Alternative splicing of tropomyosin pre-mRNAs in vitro and in vivo

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A single rat gene encodes both fibroblast TM-1 and skeletal muscle β -tropomyosin by an alternative RNA-processing mechanism. The gene contains 11 exons: Exons 1–5 and exons 8 and 9 are constitutive exons common to all mRNAs expressed from this gene; exons 6 and 11 are used in fibroblasts as well as smooth muscle; exons 7 and 10 are used exclusively in skeletal muscle. We have studied the internal alternative RNA splice choice (exons 6 and 7) of the rat tropomyosin 1 gene in vitro, using nuclear extracts obtained from HeLa cells. Use of alternative splice sites in vitro is dependent on the ionic conditions of the assay, and correct splicing occurs only under well-defined salt conditions. Splicing of exon 5 to exon 6 (fibroblast-type splice) and exon 5 to exon 7 (skeletal muscle-type splice) was dependent on precursors in which exon 6 or 7 was first joined to exon 8. The same patterns of alternatively spliced RNAs were formed when similar templates were introduced in HeLa cells by transfection. Thus, there appears to be an ordered pathway of splicing in which the internal alternatively spliced exons must first be joined to the downstream constitutive exon before they can be spliced to the upstream constitutive exon. The data are consistent with a model in which the critical event in alternative splicing occurs during the joining of exon 6 to exon 8 (fibroblast-type splice) or exon 7 to exon 8 (skeletal muscle-type splice).

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The generation of protein isoform diversity by alternative RNA splicing is a fundamental mechanism of eukaryotic gene expression, which contributes to tissue-specific and developmentally regulated patterns of gene expression (for reviews, see Leff et al. 1986; Breitbart et al. 1987). Alternative RNA splicing pathways have also been demonstrated for a number of viral genes (for review, see Ziff 1980). At present, little is known about the mechanisms that determine alternative RNA splicing of complex transcription units. In particular, it is not known how alternative splice sites are selected and whether the splicing signals in complex transcription units differ from those in simple transcription units. The identified sequence elements required for pre-mRNA splicing include the consensus sequences found at the 5' and 3' splice sites and lariat branch points (Mount 1982; Green 1986; Padgett et al. 1986). Sequence comparisons between splice junctions of alternative and constitutive exons have failed to identify any significant differences, suggesting that these sequences alone do not account for the choice between alternative splice sites (Breitbart et al. 1987). It seems likely that regulation of splice site selection in transcripts containing alternative 5' or 3' splice sites will involve other *cis*-acting elements. In addition, a number of studies have suggested the existence of *trans*-acting factors that interact with sequence elements in the pre-mRNA to promote differential splice site selection (Breitbart and

Nadal-Ginard 1987; Leff et al. 1987). However, the identity of these putative *trans*-acting factors and the signals they recognize remains unknown.

One example of the use of alternative RNA processing for the generation of protein diversity is found in the tropomyosin gene family. Tropomyosins comprise a family of related actin-binding proteins present in muscle (skeletal, cardiac, and smooth) and nonmuscle cells. Tropomyosin isoform diversity involves the expression of multiple genes, each of which codes for more than one isoform by the use of alternatively spliced exons. In addition, alternative RNA splicing for the generation of tropomyosin isoform diversity appears to be a fundamental mechanism conserved throughout evolution since it has been characterized in various species, including *Drosophila* (Basi et al. 1984; Karlik and Fyberg 1986), chicken (Fizman et al. 1986), quail (Flach et al. 1986; Hallaeur et al. 1987), rat (Ruiz-Opazo et al. 1985; Helfman et al. 1986; Ruiz-Opazo and Nadal-Ginard 1987), and human (MacLeod et al. 1985; Reinach and MacLeod 1986). Work from our laboratory has previously demonstrated that a single rat gene encodes both rat fibroblast TM-1 and skeletal muscle β -tropomyosin by alternative RNA splicing (Helfman et al. 1986). This gene contains 11 exons (see Fig. 1): Exons 1–5 and exons 8 and 9 are constitutive exons common to all mRNAs expressed from this gene; exons 6 and 11 are used in fibroblasts as well as smooth muscle cells; exons 7 and 10

are used exclusively in skeletal muscle. In addition, sequence analysis did not reveal any obvious nucleotide differences between the common and tissue-specific splice junctions that may be important for alternative splicing (Helfman et al. 1986). The structural features of this gene raise a number of questions concerning the mechanisms involved in tissue-specific processing. The observation that the same gene can generate unique isoforms in cells as different as skeletal muscle and fibroblasts underscores the importance of regulatory factors (*cis* and *trans*) that must be implicated in alternative RNA processing. However, the nature of these factors is not known.

In this paper we report a series of experiments designed to study the alternative splicing pathways of tropomyosin pre-mRNAs in vitro, using nuclear extracts obtained from HeLa cells, and in vivo by transient expression of minigenes in HeLa cells. Using nuclear extracts, the selection of alternative splice sites depends on the ionic conditions of the assay, and correct splicing occurs only under well-defined conditions. In addition, we demonstrate that the internal alternatively spliced exons must first be joined to the downstream constitutive exon before they can be spliced to the upstream constitutive exon. These studies suggest the existence of an ordered pathway of splicing involved in utilization of the internal alternative exons.

Results

Tropomyosin gene constructs used for generation of pre-mRNAs for in vitro splicing

The rat tropomyosin 1 gene gives rise to two mRNAs that result from alternative internal exon selection, 3' exon selection, and poly(A) site utilization (Fig. 1). We prepared a number of DNA templates designed to analyze the internal alternatively spliced region of the rat tropomyosin 1 gene. These constructions contain 1, 2, or 3 introns of the region between exons 5 through 8, inserted downstream of the SP6 promoter (see Fig. 2). Precursors extending to the *Sal*I site in the polylinker of SP64 or to a number of other convenient restriction sites can thus be synthesized with SP6 polymerase.

Splicing of exon 5 to exon 6 (fibroblast-type splice) requires sequences in exon 8

We used a HeLa cell nuclear splicing extract to study processing of a series of pre-mRNAs derived from the internal alternatively spliced region of the tropomyosin gene. We used nuclear extracts derived from HeLa cells because human cells contain the same isogene as the rat, and HeLa cells express exclusively the same isoform as rat fibroblasts, namely TM-1 (MacLeod et al. 1985; Helfman et al. 1986). Thus, at the onset of these studies, there was good potential for using the HeLa cell system to study the *cis*-acting elements and *trans*-acting factors involved in alternative splice-site selection. We first tested the ability of HeLa cell nuclear extracts to splice pre-mRNAs that contained exons 5 and 6. Pre-mRNAs labeled with ³²P were generated from pSP64-p2 linearized with restriction endonucleases *Nco*I and *Stu*I (see Fig. 3). Precursors prepared from templates linearized with *Nco*I contain 40 nucleotides of exon 6, whereas those prepared from templates linearized with *Stu*I contain all of exon 6 and 17 nucleotides of downstream intron sequences. When these precursors were processed in vitro, no detectable splice products were generated (Fig. 3, left and middle panels). Similar results were also obtained with precursors obtained from pSP64-p2 linearized with *Pst*I or *Xmn*I (data not shown). Because the MgCl₂ and KCl concentrations were found to strongly affect in vitro splicing patterns (see below), we also tested precursors derived from pSP64-p2 linearized with *Nco*I and *Stu*I under varying ionic conditions. Under the incubation conditions examined, we did not detect splicing of exon 5 to exon 6 using these precursors (data not shown). The lack of splicing of exon 5 to exon 6 using these pre-mRNAs was unexpected because studies of a number of different pre-mRNA substrates have established that removal of most of the 3' exon sequences has little apparent effect in the splicing reaction in vitro (Furdon and Kole 1986, 1988; Reed and Maniatis 1986; Parent et al. 1987; Turnbull-Ross et al. 1988). Thus, the results obtained using tropomyosin pre-mRNAs are unique when compared with the results obtained in other systems. In addition, these results suggest that the nucleotide sequences within exon 6 do not contain suf-

Figure 1. Schematic diagram of the intron/exon organization of the rat tropomyosin 1 gene (Helfman et al. 1986). (□) Constitutive exons; (▨) and (■) tissue-specific exons, as indicated; horizontal lines represent introns (not drawn to scale). Amino acids encoded by each exon are indicated. The cap site and polyadenylation sites are also indicated.

Figure 2. SP6/tropomyosin transcriptional templates. The DNA templates for use in in vitro transcription with SP6 polymerase are shown. All are derived from the internal region of the TM-1 gene (exons 5–8). The arrows indicate the sites of transcription initiation with the SP6 sequences (crosshatched regions) adjacent to the SP6 promoter. In vitro transcription terminates at indicated restriction sites. In addition, a number of internal restriction sites are also indicated that can be used for synthesis of truncated substrates. Boxes indicate positions of exon 5 (constitutive), exon 6 (fibroblast and smooth muscle-specific), exon 7 (skeletal muscle-specific), and exon 8 (constitutive). Lines represent each intron. The number of nucleotides in each exon and intron are indicated. All constructions are derivatives of pSP64-p2.

ficient information to be recognized as a 3' splice site by the splicing machinery.

The order of intron removal in the tropomyosin pre-mRNA in vivo is not known. To determine whether splicing of exon 5 to exon 6 required that exon 6 first be spliced to exon 8, we constructed plasmid pSP64-p4, which contains the region of the cDNA clone encoding all the sequences downstream of exon 6 through the poly(A) tract used in rat fibroblast TM-1 (see Fig. 2). Surprisingly, in precursors prepared from pSP64-p4 linearized with *Cla*I, exon 5 was spliced efficiently to exon 6 under standard splicing conditions (Fig. 3, right panel). To determine whether the products indicated in Figure 3 are associated with accurate splicing of exon 5 to exon 6, we carried out primer-extension analysis using a 20-nucleotide-long ³²P-labeled oligodeoxynucleotide corresponding to sequences in the middle of exon 6 as a primer. Sequencing of the primer-extension product confirmed that exon 5 was spliced accurately to exon 6 (data not shown). Virtually identical results were obtained with precursors derived from pSP64-p4 linearized with *Sal*I (data not shown). The splicing of exon 5 to exon 6 observed in Figure 3 was not due simply to the length of sequences downstream of exon 6, because pre-mRNAs prepared from templates derived from pSP64-p2 linearized with *Xmn*I and *Pst*I did not exhibit splicing of exon 5 to exon 6 (data not shown). In addition, a point mutation was introduced in the 3' end of intron 7 in the plasmid pSP64-p2, whereby the consensus AG was changed to AA. Incubation of precursors derived from this template did not result in splicing of exon 5 to exon 6 nor splicing of exon 5 to exon 7 (data not shown). Thus, splicing of exon 5 to exon 6 is dependent on the

Figure 3. Analysis of in vitro splicing products of tropomyosin pre-mRNAs. Time course (min) of formation of spliced products from transcripts terminating at the *Nco*I site (left), *Stu*I site (middle) of pSP64-p2, and *Cla*I site of pSP64-p4 (right). The assay conditions used for these studies consisted of 15 μ l of nuclear extract in a final reaction volume of 25 μ l containing 1 mM $MgCl_2$, 500 μ M ATP, 20 mM creatine phosphate, 2.7% (wt/vol) polyvinyl alcohol, 15–30 ng pre-mRNA, 12.8 mM HEPES (pH 8), 14% (vol/vol) glycerol, 60 mM KCl, 0.12 mM EDTA, and 0.7 mM DTT. Schematic representation of products are indicated; boxes represent exon sequences, and lines represent intron sequences.

alternative exon first being joined to downstream exon sequences.

To determine how much sequence downstream of exon 6 in pSP64-p4 was required for splicing of exon 5 to exon 6, a series of *Bal31* constructions were prepared from the *ClaI* site in pSP64-p4. The various deletions were tested for their ability to be spliced *in vitro*. Figure 4 (lanes 1–8) shows the results of processing pre-mRNAs containing 51–195 nucleotides downstream of the 3' splice site. The full-length pre-mRNA derived from pSP64-p4 linearized with *ClaI* (Fig. 4, lane 1) contains 76 nucleotides of exon 6, 63 nucleotides of exon 8, and 54 nucleotides of exon 9. The length of the corresponding *Bal31* deletions are indicated in Figure 4. Transcripts terminating 102–195 nucleotides (Fig. 4, lanes 1–5) downstream of the 3' splice site were processed in the splicing extract, as indicated by a band corresponding to the 5' exon (e), free intron 5 lariat (d), splice product (c), and intron–exon lariat intermediate (b). On the other hand, pre-mRNAs containing 51–88 nucleotides downstream of the 3' splice site were spliced very inefficiently, if at all, as indicated by the lack of free 5' exon (e) and free intron 5 lariat (d). Pre-mRNAs containing <26 nucleotides of exon 8 did not appear to be processed (Fig. 4). Thus, splicing of exon 5 to exon 6 did not require sequences contained in exon 9 but did require some, but not all, of exon 8.

Alternative splicing *in vitro* is influenced by ionic conditions

The data presented above in Figures 3 and 4 suggested that there may be an ordered pathway involved in utilization of exon 6 in which splicing of exon 6 to exon 8 will precede splicing of exon 5 to exon 6. The alternative splicing pathways of tropomyosin pre-mRNAs were examined in greater detail using pre-mRNAs containing

exons 5–8 and following the formation of splice products over time. A full-length precursor derived from pSP64-p2 was spliced in HeLa cell nuclear extracts. A time course analysis of the products is shown in Figure 5 (left). We observed three processing products corresponding to (1) free exon 5, (2) exon 5 spliced to exon 8, and (3) the free lariat released after ligation of exon 5 to exon 8. The intermediates corresponding to the lariat and downstream exon sequences expected from the splicing of exon 5 to exon 8 did not resolve from the precursor on these 4% gels. To determine whether the products indicated in Figure 5 are associated with accurate splicing of exon 5 to exon 8, we carried out primer extension analysis using a 20-nucleotide-long ³²P-labeled oligodeoxynucleotide corresponding to sequences in the middle of exon 8 as a primer (Fig. 5, right). Figure 5 (right) shows the time course of the reaction products analyzed by primer-extension analysis. One product of 127 nucleotides appeared after 45 min incubation and increased in intensity with longer incubation times. This product corresponded to the size of the primer-extension product expected if exon 5 was correctly joined to exon 8. Sequence analysis of the primer-extension product confirmed that exon 5 was spliced accurately to exon 8 (data not shown). A number of shorter primer-extension products accumulated in a time-dependent manner. One of these (70 nucleotides long; see Fig. 5, right) corresponds to the position of a branch point sequence located 24 nucleotides upstream of the 3' splice junction of exon 8 (D. Helfman and W.M. Ricci, unpubl.). We also analyzed the pre-mRNA generated from pSP64-p2 spliced *in vitro*, by primer-extension analysis, using ³²P-labeled oligodeoxynucleotide primers specific for exons 6 and 7. In agreement with the data presented in Figure 5, no splice products were detected, further indicating a lack of splicing of exon 5 to exon 6 or 7 (data not shown). Thus, the only processing products detected

Figure 4. *In vitro* splicing of exon 5 to exon 6 (fibroblast-type splice) requires sequences in exon 8. *In vitro* splicing reactions were carried out for 2 hr, and the ³²P-labeled RNA products were separated in 4% denaturing polyacrylamide gels. (Right) DNA templates derived from the *ClaI* site of pSP64-p4 by *Bal31* nuclease treatment. The assay conditions used for these studies consisted of 15 μ l of nuclear extract in a final reaction volume of 25 μ l containing 1 mM MgCl₂, 500 μ M ATP, 20 mM creatine phosphate, 2.7% (wt/vol) polyvinyl alcohol, 15–30 ng pre-mRNA, 12.8 mM HEPES (pH 8), 14% (vol/vol) glycerol, 60 mM KCl, 0.12 mM EDTA, and 0.7 mM DTT. The positions of the precursors and products are indicated: (a) Precursors; (b) exon/intron lariat intermediates; (c) spliced RNAs; (d) free intron 5 lariat; (e) free exon 5.

Figure 5. Analysis of in vitro splicing products of tropomyosin pre-mRNAs. (Left) Time course (min) of formation of spliced products from transcripts terminating at the *SalI* site of plasmid pSP64-p2. The ^{32}P -labeled RNA products were separated in 4% denaturing polyacrylamide gels. The assay conditions used for these studies consisted of 15 μl of nuclear extract in a final reaction volume of 25 μl containing 1 mM MgCl_2 , 500 μl ATP, 20 mM creatine phosphate, 2.7% (wt/vol) polyvinyl alcohol, 15–30 ng pre-mRNA, 12.8 mM HEPES (pH 8), 14% (vol/vol) glycerol, 60 mM KCl, 0.12 mM EDTA, and 0.7 mM DTT. Schematic representation of products is indicated; boxes represent exon sequences, and lines represent intron sequences. (Right) The in vitro processing products shown at left were analyzed by primer extension using a ^{32}P -labeled oligodeoxynucleotide primer specific for sequences in exon 8. Position of primer-extension products corresponding to splice products and intermediates are indicated.

using this substrate were the result of splicing exon 5 to exon 8. It was unexpected that exon 5 would be spliced to exon 8 using full-length precursors derived from pSP64-p2. Because HeLa cells do not express the skeletal muscle isoform from the endogenous gene, the lack of utilization of exon 7 in vitro was not surprising. However, the lack of utilization of exon 6 was unexpected. The absence of products utilizing the 5' or 3' splice sites of exon 6 suggests that either additional factors are required that the in vitro system is lacking, or that the incubation conditions used in Figure 5 (1 mM MgCl_2 and 60 mM KCl) are not adequate for alternative splicing.

For alternatively spliced pre-mRNA, variations in ionic conditions, extract concentration, and extract preparation have been reported to affect the choice of splice site (Hartmuth and Barta 1987; Reed and Maniatis 1986; Schmitt et al. 1987). Therefore, we examined the effects of changing the concentrations of KCl and MgCl_2 on splice-site utilization of tropomyosin pre-mRNAs. We analyzed the splicing of pre-mRNAs derived from pSP64-p2p6 linearized with *ClaI*. Plasmid pSP64-p2p6 is similar to plasmid pSP64-p2, except exon 9 sequences have been joined to exon 8. Virtually identical results were obtained with both pre-mRNAs (data not shown). We first analyzed splicing of the pSP64-p2p6 transcript by changing the concentration of MgCl_2 (Fig. 6). In comparison with the exon skipping shown in Figure 5, we detected splicing of exon 6 to exon 8 as the MgCl_2 was increased from 1 mM to 3 mM. In agreement with the results presented in Figure 5, exon 5 was spliced to exon 8 at 1 mM MgCl_2 (Fig. 6). In contrast, as the MgCl_2 concentration was increased above 1.25 mM MgCl_2 ,

Figure 6. Effect of magnesium concentration on alternative splicing in vitro. Tropomyosin pre-mRNAs derived from pSP64-p2p6 linearized with *ClaI* were processed for 2 hr in vitro, using the assay conditions described in Fig. 3, except the magnesium concentration was varied as indicated at the top. The splicing products were analyzed on a 4% gel. Schematic representation of the products is indicated. The markers are ^{32}P -labeled *MspI* fragments of pBR322 [M].

cleavage at the 5' splice site of exon 6 was detected as well as splicing of exon 6 to exon 8. Furthermore, above 2.50 mM MgCl_2 cleavage at the 5' splice site of exon 6 was the preferred choice and there was no cleavage at the 5' splice site of exon 5 and concomitant splicing of exon 5 to exon 8. We also examined the effects of changes in KCl concentration at low MgCl_2 (1.5 mM) and at high MgCl_2 (2.75 mM) concentrations (Fig. 7). As indicated in Figure 7, when the *in vitro* splicing reactions were carried out at 1.5 mM MgCl_2 , low KCl concentrations (40–70 mM) favored exon skipping wherein exon 5 was spliced to exon 8, whereas higher concentrations of KCl (70–110 mM) permitted cleavage at the 5' splice site of exon 6 and subsequent joining of exon 6 to exon 8. On the other hand, at 2.75 mM MgCl_2 , cleavage at the 5' splice site of exon 6 was readily detected over a broad

range of KCl concentrations (Fig. 7). Under any incubation conditions used, neither the 5' or 3' splice site of exon 7 was utilized when pre-mRNAs derived from pSP64-p2p6 linearized with *ClaI* were used. Thus, in HeLa cell nuclear extracts, use of the correct splice sites depends on the ionic conditions of the assay, and alternative splicing occurs only under well-defined salt conditions. If the splicing reactions are carried out for longer periods of time (3–5 hr), we can detect some splicing of exon 5 to exon 6, but only after exon 6 has first been joined to exon 8 (data not shown).

Splicing of exon 5 to exon 7 (skeletal muscle-type splice) requires sequences in exon 8

It was of interest to determine whether splicing of exon 5 to exon 7 would also be influenced by joining exon 7 to exon 8. Plasmid pSP64-p2(7/8) was constructed to study this possibility (Fig. 2). When an RNA precursor derived from pSP64-p2(7/8) was incubated *in vitro*, an RNA product of the size expected for splicing of exon 5 to exon 7 was observed (Fig. 8, left). To confirm that this product represented accurate splicing of exon 5 to exon 7, we carried out primer extension analysis using a 20-nucleotide-long ^{32}P -labeled oligodeoxynucleotide corresponding to sequences in the middle of exon 7 as a primer (Fig. 8, right). As indicated in Figure 8, a primer extension product of 129 nucleotides was obtained, corresponding to the size of the primer extension product expected if exon 5 was correctly joined to exon 7. This primer extension product was sequenced, and results confirmed that the RNA product was spliced as indicated (data not shown). Splicing of exon 5 to exon 7 was not due simply to the length of sequences downstream of exon 7 because pre-mRNAs prepared from templates derived from pSP64-p2 linearized with *XmnI* which would contain 140 nucleotides of intron 7, do not exhibit splicing of exon 5 to exon 7. In addition, there was no indication that either the 5' or 3' splice site of exon 6 was utilized with this substrate. Thus, splicing of exon 5 to exon 7 in HeLa cell nuclear extracts was found to occur if the alternative exon was first joined to exon 8.

Processing of tropomyosin minigenes in vivo

The *in vitro* experiments described above demonstrate that splicing of exon 5 to exon 6 or 7 is dependent on one of these exons first being joined to exon 8. Because the HeLa cell nuclear extracts were able to utilize the 3' splice of both exon 6 (fibroblast-type splice) and exon 7 (skeletal muscle-type splice), it is likely that 3' splice-site utilization of these exons does not require a cell-type-specific splicing machinery. However, one consideration is that 3' splice-site discrimination was lost using the *in vitro* system. This could be due to the loss of a regulatory factor during the extract preparation. For example, it is possible that *in vivo* the 3' splice site of exon 7 is subject to negative control, and the factor responsible for this was lost or is functionally inactive in

Figure 7. Effect of varying ionic conditions on alternative splicing *in vitro*. Tropomyosin pre-mRNAs derived from pSP64-p2p6 linearized with *ClaI* were processed for 2 hr *in vitro*, using the assay conditions described in Fig. 3, except 10 μl of nuclear extract was used in a final reaction volume of 25 μl , and the magnesium and potassium concentrations were varied as indicated at the top. The splicing products were analyzed on a 4% gel. Schematic representation of the products is indicated. The markers are ^{32}P -labeled *MspI* fragments of pBR322 [M].

Figure 8. In vitro splicing of exon 5 to exon 7 (skeletal muscle-type splice) requires sequences in exon 8. (*Left*) Time course (min) of formation of spliced products from transcripts terminating at the *SalI* site of plasmid pSP64-p2(7/8). The ^{32}P -labeled RNA products were separated on 4% denaturing polyacrylamide gels. The assay conditions used for these studies consisted of 15 μl of nuclear extract in a final reaction volume of 25 μl containing 1 mM MgCl_2 , 500 μM ATP, 20 mM creatine phosphate, 2.7% (wt/vol) polyvinyl alcohol, 15–30 ng pre-mRNA, 12.8 mM HEPES (pH 8), 14% (vol/vol) glycerol, 60 mM KCl, 0.12 mM EDTA, and 0.7 mM DTT. Schematic representation of products and intermediates is indicated. (*Right*) In vitro processing products shown at *left* were analyzed by primer extension using a ^{32}P -labeled oligodeoxynucleotide primer specific for sequences in the middle of exon 7. Position of the extension product corresponding to spliced RNA is indicated.

the extracts used in the present study. In addition, because tissue-specific RNA processing of this gene involves alternative 3' exon selection and poly(A) site utilization, the alternative splicing patterns observed in vivo may therefore be a consequence of the different pre-mRNAs generated by using the two different 3' exons. On the other hand, splicing of exon 5 to either exon 6 or exon 7 may not be a strictly regulated event but may be dependent on either of the alternative exons being spliced to exon 8.

To address these questions in vivo, we constructed three minigenes (Fig. 9). These three minigenes contain genomic fragments from exons 5–9. HeLa cells were transfected with these three plasmid DNAs, and the resulting transiently expressed cytoplasmic RNAs were isolated. The RNA protection analyses of these RNAs,

as well as RNA isolated from mock-infected cells, were carried out using RNA probes derived from cDNA clones encoding rat fibroblast TM-1 and skeletal muscle β -tropomyosin (Fig. 9). Transient expression of plasmid pSV40-2 in HeLa cells resulted in spliced RNA that contains exons 5 + 6 + 8 + 9 (Fig. 9). No other products were observed, and no RNAs were detected that contained exon 7. Similar results were obtained with pSV40-2(6/8). These results were not unexpected because HeLa cells express the same tropomyosin isoform as rat fibroblasts, namely TM-1, and, therefore, would be expected to utilize exon 6 and not exon 7. These results also demonstrate that in the absence of the alternative 3'-end processing that involves exons 10 and 11, HeLa cells were able to process these minigenes in a manner that reflected the products expected from the endogenous gene. Interestingly, expression of pSV40-2(7/8) in HeLa cells resulted in expression of spliced RNA that contains exons 5 + 7 + 8 + 9 (Fig. 9). Thus, in agreement with the in vitro data, HeLa cells can utilize the 3' splice site of exon 7 (skeletal muscle-type splice) if exon 7 is first joined to exon 8.

Discussion

Alternative splice-site selection in vitro

The results of the present experiments demonstrate that rat tropomyosin pre-mRNAs can be spliced in HeLa cell nuclear extracts. In addition, we also show that correct splicing occurs only under well-defined salt conditions. Utilization of the alternative splice sites was exquisitely sensitive to changes in the concentrations of MgCl_2 and KCl (Figs. 6 and 7). These results are reminiscent of the results obtained on alternative splicing of adenovirus E1A gene products in vitro demonstrating changes in ionic conditions that affect the choice of splice sites (Schmitt et al. 1987). The mechanisms responsible for these variations in splice-site selection as a function of changes in ionic conditions are not known. Splicing requires multiple components that interact with the pre-mRNA to form a functional splicing complex. It is likely that different factors have different ionic requirements. For example, the interaction of a given snRNP to the same or different splice sites may vary as a function of ionic conditions. In addition, RNA structure has been suggested to play a role in alternative RNA processing by regulating the accessibility of different exons to the splicing machinery (Khoury et al. 1979; Munroe 1984; Solnick 1985; Eperon et al. 1986; Edlind et al. 1987). Thus, changes in ionic conditions may effect the structure of the pre-mRNA itself, which may, in turn, influence the availability of a given splice site. These questions can now be studied further with the development of an in vitro system that reproduces alternative processing of tropomyosin pre-mRNAs.

The in vitro experiments have revealed a number of features of alternative splicing of tropomyosin pre-mRNAs. Of particular interest was the observation that splicing of exon 5 to exon 6 (fibroblast-type splice) is dependent on exon 6 first being joined to exon 8 (Figs. 3

Figure 9. RNA analysis of HeLa cells transfected with tropomyosin minigenes. (Top) Schematic diagram of the three tropomyosin minigenes used in the HeLa cell transfections. The autoradiograph of RNA protection analyses using probes derived from cDNA clones encoding rat fibroblast TM-1 (middle left) and skeletal muscle β -tropomyosin (middle right) of cytoplasmic RNAs from HeLa cells transfected with the minigenes, as indicated. (Bottom) Schematic diagram of the structure of the RNA probe and protected fragments (S) corresponding to spliced RNA from the three minigenes.

and 4). We also determined that splicing of exon 5 to exon 7 (skeletal muscle-type splice) was observed, but only if the alternatively spliced exon was first joined to exon 8 (Fig. 8). Similar results were obtained *in vivo* using transient expression of tropomyosin minigenes in HeLa cells (Fig. 9). These data are consistent with a model in which alternative RNA processing is regulated during splicing of exon 6 or 7 to the downstream constitutive exon (see below). However, the biological significance of these studies with respect to the skeletal muscle-type splice will require development of *in vitro* systems that accurately reflect the *in vivo* splicing patterns observed in skeletal muscle. For example, skeletal muscle cells may splice exon 5 to exon 7 independent of sequences in exon 8. Accordingly, the need for the putative tissue-specific factors that permit this splice may have been circumvented in the HeLa system by fusing exon 8 to exon 7.

Role of exons and introns in alternative splice-site selection

The reason that the splicing of exon 5 to exon 6 (fibroblast-type splice) and of exon 5 to exon 7 (skeletal

muscle-type splice) is dependent on either alternative exon being joined to exon 8 remains to be determined. One explanation is that intron sequences located downstream of an exon may contain sequences that inhibit splicing of an upstream intron (Furdon and Kole 1988). Accordingly, intron sequences downstream of exon 6 or 7 could contain sequences that inhibit splicing of these exons to exon 5. However, we were unable to detect splicing of exon 5 to exon 6 using pre-mRNAs with no downstream intron sequences unless at least 26 nucleotides of exon 8 were joined to exon 6 (Figs. 3 and 4). Thus, removal of intron sequences downstream of exon 6 alone was not sufficient for splicing of exon 5 to exon 6. At present, we do not know whether the intron sequences downstream of exon 7 are inhibitory to utilization of the 3' splice site of this exon. Another explanation is that the nucleotide sequences contained in exon 6, and perhaps exon 7, do not contain sufficient information to interact with splicing factors to form a functional splicing complex with respect to utilization of their 3' splice sites. Work from a number of laboratories has established a minimum exon length of the 3' exon in the splicing reaction (Furdon and Kole 1986, 1988; Reed and Maniatis 1986; Parent et al. 1987; Turnbull-Ross et al. 1988). These studies have demonstrated a requirement

for as little as 20 nucleotides of the 3' exon. The present studies demonstrate that tropomyosin pre-mRNAs containing all 76 nucleotides of the 3' exon 6 sequences were not spliced to exon 5 (Fig. 3). The joining of exon 8 to exons 6 or 7 may be required for the formation of a 3' exon of sufficient length to interact with splicing factors and thereby be utilized by the splicing apparatus. In addition, exon 8 may contain sequences that represent a specific splicing signal.

Studies of alternative RNA splice-site selection of SV40 late transcripts and the human fibronectin gene demonstrated that mutations within exons can affect the choice of splice sites used (Somesekhar and Mertz 1985; Mardon et al. 1987). Although these studies show that alterations in exon sequences affect splice-site selection, the mechanism responsible for these results remains to be determined. Exon sequences could, in principle, interact directly with specific components of the splicing machinery, and changes in their sequences could therefore affect binding sites. In addition, exon and intron sequences may contribute to RNA secondary structures. RNA secondary structure has been suggested to play a role in alternative RNA processing by regulating the accessibility of different exons to the splicing machinery (Khouri et al. 1979; Munroe 1984; Solnick 1985; Eperon et al. 1986; Edlind et al. 1987). Whether the joining of exons 6 and 7 to exon 8 results in changes in RNA secondary structure and, subsequently, alterations in splice-site selection is unknown. It is possible that either of the alternative exons may be sequestered and unable to interact with the splicing machinery due to RNA folding. Such potential secondary structures could involve intron sequences upstream, as well as downstream, of a given exon. Alternatively, specific sequences within introns could interact with factors that facilitate or stabilize the formation of secondary structures. The joining together of two exons and thereby removing a flanking intron may prevent the formation of RNA structures that interfere with the interaction of a given 5' or 3' splice site with splicing factors.

Utilization of internal alternative 3' splice sites does not require tissue-specific factors

Regulation of alternative splice-site selection in vivo could be the result of cell-type-specific splicing machinery that can only use the 5' or 3' splice sites of the alternatively spliced exons. Using both in vitro and in vivo systems, we were able to demonstrate splicing of exon 5 to exon 7 (skeletal muscle-type splice) when the latter exon was first joined to exon 8 (Figs. 8 and 9). The ability of HeLa cell nuclear extracts and HeLa cells to splice exon 5 to exon 7 was unexpected because this splice occurs exclusively in skeletal muscle in vivo (Helfman et al. 1986). Because the HeLa cell nuclear extracts and intact cells were able to utilize the 3' splice sites of both exon 6 (fibroblast-type splice) and exon 7 (skeletal muscle-type splice), it seems likely that 3' splice-site utilization of these exons does not require a cell-type-specific splicing machinery. Hence, utilization

of the 5' splice sites of exons 6 and 7 may be the only *cis*-sequences that need to be regulated for tissue-specific splicing.

Model for alternative splice-site selection

The data presented in the present studies are consistent with a model in which the critical event in alternative splicing occurs during the joining of exon 6 to exon 8 (fibroblast-type splice) or exon 7 to exon 8 (skeletal muscle-type splice). This model proposes that the splicing machinery in fibroblasts and smooth muscle cells promotes the joining of exon 6 to exon 8, whereas the splicing machinery in skeletal muscle promotes the joining of exon 7 to exon 8. The precise nature of this regulation is not known. Alternative RNA processing may be the result of specialized factors that recognize the relevant splice site directly, or there may be tissue-specific positive or negative regulators that control the interaction of a general splicing factor with the alternative splice sites. For example, recognition of the 5' splice sites of either exon 6 or exon 7 may require tissue-specific factors that directly interact with these splice sites, i.e., a tissue-specific U1 snRNP. Alternative RNA processing may occur by the regulation of RNA structure in such a way as to favor or prevent the use of a particular splice site by the general splicing machinery. Such a model has been proposed for alternative RNA processing in calcitonin/CGRP gene expression (Leff et al. 1987). In addition, we do not know whether splicing of exon 6 to exon 8 and exon 7 to exon 8 are both regulated processes that each require cell-specific *trans*-acting factors. Splicing of exon 6 to exon 8 may simply reflect the 'constitutive' pathway of splicing, whereas splicing exon 7 to exon 8 requires tissue-specific factors expressed in skeletal muscle. For example, studies of alternative RNA processing of the calcitonin/CGRP gene have suggested that expression of calcitonin mRNA is a constitutive pathway, whereas CGRP mRNA likely involves neuron-specific splicing factors (Leff et al. 1987). On the other hand, studies of the troponin T gene have suggested that tissue-specific factors are required for use of both constitutive and alternatively spliced exons (Breitbart et al. 1987). In the future, it will be of interest to determine whether utilization of both of the alternatively spliced exons in the tropomyosin gene will require tissue-specific splicing factors.

Finally, the studies presented in this paper may have broad implications in studying alternative RNA splicing as a mechanism to regulate the expression of specific protein isoforms in different cell types. There are a number of cellular and viral genes that contain internal alternatively spliced exons such as fibronectin, growth hormone, troponin T, and tropomyosin (for references, see Leff et al. 1986; Breitbart et al. 1987). In these cases, it will be of interest to determine whether splicing will follow an ordered pathway in which the internal alternatively spliced exons must first be joined to the downstream constitutive exon before they can be spliced to the upstream constitutive exon.

Experimental procedures

Plasmid constructions

The DNA templates for use in *in vitro* transcription with SP6 polymerase are shown in Figure 2. All are derived from the rat fibroblast TM-1 gene (Helfman et al. 1986). pSP64-p2 was constructed by first deleting sequences from the unique *Clal* site in exon 9 with *Bal31* to 17 nucleotides 3' of exon 8. The DNA was then digested at a unique *BalI* site in exon 5. The DNA was modified with *HindIII* linkers and cloned into the *HindIII* site of SP64. Thus, SP64-p2 contains an SP6 promoter with 14 nucleotides of plasmid sequence, including the *HindIII* linker following the SP6 RNA initiation site and 66 nucleotides of exon 5 and genomic sequence extending 17 nucleotides 3' of exon 8. Plasmid pSP64-p4 was derived from SP64-p2 by ligating the *NcoI*-*Sall* fragment of the cDNA clone encoding rat fibroblast TM-1 (Yamawaki-Kataoka and Helfman 1985) to the *NcoI*-*Sall* sites of pSP64-p2. Plasmids pSP64-4(1-8) (Fig. 5) were obtained from pSP64-p4 by first digesting pSP64-p4 with *Clal*. The DNA was then treated with *Bal31* nuclease, then the Klenow fragment of DNA polymerase I to generate blunt ends, followed by ligation to *Clal* linkers. Plasmid pSP64-p2(7/8) was derived from pSP64-2 by removal of the intervening sequence between exons 7 and 8 using a 30-nucleotide-long deoxyoligonucleotide containing 15 nucleotides of exon 7 and 15 nucleotides of exon 8 (Eghtedarzadeh and Henikoff 1986). Plasmid pSP64-p2p6 was constructed by joining the regions of the cDNA encoding fibroblast TM-1 corresponding to exons 9-11 to pSP64-p2.

For studies designed to analyze the internal alternatively spliced region of the rat TM-1 gene *in vivo*, the initial construction contains a 1.0-kb *BalI*-*Clal* fragment that begins at the 5' region of exon 5 and extends through the 3' region of exon 9. This fragment was modified with *HindIII* linkers and cloned into the unique *HindIII* site located 70 bp downstream of the transcriptional start site in the eukaryotic expression vector pXKH (D. Hanahan, unpubl.). This plasmid, herein referred to as pSV40-2, contains the SV40 early promoter and a functional poly(A) site flanking genomic tropomyosin sequences containing exons 5-9 (Fig. 9). Plasmid pSV40-2(6/8) was derived from pSV40-2 by removal of the genomic sequences between exons 6 and 8 using a 30-nucleotide-long deoxynucleotide containing 15 nucleotides of exon 6 and 15 nucleotides of exon 8 (Eghtedarzadeh and Henikoff 1986). Similarly, plasmid pSV40-2(7/8) was derived from pSV40-2 by removal of intron sequences between exons 7 and 8 using a 30 nucleotide long deoxynucleotide containing 15 nucleotides of exon 7 and 15 nucleotides of exon 8 (Eghtedarzadeh and Henikoff 1986).

Synthesis of RNA and *in vitro* splicing

The ³²P-labeled SP6/tropomyosin transcripts were synthesized *in vitro* primed with cap analog, as described (Konarska et al. 1984). The ³²P-labeled pre-mRNAs contained a small amount of prematurely terminated transcripts, but their presence did not appear to effect the *in vitro* splicing reactions, and the RNA substrates were not purified further.

HeLa cell nuclear extracts were prepared as described (Dignam et al. 1983; Krainer et al. 1984). *In vitro* splicing reactions were carried out at 30°C for indicated times (0-240 min). Standard assay conditions used for these studies consisted of 15 µl of nuclear extract in a final reaction volume of 25 µl containing 1 mM MgCl₂, 500 µM ATP, 20 mM creatine phosphate, 2.7% (wt/vol) polyvinyl alcohol, 15-30 ng pre-mRNA, 12.8 mM HEPES [pH 8], 14% (vol/vol) glycerol, 60 mM KCl, 0.12 mM EDTA, and 0.7 mM dithiothreitol (DTT). In some experiments,

the concentrations of MgCl₂ and KCl were varied as indicated. To vary the KCl concentration, the *in vitro* reactions were performed using 10 µl of nuclear extract in a final reaction volume of 25 µl.

Analysis of processing products

After incubation for the indicated time, the reactions were stopped by addition of a solution containing SDS-proteinase K, and the RNA recovered as described (Krainer et al. 1984). The products of the reaction were analyzed on denaturing urea-polyacrylamide gels. Intermediates were distinguished from final products by their relative appearance and disappearance in time course experiments. Lariat RNAs were identified by their aberrant mobility on different percentage polyacrylamide gels (Grabowski et al. 1984; Ruskin et al. 1984). In addition, RNAs containing lariats were verified through treatment by a lariat debranching activity (Ruskin and Green 1985). Processing products were also analyzed by primer-extension analysis and S1 nuclease protection analysis using cDNA clones derived from fibroblast TM-1 and skeletal muscle β-tropomyosin (Helfman et al. 1986). Primer extension analysis was carried out essentially as described (Ghosh et al. 1978; Erster et al. 1988). Oligodeoxynucleotides complementary to sequences encoding amino acids 198-204 within exon 6 (5'-GAGAGCCTGGTC-CATGGTTC-3'), amino acids 198-204 within exon 7 (5'-CAAGTTGTTGGTAACAATT-3'), and amino acids 223-229 within exon 8 (5'-TCCTGAAGTTTGATCTCCTT-3') of the rat fibroblast TM-1 gene were used as primers. Primers were end labeled with [γ-³²P]ATP using T4 polynucleotide kinase. The primers were annealed to the RNA by incubation for 3 min at 55°C and then 30 min at 37°C in 30 µl of primer-extension buffer [50 mM Tris-HCl (pH 8.3), 140 mM KCl, 10 mM MgCl₂]. For primer extension, reactions were transferred to 42°C and supplemented with DTT (final concentration of 5 mM) and deoxynucleotide triphosphates (final concentration of 0.5 mM) and 10 units of avian myeloblastosis virus reverse transcriptase. After 30 min, the reactions were terminated by addition of EDTA to a final concentration of 25 mM, and the radiolabeled pre-mRNA was degraded by treatment with RNase A prior to gel electrophoresis. To sequence the primer extension products, the standard splicing reactions were scaled up 10- to 30-fold, the primer-extension products were resolved on sequencing gels, and the bands cut out of gels, recovered, and subjected to sequence analysis (Maxam and Gilbert 1980).

Transfections and RNA analysis

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% CS (calf serum). HeLa cells were transfected with 5 µg of plasmid DNA per 100-mm plate using a calcium phosphate coprecipitation procedure, and the cells were harvested 48 hr later. For preparation of cytoplasmic RNA, each 100-mm plate of HeLa cells was washed three times with ice-cold phosphate-buffered saline (PBS); the cells were scraped from the plates with a rubber policeman in 1 ml of PBS and transferred to a microfuge tube. The cells were centrifuged for 15 sec and resuspended in 375 µl of ice-cold buffer containing 150 mM NaCl, 1.5 mM MgCl₂, and 10 mM Tris-HCl [pH 8.0]; 10% NP-40 was then added to a final concentration of 0.5% to lyse the cells. The nuclei were pelleted by a 2-min centrifugation in the cold room, and the supernatant was transferred to a clean tube containing 4 µl of 20% SDS and 5 µl of proteinase K (10 mg/ml). The mixture was incubated at 37°C for 15 min and extracted with phenol, and the nucleic acids were precipitated with ethanol. The RNA preparations were then

treated with DNase in the presence of RNasin and reprecipitated.

RNase protection assays were carried out as described (Zinn et al. 1983). The plasmids used for synthesis of ³²P-labeled antisense RNA were derived from cDNA clones encoding rat fibroblast TM-1 and skeletal muscle β -tropomyosin. The plasmids were prepared by inserting the *Pst*I–*Cl*aI fragments of the fibroblast and skeletal muscle cDNA clones into pSP64. The *Pst*I site, corresponding to amino acids 144 in exon 4, was modified with *Eco*RI linkers, and the *Cl*aI site, corresponding to amino acids 252 in exon 9, was modified with *Hind*III linkers. The resulting *Eco*RI–*Hind*III fragments were ligated into an *Eco*RI–*Hind*III double-cut pSP64 vector. Plasmid was linearized with *Eco*RI and transcribed with SP6 polymerase to yield a transcript of ~330 nucleotides. Because the transfected genes do not contain sequences in exon 4, it was possible to distinguish RNA derived from the transfected genes from endogenous mRNA. Hybridizations contained 10 μ g of total cytoplasmic RNA and 500,000 dpm of tropomyosin probe, in a total volume of 30 μ l. All subsequent steps were carried out as described previously (Zinn et al. 1983), except the RNase digestions were carried out at 30°C for 60 min.

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