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Alternative Splicing of a Human α-Tropomyosin Muscle-Specific Exon: Identification of Determining Sequences

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The human α-tropomyosin gene hTM_{nm} has two mutually exclusive versions of exon 5 (NM and SK), one of which is expressed specifically in skeletal muscle (exon SK). A minigene construct expresses only the nonmuscle (NM) isoform when transfected into COS-1 cells and both forms when transfected into myoblasts. Twenty-four mutants were produced to determine why the SK exon is not expressed in COS cells. The results showed that exons NM and SK are not in competition for splicing to the flanking exons and that there is no intrinsic barrier to splicing between the exons. Instead, exon SK is skipped whenever there are flanking introns. Splicing of exon SK was induced when the branch site sequence 70 nucleotides upstream of the exon was mutated to resemble the consensus and when the extremities of the exon itself were changed to the corresponding NM sequence. Precise swaps of the NM and SK exon sequences showed that the exon sequence effect was dominant to that of intron sequences. The mechanism of regulation appears to be unlike that of other tropomyosin genes. We propose that exclusion of exon SK arises because its 3′ splicing signals are weak and are prevented by an exon-specific repressor from competing for splice site recognition.

Alternative splicing of pre-mRNA produces different isoforms of mRNA from a single gene, often resulting in the production of multiple protein isoforms. In many cases, the proportions of the various isoforms alter during cell differentiation or development. Mutually exclusive splicing, whereby only one of two adjacent exons is incorporated between common flanking exons, is associated particularly with transcripts which are expressed primarily in muscle cells or which exhibit muscle-specific switches in the exon selected. This pattern of splicing raises three principal problems: (i) the two alternative exons are not usually spliced to each other, and where the process appears to be regulated, (ii) one exon is preferred in most cell types but (iii) the alternative exon is incorporated in the specific tissue.

This pattern of splicing has been studied most intensively in two tropomyosin gene systems: the 5′-proximal exons 2 and 3 of rat α-tropomyosin, in which exon 2 is incorporated specifically in smooth muscle (39), and the more central exons 6 and 7 (or 6A and 6B) of the β-tropomyosin homologs in rats and chickens, in which the 3′ most of the two exons is incorporated in skeletal muscle (19, 24, 26). The results of these studies do not address all of the issues described above, but perhaps surprisingly, it is clear that mutually exclusive splicing is not determined by a common mechanism. In the case of the rat α-tropomyosin gene, it appears that exons 2 and 3 do not splice together because the long polypyrimidine tract of exon 3 forces the branch site too close to the 5′ splice site of exon 2 (39). The default exon 3 appears to be selected because both exons are in competition for splicing to exon 1, but exon 3 is preceded by a more favorable polypyrimidine tract (32); sequences near or within that exon suppress its use in smooth muscle cells (33).

Although the central exons of the β-tropomyosin genes are also preceded by long polypyrimidine tracts, with branch sites far upstream of the skeletal muscle-specific exon (14, 22), the greater distance between the two exons rules out simple steric explanations of mutual exclusion. Indeed, it has been found that the two exons can splice together in vitro (14). Most studies have focused on the reasons why the 5′-most exon is chosen in default systems. In the chicken gene, it has been shown that alterations to the muscle-specific exon and its adjacent polypyrimidine tract activate it, both in vivo and in vitro (14, 28), and compensating changes have supported the view that these sequences are involved in sequestering the exon and its splice sites within a large secondary structure (4, 29). The distance of the branch site from that exon was unimportant in vivo, but it did prevent step 2 of the splicing reaction taking place between the mutually exclusive exons in vitro (14, 28). Similarly, experiments with the homologous rat exons have shown that the muscle-specific exon is repressed, in vitro and in vivo, because of sequences just upstream of, and within, the exon; the exon was used, or step 1 of the splicing reaction took place, when exon 7 was removed or when it was joined to exon 8 (17, 20, 21), but otherwise it is noncompetitive, i.e., intrinsically unreactive in a nonmuscle cell line (17). Some observations do not, at first sight, fit the secondary structure proposals (17, 28), and another mechanism suggested for the omission of the muscle exon is that a blocking factor binds that exon in nonmuscle cells (17, 21).

The critical differences between the 5′-proximal exons and the central pair can be summarized thus: in the former case, the mutually exclusive exons cannot splice to each other, and they are in competition for splicing to the flanking exons, whereas the central exons can, in some circumstances, splice together, but the muscle-specific exon is usually sequestered and inaccessible.

We have investigated the splicing patterns of a human α_s-tropomyosin gene, hTM_{nm} (3). The organization of the central mutually exclusive exons is very similar to that of the β-tropomyosin genes, with a skeletal muscle-specific exon. However, the developmental profile of expression of the two isoforms is quite distinct from that of α- and β-tropomyosins (16), suggesting that the mechanism of the switch between the two central exons might be different. We report here that

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a systematic mutational analysis in nonmuscle cells shows that the muscle exon does not compete for splicing, and that this arises because of a novel combination of poor branch site signals and specific exon elements. An ordered pathway for splicing, proposed for β-tropomyosin (20), cannot alone determine which of the mutually exclusive exons is incorporated. Instead, we propose that both of the mutually exclusive exons are flanked by weak splicing signals, which enable a weak repressor binding to the muscle-specific exon in nonmuscle cells to sequester that exon.

MATERIALS AND METHODS

Site-directed mutagenesis. A 2.7-kb *Bcl*I-to-*Xba*I fragment of the hTM_{nm} gene (3), spanning from exon 4 to the intron between exons 6 and 7, was inserted into the M13-derived vector mICE 10 (7) and was mutated, either by using oligonucleotides (45) or, in the case of larger mutations, by the method known as sticky-feet mutagenesis (2). Mutations were screened by dideoxy sequencing (37) of templates prepared in microtiter plates (8, 10). The first mutations introduced *Bst*EII and *Xho*I sites in exons 4 and 6, respectively, to facilitate recloning for expression (see below). In addition, a number of constructs contained a deletion of nucleotides +39 to +56 from the 5′ splice site of exon SK (see Table 1). This sequence appeared to be able to sequester the exon SK 5′ splice site in a hairpin stem, and it was removed in order to test whether it affected the exclusion of exon SK (construct Δ Stem) and to facilitate mutagenesis within exon SK.

Plasmid constructions. The simian virus (SV40)-based vector pSVL (Pharmacia) was modified to introduce a unique *Eco*RI site 12 nucleotides (nt) downstream of the major transcriptional start site of the SV40 late promoter. The cDNA for the skeletal muscle-specific isoform of hTM_{nm} (hATM 1.3 [30]) was subcloned into this site. Novel *Bst*EII and *Xho*I sites were created in exons 4 and 6, respectively, allowing fragments of wild-type and mutated hTM_{nm} genomic DNA to be incorporated as tropomyosin minigenes. A *Not*I site was introduced into exon 7 just within the downstream extremity of the sequence complementary to the S1 probes. The S1 probes made from both nonmuscle (NM) and skeletal muscle (SK) cDNA clones were modified to incorporate this *Not*I sequence, which ensured that no signal would be detected during S1 mapping from endogenous tropomyosin mRNA.

Cell culture, transfection, and RNA harvesting. COS-1 cells (13) were grown in Dulbecco modified Eagle medium supplemented with 10% newborn calf serum and antibiotics. Transfection of 5 μg of each minigene construct was achieved by the calcium phosphate coprecipitation method (15), followed after 5 h by a shock with 15% dimethyl sulfoxide. Incubation in culture medium was continued for 48 h, whereupon the cells were lysed in the presence of 0.15% Nonidet P-40, and cytoplasmic RNA was isolated by extraction with chloroform and ethanol precipitation.

S1 mapping. High-specific-activity probes were made from the cDNA species for each hTM_{nm} isoform by polymerase chain reaction (PCR) amplification between exons 4 and 7; the downstream (exon 7) oligonucleotide was labelled with [γ-^{32}P]ATP for S1 nuclease protection analysis (1). Autoradiography and quantification by laser scanning densitometry were then performed.

RT-PCR. Poly(A) RNA was selected by using Hybond mAP (Amersham); this procedure was followed by synthesis of first-strand cDNA in the presence of Moloney murine

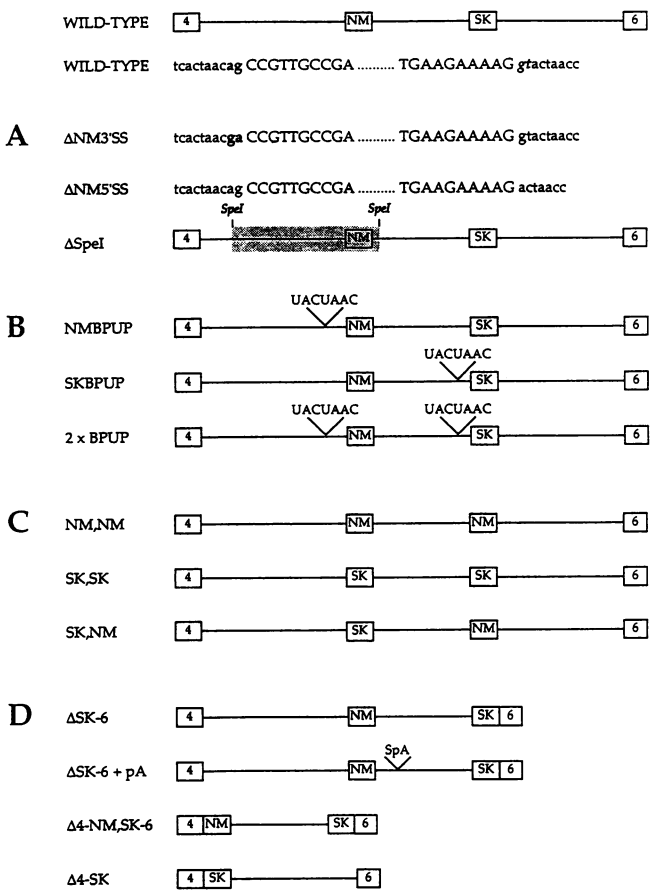
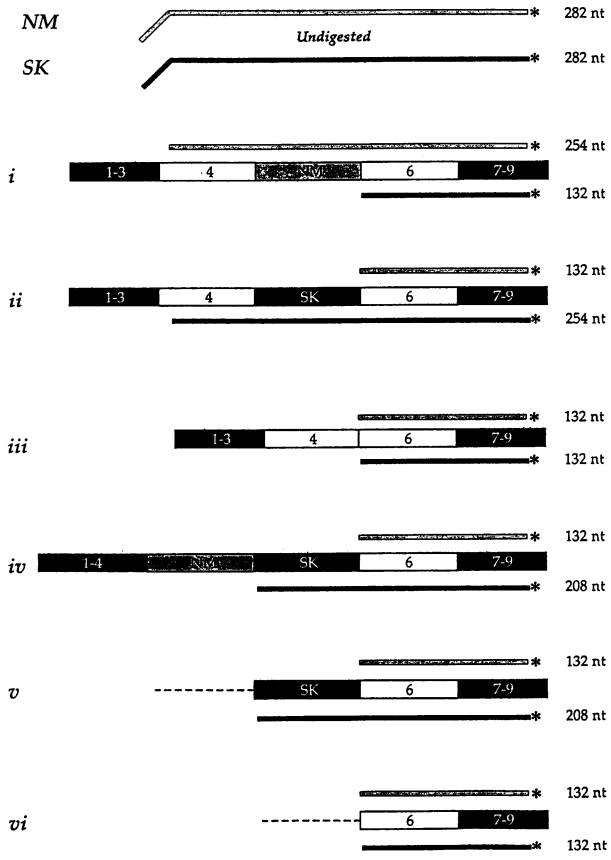


FIG. 1. Organization of the region of the hTM_{nm} gene used in this study. Exon and intron sequences are denoted by upper- and lowercase letters, respectively. A complete SK cDNA (30) was cloned within an SV40 transcription unit (see Materials and Methods). A *Bst*EII site was introduced into exon 4, 39 nt upstream of the 5′ splice site, in the cDNA and genomic clones, and an *Xho*II site was introduced into exon 6 likewise, 33 nt beyond the 3′ splice site. Neither mutation altered the length of the exon. After oligonucleotide-directed mutagenesis of the genomic clone to generate the mutants shown in panels A to D, *Bst*EII-*Xho*II fragments were inserted into the corresponding sites of the cDNA, producing the minigenes used for transfection of COS cells. The tropomyosin sequences in the minigene contained 629 nt of exons 1_{SK} to 4, the 807-nt intron downstream of exon 4, exon NM (75 nt), the 541-nt intron between exons NM and SK, exon SK (75 nt), the 706-nt intron downstream of exon SK, and 411 nt comprising exons 6 to 9. In a number of mutants (shown in Table 1), an extra 18 nt was deleted from the intron downstream of exon SK to facilitate mutagenesis of that exon. The nucleotides in the wild-type sequence which were mutated to form ΔNM3′SS are shown in boldface, and those deleted to form ΔNM5′SS are shown in italics. (A) NM deletions. Mutated nucleotides are outlined. The area in grey represents that deleted in mutation ΔSpeI. (B) Branch point mutations. The arrowheads indicate the sites of mutation to give the sequences shown above them. (C) Exon swaps. Exon positions were altered by sticky-feet mutagenesis (2). (D) Exon fusions. The natural gene contains a *Sca*I site at the 3′ splice site of exon 6. An exact fusion of exons SK and 6 was made by introduction of a *Sca*I site at the 5′ splice site of exon SK, digestion with *Sca*I, and ligation. Likewise, the gene contains an *Acc*I site at the 5′ splice site of exon 4, and introduction of a second *Acc*I site at the 3′ splice site of exon NM or SK allowed accurate intron deletions to be made. The site of insertion of a synthetic polyadenylation site (SpA [25]) is indicated.

A



B

LENGTH OF PROTECTED FRAGMENT	RNA SPECIES
NM PROBE	
282	Undigested probe
254	*i*
132	*ii-vi*
SK PROBE	
282	Undigested probe
254	*ii*
208	*iv, v*
132	*i, iii, vi*

FIG. 2. S1 mapping strategy. (A) NM and SK probes were produced from cDNA clones by PCR as described in Materials and Methods and used for mapping with S1 nuclease. The products of all possible splicing events are represented diagrammatically (i to vi), together with the extent of uninterrupted complementarity to each probe, this being the length of each of the protected fragments observed in Fig. 3, 4, 6, and 7. The dashed line in diagram v represents any sequences upstream of exon SK other than exon 4, such as exon NM (shown in diagram iv) or an unspliced intron, which could produce an SK signal of 208 nt; in diagram vi, the dashed line represents any sequences other than exon NM or SK, such as exon 4 or an unspliced intron, which would produce fragments of 132 nt with both probes. Probes and minigenes alike contained an artificial *Not*I restriction site 12 nt from the 5′ end of the probes. This generated an 8-nt tag region of noncomplementarity between probes and endogenous RNA, assuming human and monkey sequences to be identical. (B) Analysis of the derivation of each of the S1 protection fragments. RNA species are as described for panel A.

leukemia virus reverse transcriptase (RT) (10 U; Pharmacia) and PCR, from exons 4 to 7, as described previously (18). After a second round of PCR using nested primers, the products were cloned into mICE 10 (7) for sequence analysis of 48 clones derived from each mRNA preparation. Templates were prepared as described in references 8 and 10. Although maximum PCR yields were chosen, resulting in overrepresentation of shorter RNA species, this procedure gave valid ratios of NM to SK isoforms, and it was done on most samples in which SK RNA was present at detectable levels. Cleavage of the PCR products with *Xho*I, a site for which had been introduced into exon 6, was used to show that the PCR products were not derived from the otherwise identical endogenous COS cell mRNA (data not shown).

RT-PCR was done also with total RNA. In this case, the PCR used a touchdown procedure (6), with 25 cycles at the final annealing temperature and primers annealing to exon 4 and to SV40 sequences upstream of the polyadenylation site. A portion (4%) of the reaction mixture was added to a second PCR mixture, which included ^{32}P-labeled primers complementary to sequences in exon 4 (3′ of the previous primer) and exon 6; the PCR was done for just 8 cycles (50 cycles with RNA from transfected myoblasts). Part of the product was cut with *Alu*I, which cuts twice in both exon 4 and exon SK but not in exon NM. Portions of the uncut and cut material were analyzed by gel electrophoresis.

RESULTS

Strategies for expression of mutant genes and mapping transcripts. The constructs used in this series of experiments are based on a genomic fragment of the human tropomyosin gene hTM_{nm}, spanning exons 4 to 6, which was cloned within the complete cDNA for the SK isoform. Some of these constructs are represented diagrammatically in Fig. 1 and 5. Following transient expression of these minigenes from the SV40 late promoter in monkey COS-1 cells, the mRNA produced was harvested and analyzed by either the S1 mapping or RT-PCR method.

The S1 mapping strategy involved the use of 5′-end-labelled cDNA for each of the two alternative isoforms as a probe (Fig. 2). This allowed the correct products to be distinguished, but the ratio of NM to SK isoforms could not be determined from the ratio of S1 products in a single track because the shorter products could be derived from various RNA species (Fig. 2). In addition, an RNA isoform containing both exons NM and SK could produce a band of 254 nt, as if from the correct isoforms, with both probes (38).

With an unmodified minigene transfected, an SK cDNA probe produced only a band of 132 nt (protection of exon 6), and no transcripts were detectable that had incorporated the SK exon (i.e., no band at 254 nt). The corresponding NM cDNA probe produced almost exclusively a band at 254 nt (Fig. 3A). Thus, our system reproduced faithfully the expected nonmuscle splicing pattern (11, 27). The endogenous tropomyosin RNA was not detected because of the use of a tag in both the introduced genes and the probe (Fig. 2A).

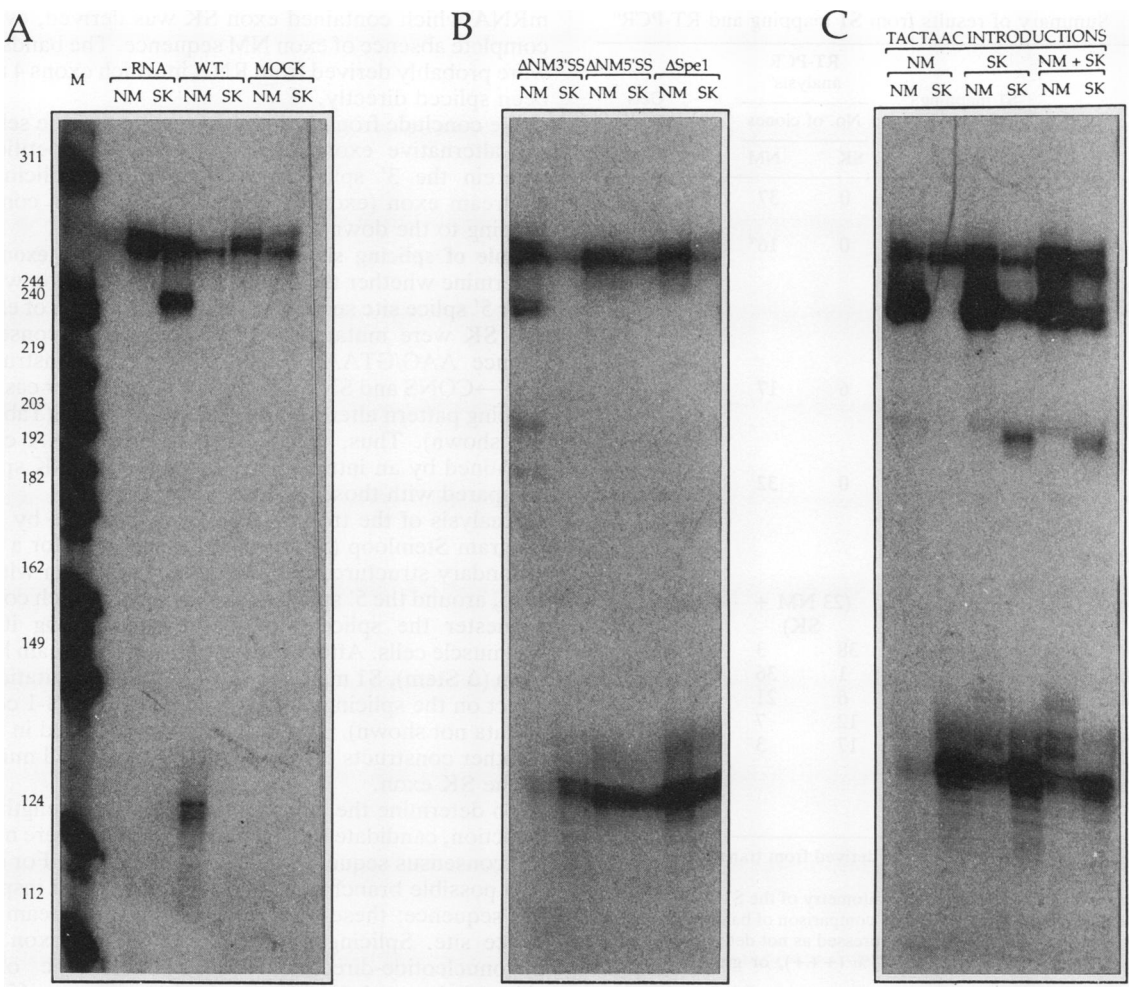


FIG. 3. S1 mapping data showing the effect on exon SK incorporation in COS cells of inactivation of exon NM splicing signals and role of the exon SK branch site. RNA samples isolated from COS-1 cells transfected with the constructs shown in Fig. 1 were mapped with S1 nuclease, using probes generated from NM or SK cDNA by PCR. Protected fragments were separated on a 6% polyacrylamide gel containing 20% (vol/vol) formamide and 8 M urea. Markers were produced by digesting pBR322 with *Hpa*II and filling in the 5′ overhangs with [α-^{32}P]dCTP and the other (unlabelled) deoxynucleoside triphosphates in the presence of the Klenow fragment of *Escherichia coli* DNA polymerase I. The resultant autoradiographs are shown. (A) Representative control transfections (-RNA, no in vivo RNA included in the reaction mixture; W.T., RNA isolated from cells transfected with the wild-type minigene; MOCK, RNA isolated from untransfected cells). (B) Test of exon competition by transfection with exon NM mutants shown in Fig. 1A. (C) Role of exon SK branch site, tested by transfection with exon SK mutants shown in Fig. 1B.

For quantitative determinations of the ratio of NM and SK isoforms, each RNA sample was amplified via a two-step RT-PCR procedure, after which 48 clones were isolated and sequenced; this procedure provided statistically valid ratios (as an example, Fig. 8 shows results with clones derived from transfection with mutant SK1-15NM). A different RT-PCR procedure was used to assess the proportions of products of different lengths when the S1 results indicated that both exons NM and SK might have been incorporated. The PCR was shortened to ensure that no products were detected from mock-transfected cells and that there was no bias toward shorter products over a range of concentrations of substrate and product. The products were analyzed by gel electrophoresis. Cleavage with *Alu*I, which cuts inside exons 4 and SK, was used to determine the ratio of use of exons NM and SK.

Is exon selection a competitive event? To distinguish between a number of possible models for the mechanism of selection of one of the pair of mutually exclusive exons, it was first essential to determine whether, in a nonmuscle system, the exon SK was excluded only because of competition from exon NM. Mutations in which either the 5′ splice site, the 3′ splice site, or the whole of exon NM was deleted were made to address this question. Figure 3B shows that when either the 5′ or 3′ splice site of exon NM (ΔNM5′SS or ΔNM3′SS) was deleted, bands at a size of 254 nt did not appear with the SK probe, showing that there had been no switch to SK RNA production.

The bands at 210 and 198 nt in the S1 protection of the NM cDNA probe by ΔNM3′SS indicate use of cryptic 3′ splice sites within exon NM, seemingly in preference to utilization of exon SK. This mRNA was also analyzed by RT-PCR. Subsequent cloning and sequencing of the products showed that the predicted cryptic 3′ splice sites 13 and 25 nt

TABLE 1. Summary of results from S1 mapping and RT-PCR[a]

Mutant	S1 mapping: level of SK[b]	RT-PCR analysis[c] No. of clones		Gels (% SK)
		SK	NM	
Wild type	−	0	37	
ΔNM5'SS	−			
ΔNM3'SS	−	0	16[d]	
ΔSpeI	−			
NM 5'SS→CONS	−			
SK 5'SS→CONS[f]	−			
Δ Stem	−			
NMBPUP	−			
SKBPUP[f]	++	6	17	15
2 × BPUP	++			40
NM,NM[f]	−			0
SK,SK	+			(All 4-6)
SK,NM[f]	−	0	32	0
ΔSK-6	+			43 SK: 57 (NM+SK)
ΔSK-6 + pA	+			
Δ4-SK	++++			
Δ4-NM,SK-6	−	(23 NM + SK)		
SK 1-15 NM[e,f]	++++	38	3	100
SK 16-30 NM[e,f]	+	1	36	0
SK 31-45 NM[e,f]	++	6	21	39
SK 46-60 NM[e,f]	+++	12	7	79
SK 61-75 NM[e,f]	++	17	3	100
SK1-15NM + Δ SKBP[f]	+			
Δ SKBP	−			

[a] Results are for the analysis of mRNA derived from transfection of COS cells with the indicated mutants.

[b] S1 mapping data derive from laser densitometry of the S1 maps shown in the figures. Levels of SK RNA, derived by comparison of bands produced by the two RNA species within tracks, are expressed as not detectable (−), less than 25% (+), 25 to 50% (++), 50 to 75% (+++), or greater than 75% (++++).

[c] RT-PCR analysis was done in two ways. In the first, RT-PCR was used to generate 48 cDNA clones from each transfection. These were analyzed by single-track sequencing; the numbers of each cDNA isoform are shown in the columns headed SK or NM. Clones containing irregular isoforms of different lengths are not included, because of bias in the PCR reaction, except in the one case where the isoform including both exons was abundant and the normal isoforms were absent. Where a track contained two sequences (NM and SK) in the region of exon V, it was assumed that, due to the density of plaques, the culture contained one representative of each class. The second method of quantitative analysis by RT-PCR involved conditions where the possibility of bias due to depletion of substrates was excluded, and analysis was by gel electrophoresis (e.g., Fig. 5). The ratio of the SK isoform to the sum of the NM and SK isoforms is given as a percentage. The irregular isoforms are not included unless they predominate. Form iii, seen at a low level in most cases, was the only form detected with mutant SK,SK. With mutant ΔSK-6, 43% of the cDNA contained the SK isoform alone and 53% contained both exons.

[d] The construct ΔNM3'SS was found to use two cryptic 3' splice sites within the NM exon to comparable levels, which account for all of the cDNA clones derived from RNA containing one of the alternative exons.

[e] Mutant including the SKBPUP mutation.

[f] Mutant including the Δ Stem mutation.

downstream of the wild-type site had been used (Table 1). The band at 254 nt probably arose from incomplete digestion of the short noncomplementary loop in the NM probe.

To determine whether the selection of exon SK is affected by the presence of exon NM sequence, a 731-nt deletion which spanned the entire exon NM (ΔSpeI; Fig. 1A) was made. It can be seen from S1 nuclease mapping of the mRNA produced from this construct (Fig. 3B) that no mRNA which contained exon SK was derived, even in the complete absence of exon NM sequence. The bands at 130 nt were probably derived from RNA in which exons 4 and 6 had been spliced directly.

We conclude from these initial results that the selection of the alternative exons is not a simple competition event wherein the 3' splice sites compete for splicing to the upstream exon (exon 4) or the 5' splice sites compete for splicing to the downstream exon (exon 6).

Role of splicing signals in the exclusion of exon SK. To determine whether the inactivity of the SK exon was due to poor 5' splice site sequences, the 5' splice sites of exons NM and SK were mutated to the 5' splice site consensus sequence AAG/GTAAGT (31) in separate constructs (NM 5'SS→CONS and SK 5'SS→CONS). In neither case was the splicing pattern altered to any detectable level (Table 1; data not shown). Thus, the absence of SK mRNA cannot be explained by an intrinsic weakness of exon SK splice sites compared with those of exon NM.

Analysis of the tropomyosin gene sequence by using the program Stemloop (5) revealed the potential for a region of secondary structure, comprising an 18-nt stem with a 23-nt loop, around the 5' splice site of exon SK, which could act to sequester the splice site, thereby preventing its use in nonmuscle cells. After deletion of the downstream half of the stem (Δ Stem), S1 mapping showed that the mutation had no effect on the splicing patterns seen in the COS-1 cell (Table 1; data not shown). This mutation was included in a number of other constructs tested because it facilitated mutagenesis of the SK exon.

To determine the effect of branch point strength on exon selection, candidate branch point sequences were mutated to the consensus sequence UACUAAC (35, 43). For exon SK, two possible branch sites were apparent from inspection of the sequence; these were 79 and 106 nt upstream of the 3' splice site. Splicing in vitro of exon 4 to exon SK, and oligonucleotide-directed RNase H cleavage of lariats, showed that a single branch site between nt −65 and −97 (probably between nt −72 and −90) is used (data not shown). Thus, the branch point is almost certainly at nt −79 (AGUUCAC), and this was the site used for mutagenesis. The branch point for exon NM has not been determined by direct experiments. S1 mapping of the products of these constructs (Fig. 3C) showed that enhancement of the NM branch point alone has no effect on exon selection. However, when the SK branch point in Δ Stem was improved, a shift in the splicing pattern occurred that gave rise to inclusion of exon SK into a proportion of transcripts (band at 254 nt with the SK probe), seemingly irrespective of the sequence at the NM branch point. Sequencing of cloned RT-PCR products from this mRNA supported these data (Table 1). A larger increase in exon SK incorporation was seen when the branch points for both exons had been improved, in the absence of the Δ Stem deletion (2× BPUP; Fig. 3C and Table 1).

The effect of the branch point mutation could be attributed to the disruption of secondary structures around exon SK. As a control, the branchpoint sequence of a wild-type minigene was mutated to GUUAGUA. No exon SK use was detected (Δ SKBP; see Fig. 9 and Table 1).

Effect of exon position. To test whether exon sequences make any contribution to selection, each exon was duplicated, or the positions were swapped, as shown in Fig. 1C (NM,NM, SK,SK, and SK,NM), by sticky-feet mutagenesis (2). By this means, exon SK was replaced accurately by exon NM, and vice versa, with the intron portions of the 5'

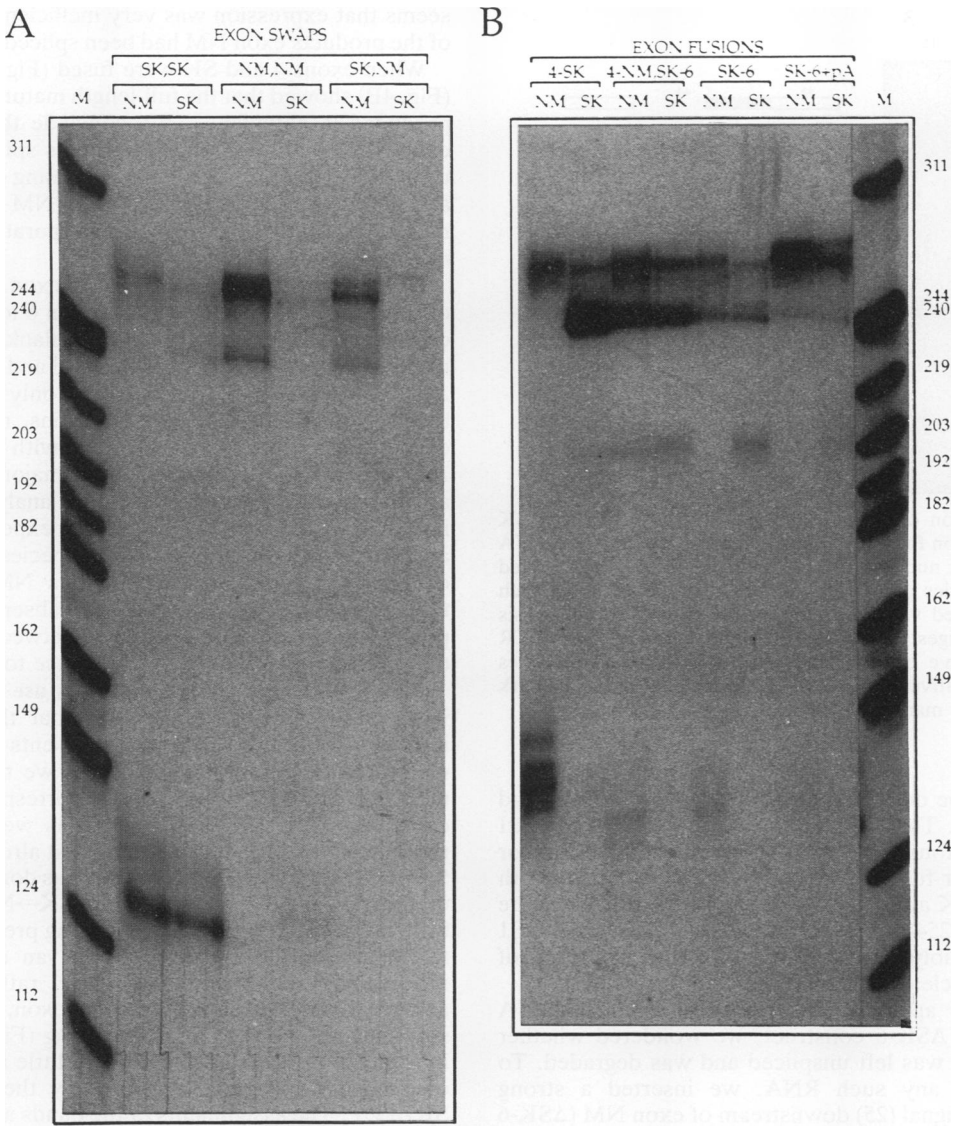


FIG. 4. S1 mapping data showing the importance of exon SK sequences, position, and order of splicing in determining sequestration of exon SK in COS cells. RNA samples were isolated from cells transfected with the constructs shown in Fig. 1. Mapping with nuclease S1 and electrophoresis were done as described in the legend to Fig. 3. (A) Transfection with exon swap mutants shown in Fig. 1C; (B) transfection with exon fusions shown in Fig. 1D.

and 3′ splice site sequences remaining unaltered. The results of expression and mapping by S1 nuclease protection are shown in Fig. 4A. Only 4% of the RNA containing exon 6 produced by mutant SK,SK had incorporated an SK exon (ratio of bands at 254 and 132 nt in lane SK, construct SK,SK). There was no detectable use of exon SK in the case of SK,NM (Fig. 4A), and all of the RNA detected had incorporated exon NM. This result was verified by RT-PCR (Table 1).

Because of the way in which this series of mutants was constructed, in order to limit the number of variables being changed, it was not possible to distinguish between the use of the pair of duplicated exons. That is, we could not tell whether the small amount of exon SK usage was due to the exon in its normal (downstream) or its abnormal (upstream) position. Thus, it cannot be said that one context was better than the other, in terms of inducing use of exon SK. From the results obtained with mutant SK,NM, we conclude that the exon sequence, rather than position or even intron sequence, is an overriding determinant in the control of default exon selection.

An ordered pathway of splicing? Helfman et al. (20) have proposed that one of the key elements in the mechanism of splicing in β-tropomyosin is the exon to which the alternative exons are first spliced. They showed that SK could be spliced to the upstream constitutive exon if it had first been fused (as if by splicing) to the downstream exon.

We have made a construct which reproduced the outcome of such an initial splice (ΔSK-6; Fig. 1D), in which exon SK was fused to exon 6. S1 mapping analysis showed (Fig. 4B) that some SK RNA was produced, together with form iv or v (Fig. 2A). RT-PCR with a range of different concentrations of ΔSK-6 RNA produced two bands, the upper being more abundant (about 57%; Fig. 5 and Table 1); these bands are of

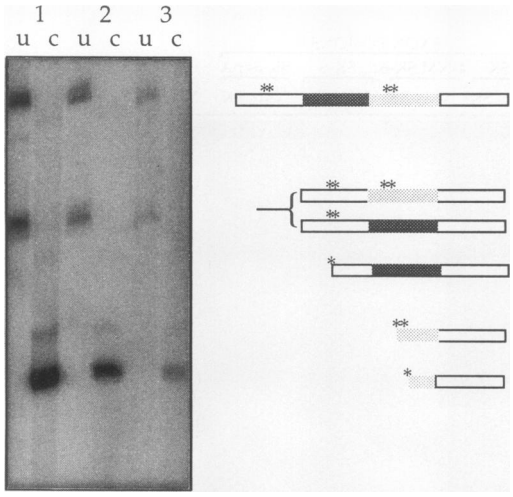


FIG. 5. Production of RNA incorporating exons NM and SK when the ΔSK-6 exon fusion was transfected. Portions of the RNA preparation used for nuclease S1 mapping (Fig. 4B) were subjected to RT-PCR in a series of 10-fold dilutions. An aliquot (c) of each product was digested with *Alu*I and analyzed by electrophoresis alongside a mock-digested aliquot (u). In the diagrams of the PCR products, constitutive exons 4 and 6 are shown as white boxes flanking the alternative exon NM (pale hatching) or SK (dark hatching). Asterisks mark the *Alu*I sites.

the right size to be derived from, respectively, form iv and normal SK RNA. This assignment was confirmed by *Alu*I cleavage within exons 4 and SK. We conclude that the major product seen after fusion of exons SK and 6 contains both exons NM and SK and that less than half the mRNA is the SK isoform. The 254-nt band seen with the NM and SK S1 probes was probably produced by formation of a loop of RNA without S1 cleavage of the probe (38).

Because of the apparent scarcity of tropomyosin RNA produced by the ΔSK-6 construct, we wondered whether most of the RNA was left unspliced and was degraded. To trap or stabilize any such RNA, we inserted a strong polyadenylation signal (25) downstream of exon NM (ΔSK-6 + pA; Fig. 1D). When the RNA produced by this construct was analyzed, it was found that there was a very low signal again from both conventional cDNA S1 probes (Fig. 4B). It seems that expression was very inefficient and that in some of the products exon NM had been spliced to exon SK again.

When exons 4 and SK were fused (Fig. 1D), S1 mapping (Fig. 4B) showed that the full-length mature SK message was produced in abundance. We conclude that the splicing of exon SK to exon 6 is facile, whereas splicing of the intron upstream of exon SK, enforced by fusing exon SK to exon 6 and removing the possibility of exon NM splicing to exon 6, may be inefficient and involves incorporation of exon NM in most of the mRNA.

Because exon NM could splice to exon SK in the absence of an alternative 3′ splice site on exon 6 for the 5′ splice site of exon NM, then removal of both flanking introns should also enhance splicing of exons NM and SK. In construct Δ4-NM,SK-6 (Fig. 1D), in which the only intron is that lying between the mutually exclusive exons, mapping would be expected to produce bands at 132 nt with the NM probe and 208 nt with the SK probe. However, major bands were found at 254 nt with both probes. RT-PCR analysis showed definitively that these bands were the expected S1 mapping artifacts and that the only mRNA species produced by this construct was that in which exons NM and SK joined together (Table 1). Thus, in the absence of alternative possibilities (even of exon skipping between exon 4 and exon 6), it appears that exon NM can splice to exon SK.

Mutation of exon SK results in its use in COS-1 cells. In view of the evidence suggesting that there is something intrinsic to the SK exon that prevents its inclusion into mature mRNA in nonmuscle cells, we mutated 15-nt segments of the exon to match the corresponding portion of exon NM (Fig. 6). These mutations were carried out on tropomyosin minigene constructs that already possessed the SKBPUP mutation (Fig. 1B). This was done so that we could determine which (if any) of the SK→NM mutants were bringing about a change in the splicing preferences, since we felt that it would be easier to see an enhancement of a reasonable level of exon SK usage, rather than trying to achieve activation of the dormant exon. When these constructs were analyzed by S1 mapping (Fig. 7), we saw that in some cases there appeared to be little increase in the use of exon SK compared with use by the parental mutant, SKBPUP. The assignments of the bands are described in the legend to Fig. 7. For constructs in which nt 1 to 15 of the exon were mutated, however, a substantial shift in splicing preference was observed. The five SK→NM mutants were

WILDTYPE	cacag TAAGTGTTCTGAGCTGGAGGAGGAGCTGAAGAATGTCACCAACAACCTCAAGTCTCTTGAGGCTCAGGCGGAGAAG gtagg
SK 1-15 NM	cacag **CCGTTGCCGAGAGAT**GGAGGAGGAGCTGAAGAATGTCACCAACAACCTCAAGTCTCTTGAGGCTCAGGCGGAGAAG gtagg
SK 16-30 NM	cacag TAAGTGTTCTGAGCT**GGATGAGCAGATTAG**GAATGTCACCAACAACCTCAAGTCTCTTGAGGCTCAGGCGGAGAAG gtagg
SK 31-45 NM	cacag TAAGTGTTCTGAGCTGGAGGAGGAGCTG**AAACTGATGGACCAGAA**CCTCAAGTCTCTTGAGGCTCAGGCGGAGAAG gtagg
SK 46-60 NM	cacag TAAGTGTTCTGAGCTGGAGGAGGAGCTGAAGAATGTCACCAACAAC**CTGAAGTGTCTGAG**GGCTCAGGCGGAGAAG gtagg
SK 61-75 NM	cacag TAAGTGTTCTGAGCTGGAGGAGGAGCTGAAGAATGTCACCAACAACCTCAAGTCTCTTGA**TGCTGCTGAAGAAAA**G gtagg

FIG. 6. SK→NM mutants. Shown is the sequence of exon SK of hTM_{nm} (uppercase letters) and its abutting splice sites (lowercase letters), which was mutated by oligonucleotide-directed mutagenesis to give the variations in sequence shown in bold, which are the corresponding nucleotides of exon NM. These mutations were in the previously described mutant SKBPUP (Fig. 1B), which incorporated the ΔStem deletion.

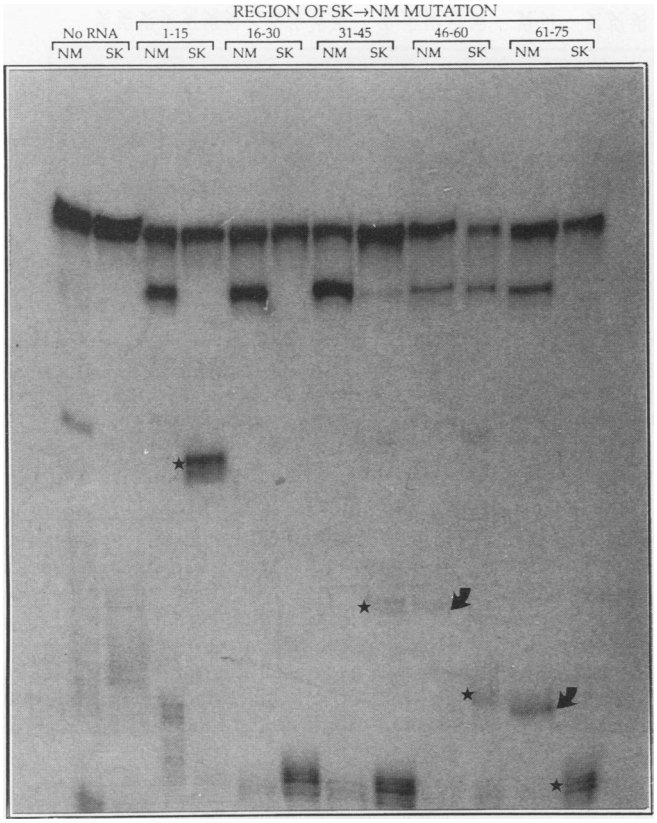


FIG. 7. S1 mapping data showing the effects of systematic replacement of exon SK sequences with corresponding portions of exon NM. COS-1 cells were transfected with the mutants shown in Fig. 6, using NM and SK cDNA probes. Because of deviation from the wild-type sequence at intervals throughout the SK exon, the sizes of bands expected to correspond to protection of the wild-type SK probe by SK RNA vary. These bands are marked with asterisks. The bands at the foot of the autoradiograph are the normal products of about 132 nt. Bands arising from protection of the NM probe by SK RNA are indicated by arrows. In the case of mutant 61-75, the SK probe is not complementary to the SK exon at its 3′ end, and thus the protected product would migrate with products of other origin at 132 nt. However, the exon NM probe would protect extra nucleotides of SK mRNA (shown by the arrowed band). The incidence of full-length protection of the SK probe (the band underneath the undigested probe at 254 nt; lanes SK) is correlated with the extent of similarity of mutant and wild-type sequences (see Fig. 6). All of these RNA samples were analyzed also by RT-PCR and single-track sequencing of a number of resultant clones (Fig. 8 and Table 1).

analyzed by RT-PCR and sequencing (48 clones from each transfection), showing definitively that three of the mutations, SK1-15NM, SK46-60NM, and SK61-75NM, gave significantly increased levels of exon SK splicing compared with the level produced by SKBPUP (Fig. 8 shows an example of the data for SK1-15NM; data for the other constructs are summarized in Table 1). Ten of the clones sequenced for mutant SK46-60NM contained both exons NM and SK (not shown in Table 1), and this form was found at lower frequencies with SK31-45NM and SK61-75NM. We conclude that several sequences within exon SK are important in selection of the default exon in nonmuscle cells.

In order to determine whether the branch point enhancement was necessary for detection of the exon sequence effect, the introduced consensus branch point sequence in SK1-15NM was replaced by the arbitrary sequence used in mutant Δ SKBP. The band at 193 nt, which is indicative of incorporation of exon SK, was reduced but not quite eliminated, despite the absence of either a strong branch point sequence or, indeed, the normal branch point sequence (Fig. 9, SK1-15NM + Δ SKBP).

Use of the SK exon in a muscle cell line. C2 myoblasts were transfected with the original minigene construct (wild type in Fig. 1 and 3A). RT-PCR followed by *Alu*I cleavage showed clearly that in myoblasts both the SK isoform and the more abundant NM isoform were produced (27 and 73%, respectively; Fig. 10). This result is consistent with the pattern of expression of the endogenous gene (16). In contrast to the result seen with construct ΔSK-6 in COS cells, no products including both exons NM and SK were detected, which suggests that ΔSK-6 did not mimic the events determining exon SK incorporation in muscle cells. In other experiments, we have shown that, as with the endogenous gene (16), the proportion of the SK isoform expressed from the minigene increases dramatically during differentiation of the myoblasts into myotubes (17a).

DISCUSSION

A systematic analysis of the *cis*-acting sequence elements that control alternative splicing is made more difficult with mutually exclusive exons by the number of steps at which the outcome could be determined: three exons (NM, SK, and 6) might compete for splicing to exon 4, three exons might compete for splicing to exon 6, the 3′ splice site of exon SK could splice to either exon 4 or exon NM, and the 5′ splice site of exon NM could splice to either exon SK or exon 6. Any of these steps could, in principle, be decisive. Nonetheless, not only have we identified some very important controlling sequences, but we have some indications of the steps that they affect.

We have used COS cells as a convenient nonmuscle cell line, with none of the indications of incorrect nonmuscle splicing patterns reported before with β-tropomyosin minigenes transfected into COS (11) or myoblast (27) cell lines. One interpretation of this difference is that the putative repressor of β-tropomyosin exon SK use (17) is different from and less abundant than any corresponding repressor of hTM_{nm}.

Our initial results showed conclusively that exon SK was not in competition with exon NM, in that deletion or inactivation of the latter did not activate the former. Thus, the basis of exclusion of exon SK in the human hTM_{nm} gene is radically different from that of the muscle-specific exon 2 of the rat α-tropomyosin gene (32) and, at least in this respect, similar to that of the SK exon in rat β-tropomyosin (17). Similar experiments with the rat skeletal alkali myosin light-chain 1/3 gene have shown that the choice between two mutually exclusive exons depends in part on competition for splicing to an upstream exon (12). We conclude that exon SK is intrinsically unreactive in COS cells, if there are flanking introns, and that, unlike the rat α-tropomyosin exons 2 and 3, exons NM and SK are not of necessity completely exclusive; it seems that these exons are not spliced together in the nonmuscle cells because exon SK is not able to compete for splicing to any exon with exon NM or any other exons (see below).

If exon SK is inert, whether it is sequestered in secondary structure or possesses incompetent splicing signals, it is likely that one of the two flanking splices would be rate

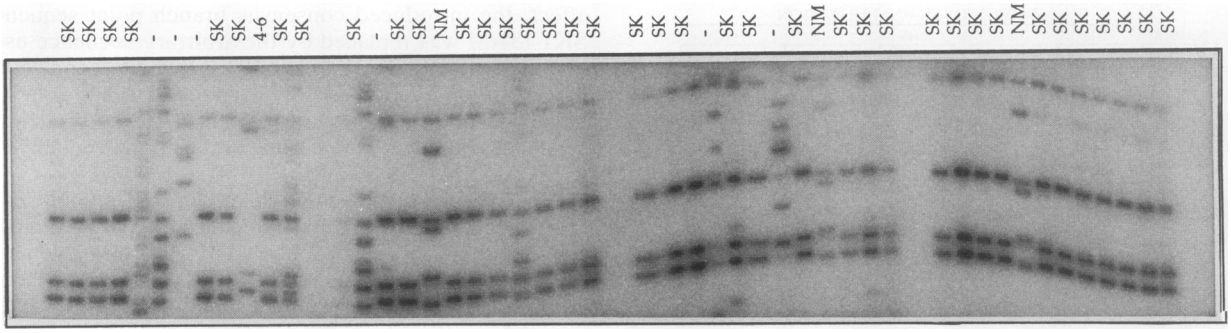


FIG. 8. Single-track sequence analysis of clones produced by RT-PCR from RNA isolated from cells transfected with mutant SK1-15NM. Poly(A) RNA was isolated from the total RNA used for S1 mapping (Fig. 7). Forty-eight clones were sequenced with ddATP and Klenow enzyme. The portion of the autoradiograph shown exhibits the differences between the exon NM and exon SK sequences (identified at the top of each lane). Some of the sequences are unrelated to tropomyosin or contain the product of splicing between exons 4 and 6.

determining, as found for the rat β-tropomyosin (20). We found that the splicing of exon SK to exon 6 is not obstructed once exon 4 has spliced to exon SK, whereas joining of exon SK to exon 6 results in a substantial loss of mutual exclusion. It seems that exon SK is not selected whenever there is a choice; exon 4 will splice to exon NM or, in its absence, exon 6 rather than use exon SK; when exon SK is fused to exon 6, exon 4 still prefers exon NM to exon SK, but exon NM can splice only to exon SK, because there are no downstream introns. It is possible that with downstream introns, the SK-6 fusion exon would itself be omitted. We conclude that it is very unlikely that the natural switch to production of the SK isoform would be determined only by splicing to the downstream exon; inclusion of exon SK rather than exon NM, and not both, could follow only from its selection by exon 4. It would be interesting to test whether inactivation of the 5′ splice site of exon 4 has any effect on exon NM-exon 6 splicing. Our conclusion suggests that the mechanism of selection is quite different from that proposed for β-tropomyosin (20).

This proposal makes several predictions. First, the 5′ splice site of exon SK, which splices so well to exon 6, should not be intrinsically weak. We have ranked a series of 37 5′ splice site sequences in terms of their competitive performance in vivo, and the exon SK site was found to be very effective (23). Furthermore, we established that it is not required for the exclusion of exon SK; altering it to the strongest 5′ splice site, a consensus sequence (9, 23), had no effect (Fig. 3B). Nonetheless, splicing of exon SK to exon 6 must be prevented in vivo before exon NM can splice to exon 6 (alternatively, splicing of exon SK to exon 6 might leave the upstream intron in the product, resulting in degradation). This might be achieved if the 3′ splice site of exon SK was intrinsically unreactive, preventing proper recognition or definition of the 5′ splice site at the other end of the exon (36). It is clear from Fig. 3C that the branch point upstream of the exon SK 3′ splice site is indeed one of the major factors limiting the use of exon SK; poor recognition of the branch point would affect recognition of the 3′ splice site as a whole (34, 40). Comparisons between the results obtained with SKBPUP, which includes the Δ Stem deletion, and 2 × BPUP, which does not, suggest that the stem deletion does not enhance the use of an exon activated by branch point mutations.

The isoform switch in muscle cells could, in principle, be achieved by promoting the splicing of exon SK, via enhancement of the exon SK 3′ splice site, or by inhibition of exon NM use (or both). The latter would be expected to act like the deletion of exon NM or its 5′ splice site and lead to joining of exon 4 to exon 6. We favor muscle-specific enhancement of exon SK use and, from the discussion above, we predict that the critical step is that in which exon 4 splices to exon NM or exon SK.

The role of exon sequences was determined by replacing exon SK sequences with those from exon NM. It is possible that these sequences contribute to the inactivity of exon SK

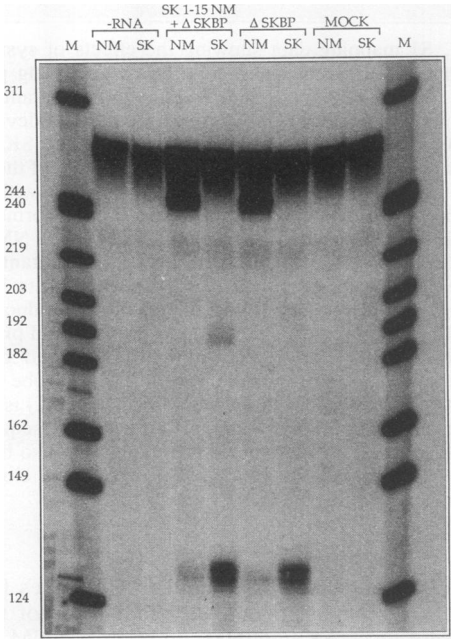


FIG. 9. Effect of exon sequence substitution in the absence of an improved branch site. Construct SK1-15NM (Fig. 6) was mutated to remove the branch point sequence, which had previously been introduced to give mutation SKBPUP (Fig. 1B), producing SK1-15NM + ΔSKBP, and the same branch point change was made to a wild-type construct to give ΔSKBP. The RNA isolated following transfection of COS-1 cells with these constructs was mapped by using NM and SK cDNA probes. The band at 193 nt corresponds to complete protection of the SK probe by RNA containing the mutant SK exon. S1 mapping was also performed in the absence of in vivo RNA (-RNA) and in the presence of RNA isolated from untransfected COS-1 cells (MOCK).

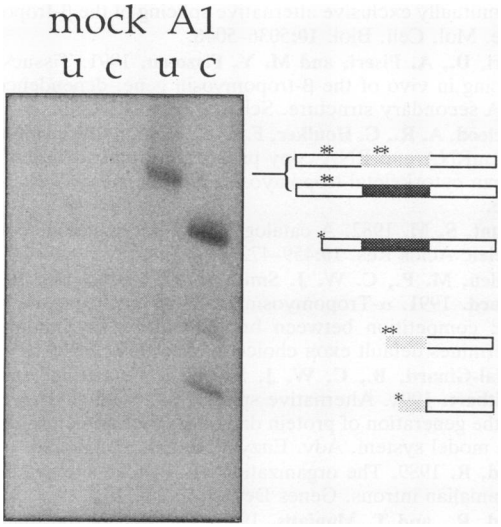


FIG. 10. Expression of the minigene in C2 rodent myoblasts. Total RNA isolated from C2 myoblasts, transfected (A1) or mock transfected (mock), was used for RT-PCR. The second PCR reaction included ^{32}P-labelled primers. An aliquot (c) of each product was digested with *Alu*I and analyzed by electrophoresis alongside a mock-digested aliquot (u). In the diagrams of the PCR products, constitutive exons 4 and 6 are shown as white boxes flanking the alternative exon NM (pale hatching) or SK (dark hatching). Asterisks mark the *Alu*I sites.

or, in exon NM, are stimulatory. The latter possibility seems less likely simply because exon use is the default option for splicing in COS and other nonmuscle cells. These sequences appeared, from the experiment in which the exons were swapped precisely, to be very important; they sufficed to determine that the SK exon was not used even when it was in an environment in which branch site signals were not limiting; conversely, an environment with limiting branch site signals was not sufficient to eliminate use of exon NM. Such an effect might be expected if the exon could form a secondary structure encompassing both splice sites, but it is rather unlikely that similar secondary structures could form both when the exon is in its normal position and when it is flanked by the 4-NM and NM-SK introns. Indeed, an extensive analysis by computer of potential secondary structures showed no features akin to those seen in chick β-tropomyosin (4, 29). An alternative possibility, that the exon sequences might restrict transcription such that splicing complexes assemble first on exon NM and commit it to splicing, is ruled out by the results with mutant SK,NM. Instead, it seems most likely that the exon sequence or structure is a target for the action of repressor proteins in nonmuscle cells.

The effect of branch point replacement also might be explained by a disruption of repressive secondary structures, although an interaction between the branch point and the exon sequences would seem to be rather unlikely in view of the additive effect of mutations in these two regions. The most informative way of testing this possibility, and of indicating the stage of the splicing reaction at which the branch point sequence is restrictive, would be by cotransfection of a wild-type minigene with a U2 small nuclear RNA gene, mutated in an appropriate way to enhance recognition of the exon SK branch point sequence (42, 44). Such experiments are in progress.

Our results suggest that the mechanisms of regulating the incorporation of hTM_{nm} exon SK are different from those proposed for the analogous exon in β-tropomyosin (see the introduction) in that proper expression was seen in COS cells, the branch point is closer to the SK exon and the sequences in between are clearly less important than the exon sequence, secondary structures extending into the intron are unlikely to be involved, and splicing to the downstream exon does not define proper exon use. On the basis of our present results, we interpret the results of the exon swaps, the branch point mutations, and the exon sequence mutations in terms of a model in which exon SK is suppressed by a nonmuscle repressor active only when the exon is flanked by intrinsically weak splicing signals (and not when the branch site of the normal exon SK is improved to the consensus sequence). Given that exon SK use was suppressed when the exon was at the exon NM position, we predict that the splicing signals flanking exon NM are weak also, which may be of importance in the suppression of this exon in muscle cells.

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REFERENCES

1. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
2. **Clackson, T., and G. Winter.** 1989. Sticky feet-directed mutagenesis and its application to swapping antibody domains. Nucleic Acids Res. **17**:10163–10170.
3. **Clayton, L., F. C. Reinach, G. M. Chumbley, and A. R. Macleod.** 1988. Organization of the hTM_{nm} gene: implications for the evolution of muscle and non-muscle tropomyosins. J. Mol. Biol. **201**:507–515.
4. **Clouet d'Orval, B., Y. d'Aubenton Carafa, P. Sirand-Pugnet, M. Gallego, E. Brody, and J. Marie.** 1991. RNA secondary structure repression of a muscle-specific exon in HeLa cell nuclear extracts. Science **252**:1823–1828.
5. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
6. **Don, R. H., P. T. Cox, B. J. Wainwright, K. Baker, and J. S. Mattick.** 1991. 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res. **19**:4008.
7. **Eperon, I. C.** 1986. M13 vectors with T7 polymerase promoters: transcription limited by oligonucleotides. Nucleic Acids Res. **14**:2830.
8. **Eperon, I. C.** 1986. Rapid preparation of bacteriophage DNA for sequence analysis in sets of 96 clones, using filtration. Anal. Biochem. **156**:406–412.
9. **Eperon, L. P., J. P. Estibeiro, and I. C. Eperon.** 1986. The role of nucleotide sequences in splice site selection in eukaryotic pre-messenger RNA. Nature (London) **324**:280–282.
10. **Eperon, L. P., I. R. Graham, A. D. Griffiths, and I. C. Eperon.** 1988. Effects of RNA secondary structure on alternative splicing of pre-mRNA: is folding limited to a region behind the transcribing RNA polymerase? Cell **54**:393–401.
11. **Erster, S. H., L. A. Finn, D. A. Frendewey, and D. M. Helfman.** 1988. Use of RNase H and primer extension to analyze RNA splicing. Nucleic Acids Res. **16**:5999–6014.
12. **Gallego, E. M., and B. Nadal-Ginard.** 1990. Myosin light chain

1/3 gene alternative splicing: *cis* regulation is based upon a hierarchical compatibility between splice sites. Mol. Cell. Biol. **10**:2133–2144.
13. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
14. **Goux-Pelletan, M., D. Libri, Y. d'Aubenton-Carafa, M. Y. Fiszman, E. Brody, and J. Marie.** 1990. *In vitro* splicing of mutually exclusive exons from the chicken β-tropomyosin gene: role of the branch point location and very long pyrimidine stretch. EMBO J. **9**:241–249.
15. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus IV DNA. Virology **52**:456–467.
16. **Gunning, P., M. Gordon, R. Wade, R. Gahlmann, C.-S. Lin, and E. Hardemann.** 1990. Differential control of tropomyosin mRNA levels during myogenesis suggests the existence of an isoform competition-autoregulatory compensation control mechanism. Dev. Biol. **138**:443–453.
17. **Guo, W., G. J. Mulligan, S. Wormsley, and D. M. Helfman.** 1991. Alternative splicing of β-tropomyosin pre-mRNA: *cis*-acting elements and cellular factors that block use of a skeletal muscle exon in non-muscle cells. Genes Dev. **5**:2096–2107.
17a. **Hamshere, M.** Unpublished data.
18. **Hamshere, M., G. Dickson, and I. C. Eperon.** 1991. The muscle specific domain of N-CAM: structure and alternative splicing patterns. Nucleic Acids Res. **19**:4709–4716.
19. **Helfman, D. M., S. Cheley, E. Kuismanen, L. A. Finn, and Y. Yamawaki-Kataoka.** 1986. Nonmuscle and muscle tropomyosin isoforms are expressed from a single gene by alternative RNA splicing and polyadenylation. Mol. Cell. Biol. **6**:3582–3595.
20. **Helfman, D. M., W. M. Ricci, and L. A. Finn.** 1988. Alternative splicing of tropomyosin pre-mRNAs in vitro and in vivo. Genes Dev. **2**:1627–1638.
21. **Helfman, D. M., R. F. Roscigno, G. J. Mulligan, L. A. Finn, and K. S. Weber.** 1990. Identification of two distinct intron elements involved in alternative splicing of β-tropomyosin pre-mRNA. Genes Dev. **4**:98–110.
22. **Helfman, D. M., and W. M. Ricci.** 1989. Branch point selection in alternative splicing of tropomyosin pre-mRNAs. Nucleic Acids Res. **17**:5633–5650.
23. **Lear, A. L., L. P. Eperon, I. M. Wheatley, and I. C. Eperon.** 1990. A hierarchy for 5′ splice site preference determined *in vivo*. J. Mol. Biol. **211**:103–115.
24. **Lees-Miller, J. P., L. O. Goodwin, and D. M. Helfman.** 1990. Three novel brain tropomyosin isoforms are expressed from the rat α-tropomyosin gene through the use of alternative promoters and alternative RNA processing. Mol. Cell. Biol. **10**:1729–1742.
25. **Levitt, N., D. Briggs, A. Gil, and N. J. Proudfoot.** 1989. Definition of an efficient poly(A) site. Genes Dev. **3**:1019–1025.
26. **Libri, D., M. Lemonnier, T. Meinnel, and M. Y. Fiszman.** 1989. A single gene codes for the β subunits of smooth and skeletal muscle tropomyosin in the chicken. J. Biol. Chem. **264**:2935–2944.
27. **Libri, D., J. Marie, E. Brody, and M. Y. Fiszman.** 1989. A subfragment of the β tropomyosin gene is alternatively spliced when transfected into differentiating muscle cells. Nucleic Acids Res. **17**:6449–6462.
28. **Libri, D., M. Goux-Pelletan, E. Brody, and M. Y. Fiszman.** 1990. Exon as well as intron sequences are *cis*-regulating elements for the mutually exclusive alternative splicing of the β-tropomyosin gene. Mol. Cell. Biol. **10**:5036–5046.
29. **Libri, D., A. Piseri, and M. Y. Fiszman.** 1991. Tissue-specific splicing in vivo of the β-tropomyosin gene: dependence on an RNA secondary structure. Science **252**:1842–1845.
30. **Macleod, A. R., C. Houlker, F. C. Reinach, and K. Talbot.** 1986. The mRNA and RNA-copy pseudogenes encoding $TM30_{nm}$, a human cytoskeletal tropomyosin. Nucleic Acids Res. **14**:8413–8426.
31. **Mount, S. M.** 1982. A catalogue of splice junction sequences. Nucleic Acids Res. **10**:459–472.
32. **Mullen, M. P., C. W. J. Smith, J. G. Patton, and B. Nadal-Ginard.** 1991. α-Tropomyosin mutually exclusive exon selection: competition between branchpoint/polypyrimidine tracts determines default exon choice. Genes Dev. **5**:642–655.
33. **Nadal-Ginard, B., C. W. J. Smith, J. G. Patton, and R. E. Breitbart.** 1991. Alternative splicing is an efficient mechanism for the generation of protein diversity: contractile protein genes as a model system. Adv. Enzyme Regul. **31**:261–286.
34. **Reed, R.** 1989. The organization of 3′ splice-site sequences in mammalian introns. Genes Dev. **3**:2113–2123.
35. **Reed, R., and T. Maniatis.** 1988. The role of the mammalian branchpoint sequence in pre-mRNA splicing. Genes Dev. **2**:1268–1276.
36. **Robberson, B. L., G. J. Cote, and S. M. Berget.** 1990. Exon definition may facilitate splice site selection in RNAs with multiple exons. Mol. Cell. Biol. **10**:84–94.
37. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
38. **Sisodia, S. S., D. W. Cleveland, and B. Sollner-Webb.** 1987. A combination of RNase H and S1 nuclease circumvents an artefact inherent to conventional S1 analysis of RNA splicing. Nucleic Acids Res. **15**:1995–2011.
39. **Smith, C. W. J., and B. Nadal-Ginard.** 1989. Mutually exclusive splicing of α-tropomyosin exons enforced by an unusual lariat branch point location: implications for constitutive splicing. Cell **56**:749–758.
40. **Smith, C. W. J., E. B. Parro, J. G. Patton, and B. Nadal-Ginard.** 1989. The 3′ splice site of mammalian introns is defined by scanning from an independently specified branchpoint. Nature (London) **342**:243–247.
41. **Wieczorek, D. F., C. W. J. Smith, and B. Nadal-Ginard.** 1988. The rat α-tropomyosin gene generates a minimum of six different mRNAs coding for striated, smooth, and nonmuscle isoforms by alternative splicing. Mol. Cell. Biol. **8**:679–694.
42. **Wu, J., and J. L. Manley.** 1989. Mammalian pre-mRNA branch site selection by U2 snRNP involves base-pairing. Genes Dev. **3**:1553–1561.
43. **Zhuang, Y., A. M. Goldstein, and A. M. Weiner.** 1989. UAC-UAAC is the preferred branch site for mammalian mRNA splicing. Proc. Natl. Acad. Sci. USA **86**:2752–2756.
44. **Zhuang, Y., and A. M. Weiner.** 1989. A compensatory base change in human U2 snRNA can suppress a branch site mutation. Genes Dev. **3**:1545–1552.
45. **Zoller, M., and M. Smith.** 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol. **100**:468–500.