions of the putative secondary structure did not activate its 5' splice site (Fig. 3). Our results are consistent with the conclusion that the splicing of the alternative exons of the chicken and rat  $\beta$ -TM genes requires different regulatory elements (Balvay et al., 1994). Expression of the chicken minigene in mouse cells results in inclusion of both mutually exclusive exons, and expression of the rat minigene in quail myoblasts causes the usage of exon 7.

Our present data extend our model that exon 6 has to be joined to the downstream common exon 8 before it can be spliced to the upstream common exon 5 in nonmuscle cells (Guo & Helfman, 1993), and further our understanding of how exon 7 is regulated. Two *cis*-acting elements, one in the intron upstream of exon 7 and the other at the 5' end of exon 7, are involved in blocking the utilization of the 3' splice site of exon 7 (Helfman et al., 1990; Guo et al., 1991; Guo & Helfman, 1993). We show that the 5' splice site of exon 7 is suppressed by competition of the 5' splice site of exon 6 in this study. The inability to use both the 5' and 3' splice sites of exon 7 allows exon 6 to be spliced to exon 8, fulfilling the requirement that joining of exon 8 to exon 6 is necessary for the usage of exon 6. These data also suggest that splicing of exon 6 to exon 8 is a default pathway in nonmuscle cells, and that tissue-specific factors may be present in skeletal muscle cells. These factors either suppress the 5' splice site of exon 6, activate the 5' splice site of exon 7, or both. Alternatively, skeletal muscle-specific factors may activate the 3' splice site of the skeletal muscle-specific exon 7, resulting in joining of exon 7 to exon 5 and exclusion of exon 6. We cannot exclude other possible mechanisms that may be involved in the activation of exon 7 in skeletal muscle cells. We have recently developed an *in vitro* system to identify skeletal muscle-specific factors (Wang Y-C, Liu J-P, Krainer AR, Helfman DM, in prep.) and experiments are in progress to identify these proteins.

## MATERIALS AND METHODS

### Plasmid construction

The DNA templates for synthesizing *in vitro* splicing substrates were all in an pSP64 vector that contains an SP6

promoter, and derived from the rat  $\beta$ -TM gene (Helfman et al., 1988). p6(8/9) was constructed from pSP64-p2p6(8/9) (Tsu-kahara et al., 1994) by deleting sequences from *HindIII* site in polylinker to *NcoI* site in exon 6, filled in by the Klenow fragment, and re-ligated with a *HindIII* linker. The resulting construct contained 43 nt of the 3' part of exon 6. p6(8/9)d25 was derived from p6(8/9) by switching the respective PCR fragment from *NcoI* in exon 6 to *PstI* at the 3' end of intron 6 using pSp64-p2(7/8)d25 (Helfman et al., 1990) as a template; the *NcoI* site was changed to *HindIII* site. The ex-1 mutation, mutations that alter the 5' splice site of exon 6 from GU to GG, GA, or AU (ex6gg, ex6ga, ex6au), and the mutation that converts the sequence GUAUAG at the 5' splice site of exon 7 to the consensus sequence GUAAGU were generated by site-directed mutagenesis (Kunkel, 1985). The constructs were verified by sequencing.

The plasmids used in *in vivo* transfection experiments have been described previously (Helfman et al., 1988).

### Synthesis of RNA and *in vitro* splicing

The  $^{32}$ P-labeled splicing substrates were synthesized *in vitro* and primed with Cap analog, as described by Konarska et al. (1984). The  $^{32}$ P-labeled pre-mRNAs contained a small amount of prematurely terminated transcripts, but their presence did not affect the *in vitro* splicing reaction, and the RNA substrates were not purified further.

HeLa cell nuclear extracts were prepared as described earlier (Dignam et al., 1983; Krainer et al., 1984). *In vitro* splicing reactions were carried out at 30°C for 2 h unless otherwise indicated. Standard splicing conditions used for these studies consisted of 15  $\mu$ L of HeLa cell nuclear extract in a final reaction volume of 25  $\mu$ L, containing 2 mM MgCl<sub>2</sub>, 0.5 mM ATP, 20 mM creatine phosphate, 15 ng pre-mRNA, 12.8 mM HEPES (pH 8), 14% (v/v) glycerol, 60 mM KCl, 0.12 mM EDTA, and 0.3 mM dithiothreitol.

### Transfection and RNA analysis

HeLa cells were grown in Dulbecco's modified Eagle medium supplemented with 5% fetal bovine serum, and transfected with 5  $\mu$ g of plasmid DNA per 100-mm plate by using a calcium phosphate coprecipitation procedure; cells were harvested 48 h later, and whole-cell RNA was isolated as described (Gilman, 1988). Reverse transcription was carried out using 10  $\mu$ g whole-cell RNA and 100  $\mu$ mol random primers in 30  $\mu$ L reaction. To determine the spliced products, a sense primer SV40: TTTTGGAGGCCTAGGCTTTT, homologous to the SV40 promoter, and an antisense primer Ex8: TCCAGAAGTTTGATCTC-TTC, complementary to exon 8, were used in PCR reactions. In some experiments, the primers were  $^{32}$ P-labeled by T4 polynucleotide kinase. Half of each PCR product was directly mixed with *PstI* for digestion, and the undigested and digested PCR products were run in a denaturing gel and visualized by either ethidium bromide staining or autoradiograph. To amplify the intermediates, a sense primer Int6: CTGTCTCACCTCACTGTGC, homologous to the intron 6 sequence, and the antisense primer Ex8 were used in PCR reactions. Half of each PCR product was directly mixed with *NcoI* for digestion, and the undigested and digested PCR products were run in a denaturing gel and

visualized by ethidium bromide staining. To determine the amount of the exogenous RNA used in the RT-PCR reactions, PCR reactions were carried out by using the sense primer SV40 and an antisense primer Ex5: GCCTCCTCGGCTCTCTCT, complementary to the exon 5 sequence. PCR products were run in an agarose gel and visualized by ethidium bromide staining. All of the PCR reactions were run for 20 cycles (94 °C, 55 °C, and 72 °C) to maintain linear amplification.

To quantify the *in vivo* spliced products, ethidium bromide staining or autoradiograph films were scanned on a UMAX UC630 MaxColor scanner using Adobe Photoshop™ 2.5 software at 300 dpi and quantitation was made using NIH image 1.6 software with the built-in logarithmic calibration.

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