MOLECULAR AND CELLULAR BIOLOGY, Nov. 1986, p. 3582–3595
0270-7306/86/113582-14$02.00/0

Vol. 6, No. 11

Nonmuscle and Muscle Tropomyosin Isoforms Are Expressed from a Single Gene by Alternative RNA Splicing and Polyadenylation

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Received 16 April 1986/Accepted 23 June 1986

The molecular basis for the expression of rat embryonic fibroblast tropomyosin 1 and skeletal muscle beta-tropomyosin was determined. cDNA clones encoding these tropomyosin isoforms exhibit complete identity except for two carboxy-proximal regions (amino acids 189 to 213 and 258 to 284) and different 3′-untranslated sequences. The isoform-specific regions delineate the troponin T-binding domains of skeletal muscle tropomyosin. Analysis of genomic clones indicates that there are two separate loci in the rat genome that contain sequences complementary to these mRNAs. One locus is a pseudogene. The other locus contains a single gene made up of 11 exons and spans approximately 10 kilobases. Sequences common to all mRNAs were found in exons 1 through 5 (amino acids 1 to 188) and exons 8 and 9 (amino acids 214 to 257). Exons 6 and 11 are specific for fibroblast mRNA (amino acids 189 to 213 and 258 to 284, respectively), while exons 7 and 10 are specific for skeletal muscle mRNA (amino acids 189 to 213 and 258 to 284, respectively). In addition, exons 10 and 11 each contain the entire 3′-untranslated sequences of the respective mRNAs including the polyadenylation site. Although the gene is also expressed in smooth muscle (stomach, uterus, and vas deferens), only the fibroblast-type splice products can be detected in these tissues. S1 and primer extension analyses indicate that all mRNAs expressed from this gene are transcribed from a single promoter. The promoter was found to contain G-C-rich sequences, a TATA-like sequence TTTTA, no identifiable CCAAT box, and two putative Sp1-binding sites.

Tropomyosins are components of the contractile systems of skeletal, cardiac, and smooth muscles and the cytoskeleton of nonmuscle cells. Although they are present in all cells, different forms of the protein are characteristic of specific cell types. Tropomyosin is a rodlike protein composed of two highly alpha-helical subunits wrapped around each other to form a coiled-coil structure (87). Skeletal muscle contains two forms of tropomyosin termed alpha and beta, the proportions of which vary with fiber type (9, 10, 19, 20, 81, 88). Both of these subunits are 284 amino acids long and differ only slightly in amino acid sequence (55). In cardiac muscle of small mammals such as rodents, only alpha-tropomyosin is expressed (19). In striated muscle, tropomyosin is localized to the thin filaments, where it is found along both grooves of the actin filaments (87). The function of tropomyosin in skeletal and cardiac muscle is in association with the troponin complex (troponin I, T, and C) to regulate the calcium-sensitive interaction of actin and myosin (87, 94).

Tropomyosins are found in all smooth muscle and nonmuscle cells (16, 18, 21, 22, 28, 30, 31, 41–43, 45, 64). Multiple forms of tropomyosin have been detected in many cultures of nonmuscle cells (33, 34, 38, 50, 51, 59, 60, 85, 92). For example, rat embryonic fibroblasts contain three major tropomyosins termed 1, 2, and 4 (apparent molecular weights of 40,000, 36,500, and 32,400, respectively) and two relatively minor tropomyosins termed 3 and 5 (apparent molecular weights of 35,000 and 32,000, respectively) (59, 60). The multiplicity of tropomyosin isoforms in fibroblasts raises questions as to the structure and function of each isoform in nonmuscle cells. In addition, nonmuscle and smooth muscle cells do not contain a troponin complex. In these cell types the phosphorylation of the light chains of myosin by the enzyme myosin light chain kinase appears to be the major calcium-sensitive regulatory mechanism controlling the interaction of actin and myosin (1). These differences in the regulation of the contractile apparatus of smooth muscle and nonmuscle cells compared with skeletal and cardiac muscle appear to require structurally as well as functionally distinct forms of tropomyosin.

Amino acid and nucleic acid sequence analyses of tropomyosins from skeletal muscle (53, 55, 77, 80, 91), cardiac muscle (49, 77), smooth muscle (37, 46, 80, 82), and some nonmuscle cells (38, 48, 53, 102) show them to be highly homologous. Nevertheless, structural differences do exist among the various protein isoforms. These divergent regions appear to delineate functional domains of the proteins, including troponin-binding domains, actin-binding properties, and head-to-tail polymerization (for example, see references 48 and 72).

In addition to questions relating to tropomyosins at the protein and cellular levels, the structure and organization of the genes encoding tropomyosins in vertebrates remain to be elucidated. For example, the molecular bases for the occurrence of the multiple forms of tropomyosin in fibroblasts are not fully understood. In vitro translation of mRNA isolated from fibroblasts indicates that each form is encoded by a separate mRNA rather than formed by posttranslational modifications of the proteins (39, 50, 59, 92). The mRNA for each species of tropomyosin could be encoded by a separate gene. Alternatively, some of the mRNAs may be expressed from a single gene via differential processing of the transcript.

Recently, we reported the isolation and characterization of cDNA clones encoding the entire coding region for rat embryonic fibroblast tropomyosin 1 (apparent molecular weight, 40,000) (102). Northern blot analysis demonstrated that fibroblasts and smooth muscle (stomach and uterus)

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contained a single species of mRNA of approximately 1.1 kilobase (kb). In contrast, skeletal muscle was found to contain a single species of mRNA of approximately 1.3 kb. These studies indicated a high degree of homology between the fibroblast and skeletal transcripts and prompted structural studies on the skeletal muscle mRNA.

In the present study, we report the isolation and characterization of cDNA clones containing the entire coding region for skeletal muscle beta-tropomyosin. The nucleic acid and deduced amino acid sequences are compared with those of rat embryonic fibroblast tropomyosin 1. Northern transfer and S1 analysis were performed to study the expression of these tropomyosin isoforms in different tissues. We also demonstrated that a single gene encodes these two mRNAs, both generated by tissue-specific processing of the primary transcript.

MATERIALS AND METHODS

Construction and screening of rat skeletal muscle cDNA library. Starting with 20 μg of poly(A)$^+$ RNA obtained from adult rat skeletal muscle, a cDNA expression library was constructed by sequential addition of *Eco*RI and *Sal*I linkers and insertion into the vectors pUC8 and pUC9 (36, 97). Transformation of *Escherichia coli* DH-5 was performed as described previously (35). The bacteria were plated onto 82-mm nitrocellulose filters (Triton-free HATF; Millipore Corp., Bedford, Mass.) overlaid on ampicillin plates. Colonies were replica plated onto 82-mm nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.). Ampicillin-resistant colonies were screened for the presence of tropomyosin sequences with a ^{32}P-labeled DNA probe prepared by nick translation (74) of rat embryonic fibroblast tropomyosin 1 cDNA (102). Colonies were lysed in situ by the method of Grunstein and Hogness (34). Hybridizations were carried out in buffer containing 50% formamide, 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.0), 1× Denhardt solution (21), 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (pH 7.0), 160 μg of carrier DNA per ml, and 20 μg of yeast tRNA per ml. Filters were washed in 0.1× SSC containing 0.1% sodium dodecyl sulfate (SDS) at 50°C and autoradiographed for 1 to 2 days with Du Pont Cronex Lightning-Plus intensifying screens.

Screening of phage genomic libraries. A rat genomic DNA library was kindly provided by Richard O. Hynes and has been described in detail elsewhere (93). Genomic clones were screened for inserts corresponding to rat embryonic fibroblast tropomyosin 1 by in situ plaque hybridization (4). Approximately two genomic equivalents were screened with ^{32}P-labeled nick-translated (74) cDNA clones encoding rat embryonic fibroblast tropomyosin 1 (102). Phage DNA was prepared as described by Yamamoto et al. (101).

Southern blotting analysis. High-molecular-weight DNA was isolated from rat fibroblasts and adult rat liver, skeletal muscle, stomach, heart, and brain (Fischer strain) and rat embryonic fibroblasts as described previously (57). The purified DNAs were digested with various restriction enzymes, and the DNA fragments were separated on agarose gels (0.8 to 1.2%). DNA blotting was carried out by the method of Southern (89). The filters were hybridized for 24 h to nick-translated probes in the same formamide buffer described above for screening the cDNA library. The filters were washed in 0.1× SSC–0.1% SDS at 50 to 65°C and autoradiographed for 1 to 3 days with Du Pont Cronex Lightning-Plus X-ray intensifying screens.

Restriction mapping and DNA sequencing. For analysis of cDNA clones, a series of overlapping clones from the rat cDNA library were identified. Appropriate DNA fragments were ligated to compatible plasmids of bacteriophage M13mp18 or M13mp19 (103). DNA sequences were determined by the dideoxy chain termination procedure (83).

For detailed mapping, genomic DNA fragments were subcloned into pUC18 or pUC19 by standard procedures (57, 103). Subcloned fragments were mapped by complete digestions. Appropriate DNA fragments were isolated and ligated into compatible plasmids of bacteriophage M13mp8, M13mp9, M13mp18, and M13mp19 (97, 103). The DNA sequence was determined by the dideoxy chain termination procedure (83). Alternatively, fragments were sequenced by the method of Maxam and Gilbert (61).

Preparation of RNA. Total cellular RNA was prepared from rat embryonic fibroblasts and adult rat stomach, uterus, vas deferens, heart, and skeletal muscle (thigh) as described previously (12, 29). Poly(A)$^+$ RNA was prepared by oligo (dT)-cellulose chromatography (2).

Northern transfer analysis of rat tissue RNA. Total cellular RNA (10 μg) from rat skeletal muscle (thigh), stomach, uterus, vas deferens, heart, and rat embryonic fibroblasts was separated on a 1.2% formaldehyde–agarose gel (57), transferred to nitrocellulose paper (95), and hybridized with a ^{32}P-labeled probe prepared by nick translation of skeletal muscle clone pRSkM-22 in the formamide buffer described above. Filters were washed in 0.1× SSC containing 0.1% SDS at 65°C and autoradiographed for 4 days with Du Pont Cronex Lightning-Plus intensifying screens.

S1 nuclease mapping of RNA. S1 nuclease analysis was performed essentially as described by Berk and Sharp (5). Full-length skeletal muscle beta-tropomyosin cDNA (clone pRSkM-22) was digested with *Nco*I and end labeled with the Klenow fragment of DNA polymerase I (New England BioLabs, Inc., Beverly, Mass.) and [α-^{32}P]dCTP (specific activity, 800 Ci mmol^{-1}; New England Nuclear Corp., Boston, Mass.). After digestion with *Cla*I, a 757-base-pair (bp)-long *Nco*I-*Cla*I fragment was isolated by agarose gel electrophoresis. Full-length rat embryonic fibroblast tropomyosin 1 (clone pTM-1) was digested with *Acc*I and end labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP (specific activity, 800 Ci mmol^{-1}; New England Nuclear Corp.). After digestion with *Cla*I, an 804-bp-long *Acc*I-*Cla*I fragment was isolated by agarose gel electrophoresis. Full-length skeletal muscle beta-tropomyosin cDNA (clone pRSkM-22) was digested with *Pst*I and end labeled with polynucleotide kinase (New England BioLabs) and [γ-^{32}P]ATP (specific activity, 3,000 Ci mmol^{-1}; New England Nuclear Corp.). After digestion with *Nco*I, a 424-bp-long fragment was isolated by agarose gel electrophoresis.

The probe for the 5'-end analysis of the gene was prepared by end labeling the genomic *Nco*I fragment at the ATG start codon of exon 1 (see Fig. 6) with [γ-^{32}P]ATP and T4 polynucleotide kinase. The end-labeled fragment was digested with *Bam*HI. The resulting end-labeled fragments (approximately 270 bp) were isolated from polyacrylamide gels.

The purified end-labeled fragments were then denatured in the presence of 10 μg of total RNA at 85°C for 15 min in 30 μl of buffer containing 80% formamide, 0.4 M NaCl, and 10 mM 1,4-piperazinediethanesulfonic acid (pH 6.4). Hybridizations were carried out in the same buffer at 60°C for 5 h. After hybridization, the samples were diluted to 300 μl with ice-cold buffer containing 0.28 M NaCl, 0.05 M sodium

acetate (pH 4.6), 4.5 mM $ZnSO_4$, 20 μg of carrier DNA per ml, and approximately 400 U of S1 nuclease. The samples were incubated at 37°C for 60 min. S1 nuclease-resistant products were analyzed by electrophoresis on an 8% polyacrylamide gel containing 8 M urea and detected by autoradiography.

Primer extension analysis. Primer extension analysis was carried out essentially as previously described (32). For primer extension analysis, a 49-bp fragment corresponding to a 5′-untranslated region of the mRNA was obtained starting from amino acid 1 of exon 1 (see Fig. 6). DNA was digested with *Nco*I and end labeled with [γ-^{32}P]ATP, using T4 polynucleotide kinase. The end-labeled fragments were digested to completion with *Acc*I. The labeled fragments were isolated from polyacrylamide gels. The labeled fragments were denatured in the presence of total RNA at 85°C for 15 min in 20 μl of buffer containing 80% formamide, 0.4 M NaCl, and 10 mM 1,4-piperazinediethanesulfonic acid (pH 6.4). Hybridizations were carried out in the same buffer at 60°C for 3 h. For primer extension, the mixture was diluted to 200 μl in a buffer containing 50 mM Tris chloride (pH 8.3), 20 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM deoxynucleotide triphosphates, and 5 U of reverse transcriptase. The samples were incubated at 42°C for 1 h. The products were then analyzed by electrophoresis on an 8% polyacrylamide gel containing 8 M urea and detected by autoradiography.

RESULTS

Isolation of cDNA clones encoding skeletal muscle beta-tropomyosin. To isolate cDNA clones encoding skeletal muscle tropomyosin, we constructed a cDNA library in the plasmid expression vectors pUC8 and pUC9. The library was screened with a ^{32}P-labeled DNA probe prepared from cDNA clone pTM-1 that encodes the entire rat embryonic fibroblast tropomyosin 1 that we characterized previously (102). Twenty-two colonies hybridized to the ^{32}P-labeled probe. These positive colonies were isolated, and the sizes of the cDNA inserts were determined. Five clones with insert sizes ranging from 700 to 1,200 bp were further characterized by restriction enzyme mapping. Subcloning of the overlapping cDNA clones into M13mp18 and M13mp19 yielded sequencing substrates in both orientations. One clone, pRSkM-22, had the largest-size cDNA insert (approximately 1,200 bp) and contained the entire coding region and 3′-untranslated region through the poly(A) track, as well as a portion (16 bp) of the 5′-untranslated region (Fig. 1).

The complete nucleotide sequence of the skeletal muscle cDNA and the deduced amino acid sequence are shown in Fig. 1. The amino acid sequence of the rat skeletal muscle cDNA is identical to that of rabbit skeletal muscle beta-tropomyosin (55). Comparison of the rat skeletal muscle beta-tropomyosin coding sequence with that of the rat embryonic fibroblast tropomyosin 1 sequence revealed a number of interesting structural features (Fig. 1). The skeletal muscle tropomyosin is identical to the fibroblast isoform except for two distinct regions: one internal (amino acids 189 to 213) and the other at the carboxy terminus (amino acids 258 to 284). In this regard, biochemical studies have indicated the presence of two troponin T-binding sites on skeletal muscle tropomyosin (13–15, 40, 41, 54, 58, 62, 68, 69, 71, 72, 90, 100). One troponin T-binding site is located at the carboxy terminus of the molecule, and the second site is located near Cys-190. It is apparent from the sequence comparison presented in Fig. 1 that these troponin T-binding domains correspond to those regions where the sequences differ between the fibroblast and skeletal muscle isoforms. Accordingly, these isoform-specific regions appear to delineate functional domains of the proteins. It should be noted that the difference in the third base of the codon for amino acid 188 in skeletal muscle and fibroblast tropomyosins is due to split codons in separate exons encoding amino acids 188 to 213 (see below and Table 1). The skeletal muscle cDNA had a relatively long 3′-untranslated region (233 bp) compared with that from fibroblasts (73 bp). These differences in the 3′-untranslated region explain the size difference observed between the skeletal muscle mRNA (approximately 1.3 kb) and the fibroblast and smooth muscle mRNAs (approximately 1.1 kb). Since the nucleotide sequence of the rat skeletal muscle cDNA was identical with the sequence of the fibroblast cDNA except for the two isoform-specific regions and 3′-untranslated sequences, the data strongly suggested that both mRNAs arise from a common gene via alternative RNA splicing. Below we present the analysis of genomic clones demonstrating that the 1.1-kb mRNA observed in fibroblasts and smooth muscle cells and the 1.3-kb mRNA in skeletal muscle are expressed from a single gene.

Expression of tropomyosin mRNAs in different tissues. Since muscle and nonmuscle cells each contain different tropomyosin isoforms, we investigated the tissue specificity of the skeletal muscle cDNA clone. Northern blot analysis of RNA from various rat tissues probed with nick-translated pRSkM-22 revealed the existence of two hybridizing classes of mRNA (Fig. 2): a 1.3-kb mRNA species present in skeletal muscle and a 1.1-kb mRNA expressed in smooth muscle (stomach, uterus, and vas deferens) and nonmuscle (fibroblasts) cells. Cardiac muscle contained neither mRNA species. This is consistent with a previous report indicating that rat cardiac muscle contains a single isoform of tropomyosin that is likely encoded by the same gene that encodes rat skeletal muscle alpha-tropomyosin (80).

To determine which sequences encoding amino acids 189 to 213 in skeletal muscle or fibroblast RNA were expressed in various smooth muscle cells, we performed S1 nuclease mapping analysis. When the *Nco*I-*Cla*I fragment of the skeletal muscle cDNA was used as a probe, the expected protection of the full-length fragment (757 nucleotides) was obtained only with skeletal muscle RNA (Fig. 3a). In contrast, S1 protection experiments with fibroblast- and smooth muscle (stomach, uterus, and vas deferens)-derived RNAs yielded a 560-nucleotide fragment (Fig. 3a). This is consistent with the sequence data which show that the differences between the fibroblast and skeletal muscle cDNAs are confined to the carboxy-proximal ends. When the *Acc*I-*Cla*I fragment of the fibroblast tropomyosin 1 cDNA was used as a probe, a full-length fragment (804 nucleotides) was obtained with fibroblast-, stomach-, uterus-, and vas deferens-derived RNAs (Fig. 3b). On the other hand, S1 protection experiments with skeletal muscle RNA yielded a 607-nucleotide fragment (Fig. 3b). These results indicate that identical tropomyosin isoforms are expressed in fibroblast and smooth muscle cells. It is worth noting that by S1 nuclease mapping with the rat embryonic fibroblast cDNA we have further demonstrated that the smooth muscle RNAs have the same carboxy-terminal regions (amino acids 258 to 284) as fibroblast RNAs (data not shown). Recently, it was reported that skeletal muscle alpha-tropomyosin differs from a smooth muscle tropomyosin at both an amino-terminal region (amino acids 40 to 76) and a carboxy-terminal region (amino acids 258 to 284) (80). To determine whether any other RNAs were structurally related to the skeletal muscle

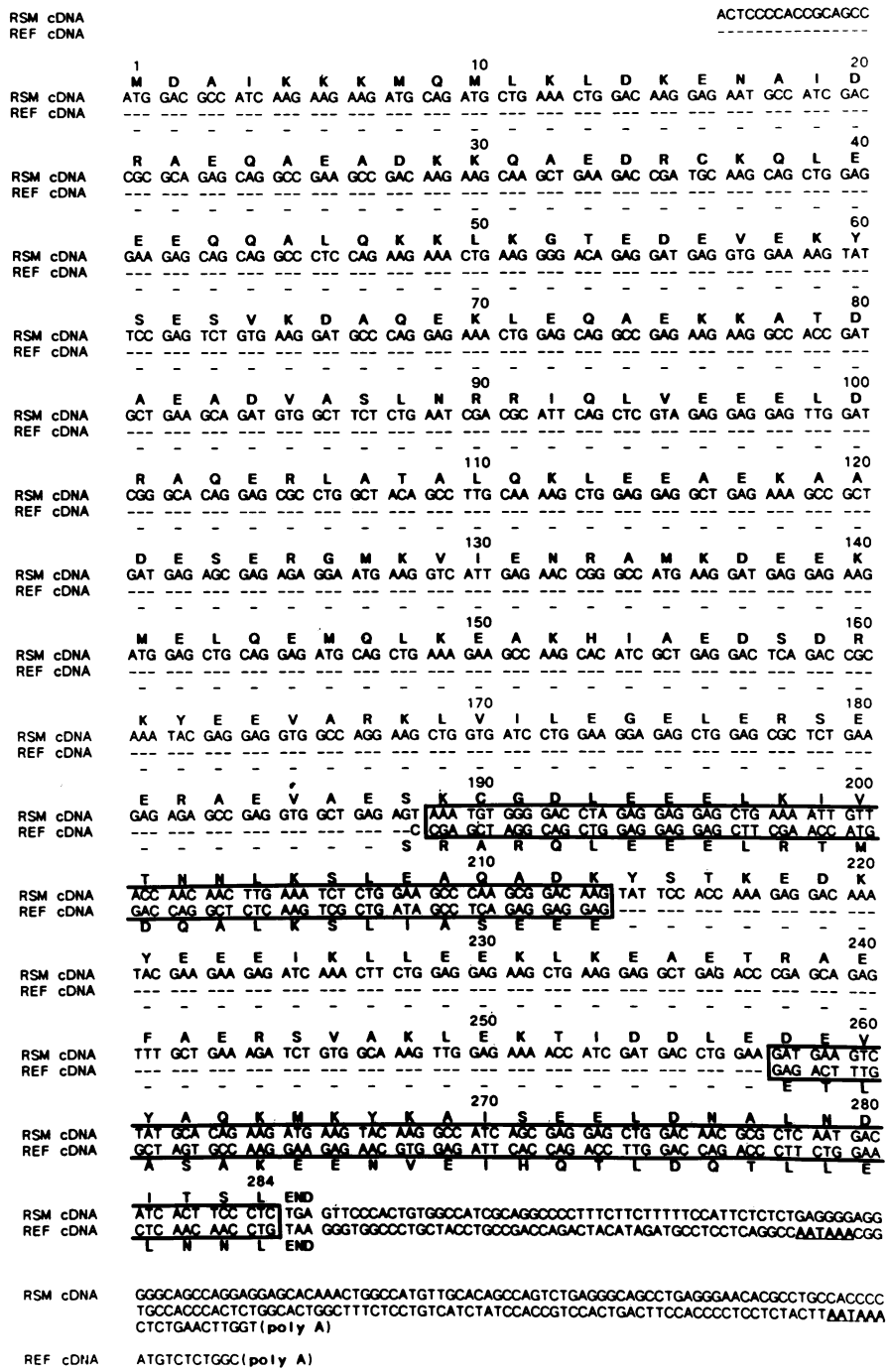

FIG. 1. Nucleotide and derived amino acid sequence comparison of cDNA clones encoding rat skeletal muscle beta-tropomyosin and rat embryonic fibroblast tropomyosin 1. Sequence analysis of rat skeletal muscle tropomyosin cDNA (RSM) was carried out by the dideoxy chain termination method (83). The rat embryonic fibroblast tropomyosin 1 (REF) sequence (102) is aligned for comparison. The deduced amino acid sequences for the skeletal muscle and fibroblast tropomyosins are shown on top and underneath, respectively, of the corresponding nucleotide sequences. The sequences specifying the entire coding region of 284 amino acids are shown as well as 5'- and 3'-untranslated regions. The polyadenylation signal AATAAA is underlined. The isoform-specific coding sequences are boxed. Most of the fibroblast cDNA sequence is shown as dashed lines indicating identity with the skeletal muscle tropomyosin isoform.

beta-tropomyosin and fibroblast tropomyosin 1 cDNAs, we carried out S1 protection experiments with an *Nco*I-*Pst*I fragment corresponding to amino acids 1 to 144. Only the full-length input fragment of 424 nucleotides was protected with RNAs obtained from fibroblasts, smooth muscle (stomach, uterus, and vas deferens), and skeletal muscle (Fig. 3c). These results demonstrate that all RNAs complementary to the fibroblast and skeletal muscle mRNAs described here contain complete identity at the amino-terminal half of the protein. In agreement with Northern blot analyses, no S1 nuclease-protected fragments were detected with RNA obtained from cardiac muscle (Fig. 3a, b, and c).

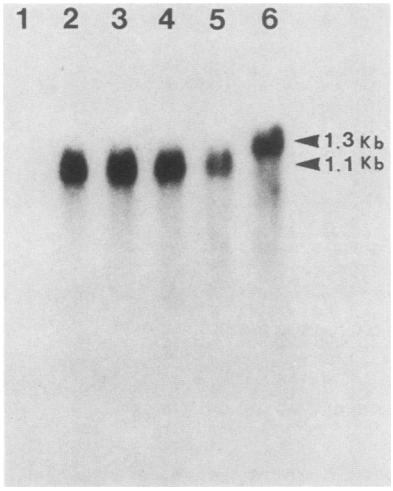

FIG. 2. Detection of RNA coding for tropomyosin in different rat tissues. Total cellular RNAs (10 μg per lane) were isolated from various rat tissues, separated in a formaldehyde-agarose gel, transferred to nitrocellulose paper, and hybridized with a ^{32}P-labeled probe prepared from pRSkM-22. Lanes: 1, heart, as a negative control; 2, vas deferens; 3, uterus; 4, stomach; 5, rat embryonic fibroblasts; 6, skeletal muscle. Filters were washed in 0.1% SDS–0.1× SSC at 65°C for 1 h and autoradiographed for 4 days.

Identification of recombinant DNA clones containing a tropomyosin gene. Two possibilities exist to explain the expression of the fibroblast and skeletal muscle mRNAs described in the previous section. Either a single gene codes for these two isoforms via tissue-specific RNA processing, or both fibroblast and skeletal muscle tropomyosins are encoded by two genes that are highly conserved. The cDNA clones to rat embryonic fibroblast tropomyosin 1 and skeletal muscle beta-tropomyosin were used to assess the number and structure of the corresponding genes. Southern blot analysis of genomic DNA was used to study the genetic complexity of the genes encoding these tropomyosin isoforms. ^{32}P-labeled probes made from cDNA inserts containing sequences encoding rat embryonic fibroblast tropomyosin 1 hybridized to several bands with *Eco*RI, *Hin*dIII, *Bam*HI, or *Kpn*I (Fig. 4). The identical patterns of hybridizing restriction enzyme fragments were obtained with a full-length cDNA clone for rat skeletal muscle beta-tropomyosin (data not shown). Since neither cDNA clone encoding the fibroblast or the skeletal muscle tropomyosin contains *Eco*RI, *Hin*dIII, *Bam*HI, or *Kpn*I restriction sites, it is possible that each hybridizing fragment represents a different tropomyosin gene. Alternatively, the restriction fragments observed could have been produced by a single tropomyosin gene containing appropriate restriction sites at intervening sequences.

To fully characterize the genomic sequences coding for

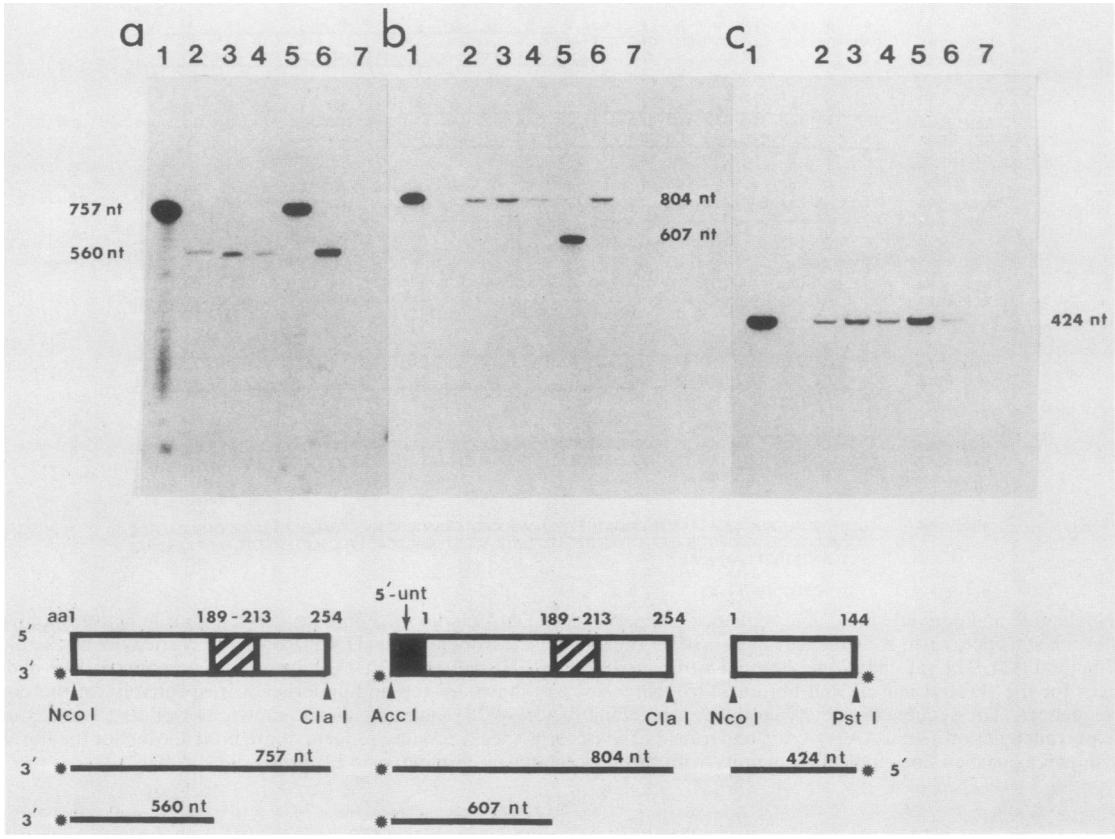

FIG. 3. Tissue-specific expression of two rat tropomyosin mRNAs. S1 nuclease mapping analysis was performed as described in Materials and Methods. Three ^{32}P-labeled DNA fragments were used for S1 protection experiments: an *Nco*I-*Cla*I fragment (757 bp) derived from the skeletal muscle beta-tropomyosin cDNA (A); an *Acc*I-*Cla*I fragment (804 bp) derived from the embryonic fibroblast cDNA (B); and an *Nco*I-*Pst*I fragment (424 bp) derived from the skeletal muscle beta-tropomyosin cDNA (C). Lane 1, ^{32}-P-end-labeled input fragments as described. Lanes 2 to 7, S1 nuclease protection fragments, using RNA from various sources: 2, rat embryonic fibroblasts; 3, stomach; 4, uterus; 5, skeletal muscle; 6, vas deferens; 7, heart. aa, Amino acid; nt, nucleotides; *, labeled end.

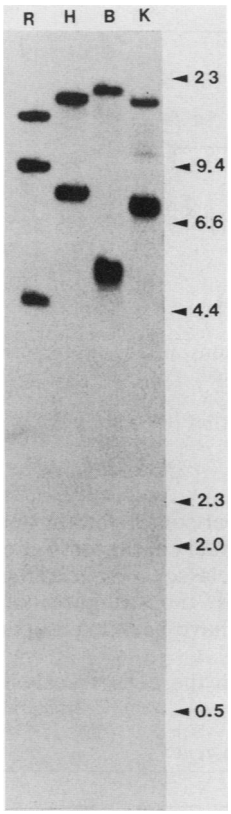

FIG. 4. Genomic representation of tropomyosin genes. Rat liver DNA was digested with *Eco*RI (R), *Hin*dIII (H), *Bam*HI (B), or *Kpn*I (K), fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to nick-translated ^{32}P-labeled probes made from full-length cDNA clones encoding rat embryonic fibroblast tropomyosin 1. The filters were washed extensively in 0.1× SSC–0.1% SDS at 65°C and autoradiographed for 3 days with Du Pont Cronex Lightning-Plus X-ray intensifying screens. Molecular weight markers used were bacteriophage lambda DNA digested with *Hin*dIII.

fibroblast and skeletal muscle mRNAs, two genomic equivalents of a partial *Sau*3AI rat genomic library were screened with ^{32}P-labeled rat embryonic fibroblast tropomyosin 1 cDNA. Nine recombinant DNA clones were selected by this procedure. Restriction enzyme analysis indicated that the nine clones represented two distinct classes (Fig. 5). Comparison by Southern blot analysis of genomic DNA indicated that they represent all the sequences in the rat genome that contain sequences to the cloned cDNAs. As indicated, there are two separate loci in the rat genome; one locus contains a functional gene (Fig. 5A), and the other locus appears to represent a pseudogene (Fig. 5B). Features that suggest that the latter clones represent a pseudogene include the following: (i) only a relatively small region of DNA (less than 1.5 kb) contains sequences complementary to our cloned cDNAs; (ii) the DNA contains sequences that hybridize to ^{32}P-labeled probes prepared to both the 5′- and 3′-untranslated regions of the fibroblast cDNA; and (iii) restriction enzyme polymorphisms compared with cloned cDNA indicate that these genomic sequences could not encode the cDNAs we isolated. The characteristics of this pseudogene suggest that a mature fibroblast tropomyosin 1 mRNA was likely involved in its origin.

Mapping of exons and introns in the tropomyosin gene. The clones indicated in Fig. 5A were further characterized. The direction of transcription was determined by Southern blot analysis with probes to both the 5′ and 3′ regions of the cloned cDNAs. One clone (lambda 17) was found to contain sequences complementary to both the 5′- and 3′-untranslated sequences of the fibroblast and skeletal muscle cDNAs. Since this clone appeared to contain sequences to the entire functional gene, including the 5′- and 3′-flanking regions, lambda 17 was selected for detailed analysis. The exact sequences of the exons and the flanking regions of the intervening sequences were determined by reference to the known cDNA sequences (102; this study) and the consensus sequence of the intron-exon boundary (7, 65, 86). The gene is divided into 11 exons and spans approximately 10 kb (Fig. 6). Table 1 lists the sequences of the 5′ and 3′ boundaries of each intron. These sequences are in good agreement with the consensus sequences for exon-intron boundaries of other eucaryotic genes (65, 86). All introns begin with the sequence G-T at the 5′ end and end with the sequence A-G. With respect to the reading frame, all three classes of exon-intron junctions were present. Class 0 junctions (those between codons) occur six times, and class 1 and 2 junctions, which interrupt codons after the first and second nucleotide, each occur two times. It is worth noting that owing to split codons encoding amino acid 188 at exons 6 and 7, splicing of exon 6 (fibroblast-specific exon) with exon 7 (skeletal muscle-specific exon) would result in an mRNA that contained an in-frame stop codon following amino acid

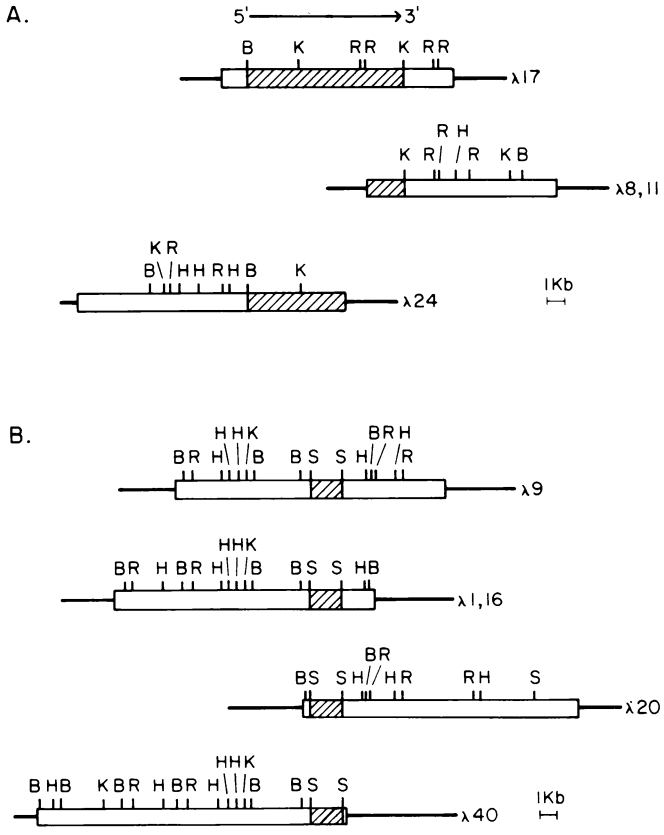

FIG. 5. Physical map of the two types of overlapping genomic recombinant phage encoding tropomyosin. The hatched regions indicate the position of the regions complementary to tropomyosin cDNA. The direction of transcription is indicated in panel A by the upper bar. The restriction sites for the enzymes *Bam*HI (B), *Eco*RI (R), *Hin*dIII (H), *Kpn*I (K), and *Sal*I (S) are indicated.

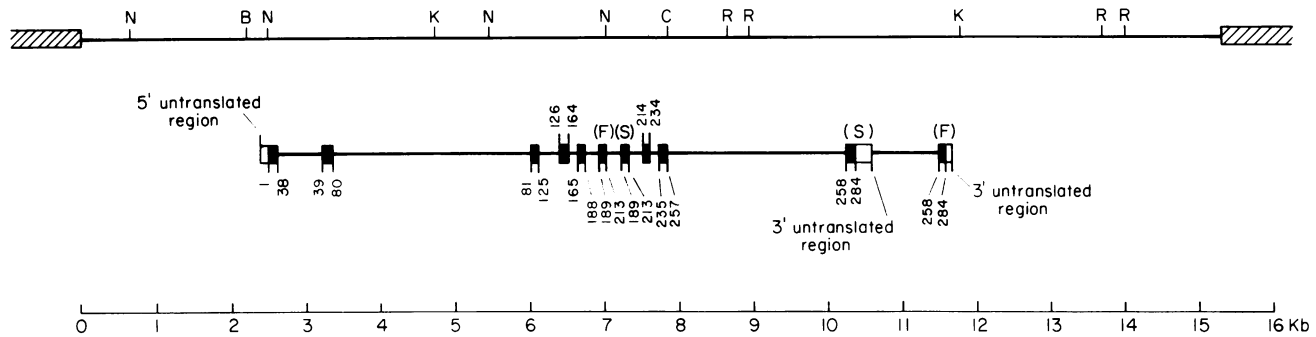

FIG. 6. Physical map of tropomyosin gene contained in lambda clone 17. Boxes represent exons, while lines represent introns. The amino acids encoded by each exon were directly determined by sequence analysis and are indicated. Positions of the fibroblast- and skeletal muscle-specific exons encoding amino acids 189 to 213 and 258 to 284 are indicated by (F) and (S), respectively. All other exons are common to the fibroblast and skeletal muscle RNAs. The restriction sites for the enzymes *Bam*HI (B), *Cla*I (C), *Eco*RI (R), *Kpn*I (K), and *Nco*I (N) are indicated.

213 of the fibroblast sequences and would not encode a functional protein. In addition, there appear to be no obvious differences in the nucleotide sequences of the introns flanking the common and tissue-specific exons (Table 1). Recently, Karlik and Fyrberg (44) reported the structure of a *Drosophila* tropomyosin gene that has basically the same intron-exon organization as the rat tropomyosin gene described here. Comparison of the rat and *Drosophila* genes revealed that the borders of the various exons were identical with respect to the classes of splice junctions characteristic of each exon. Thus, the structures of these two related tropomyosin genes have been highly conserved throughout evolution.

In agreement with the cDNA analysis presented above,

TABLE 1. Exon-intron organization of rat tropomyosin gene[a]

Intron no.	Sequence of exon-intron junctions		
	5' boundary		3' boundary
1	TGC AAG CAG 38 Gln	gtggggccca. . . ccccttcttccacag	CTG GAG GAA 39 Leu
2	GCC ACC GAT 80 Asp	gtgagtgtgg. . . ccctctgacccacag	GCT GAA GCA 81 Ala
3	AGC GAG AG 125 Arg	gtggggaagg. . . gtccctgcttctcag	A GGA ATG 125 Arg
4	TAC GAG GAG 164 Glu	gtgacagcct. . . cccgcttcccctcag	GTG GCC AGG 165 Val
5	GCT GAG AG 188 Ser	gtagggatac. . . gccccctgcccccag	C CGA GCT 188 Ser
6	GAG GAG GAG 213 Glu	gtagtagcct. . . cacacgcccctgcag	T AAA TGT 188 Ser
7	GCG GAC AAG 213 Lys	gtatagggggg. . . gtttgtctcttaaag	TAT TCC ACC 214 Tyr
8	CTG AGG GAG 234 Glu	gtgagactac. . . ccttctttatttcag	GCT GAG ACC 235 Ala
9	CTG GAA G 258 Asp/Glu	gtaaaaagga. . . cttcggactccccag	AT GAA GTC 258 Asp
10		tgcttcccctcacag	AG ACT TTG 258 Glu

[a] The nucleotide sequences of exon-intron junctions were determined from genomic subclones. Exon sequences are in capital letters; intron sequences are in lower-case letters. The number shown below the DNA sequence denotes the amino acid in the corresponding tropomyosin proteins.

common exons were found for amino acids 1 to 188 (exons 1 through 5) and for amino acids 214 to 257 (exons 8 and 9). Exon 6 contained sequences for amino acids 189 to 213 in fibroblast tropomyosin, while exon 7 contained sequences for amino acids 189 to 213 in skeletal muscle tropomyosin. In addition, exon 10 encoded sequences to the carboxy-terminal region of skeletal muscle tropomyosin (amino acids 258 to 284), including all the 3'-untranslated sequences (233 bp) through the polyadenylation site. Likewise, exon 11 encoded sequences to the carboxy-terminal region of fibroblast tropomyosin (amino acids 258 to 284), including the 3'-untranslated sequences (73 bp) through the polyadenylation site. Hence, the organization of this gene indicates that a combination of both alternative RNA splicing and polyadenylation is responsible for the generation of tissue-specific protein isoforms. Southern blot analysis of high-molecular-weight DNA from a variety of cells and tissues (fibroblast, liver, heart, skeletal muscle, brain, and stomach) revealed no alterations of the DNA at this level of detection, thus excluding DNA rearrangements as a mechanism for the generation of the multiple mRNAs (data not shown).

5' end of the tropomyosin gene. The 5'-untranslated regions of our longest cDNAs for rat embryonic fibroblast tropomyosin 1 (102) and skeletal muscle beta-tropomyosin extend 61 and 16 bp, respectively, upstream of the initiator methionine codon. In addition, the 16 bp of the skeletal muscle cDNA were identical with the fibroblast cDNA. To define precisely the 5' end of the mRNAs transcribed from this gene, we carried out primer extension and S1 experiments. For primer extension analysis, a ^{32}P-labeled DNA probe was obtained from nucleotides +3 to −46 (+1 denotes the A of the AUG start codon). The ^{32}P-labeled probe was hybridized with RNA, and the primer was extended with reverse transcriptase. The sizes of the products were determined by gel electrophoresis, and the products were detected by autoradiography (Fig. 7A). With RNA from fibroblast, uterus, stomach, vas deferens, and skeletal muscle, two primer extension products were detected with lengths of 115 and 113 bp (Fig. 7A, lanes 1 to 5). This corresponded to a 5'-untranslated region of 112 or 110 bp. No primer extension products were obtained in the presence of tRNA and the primer (Fig. 7A, lane 6). To confirm these results, the 5' ends of the tropomyosin transcripts were mapped by S1 nuclease analysis. For S1 protection experiments a fragment was used extending from nucleotides +3 to approximately −270. When this fragment was 5' end labeled and hybridized with RNA from fibroblast, stomach, uterus, vas deferens, and skeletal muscle, two major protected fragments were detected (Fig. 7B). The S1-protected fragments were identical to those detected by primer extension analysis. It is worth noting that there did not appear to be any preference for the use of either CAP site in any of the tissues examined. The close proximity of the two CAP sites (112 or 110 bp) is likely due to the use of a single promoter that can utilize two different transcription initiation sites that differ by only 2 bp. This may be due to the lack of a consensus TATA-like sequence located upstream of the CAP sites (Fig. 8). The data indicated that all RNAs transcribed from this gene contain almost identical 5' ends and involve the use of the same promoter. Thus, the two different species of mRNA generated from this gene are processed from a transcript that has identical 5' ends. Furthermore, the data demonstrate that exon 1 contains the entire 5'-untranslated sequences in addition to the first 38 amino acids of the protein.

The nucleotide sequence of the 5' end of the tropomyosin gene is shown in Fig. 8. As indicated by S1 and primer extension analysis, the sites of initiation of transcription (CAP sites) are located 110 and 112 bp upstream of the ATG codon used to initiate translation. The putative promoter region is G-C rich, with very few A-T sequences. The sequence TTTTA resembling a TATA box is located 19 bp upstream of the 5'-most CAP site. No sequence resembling a CCAAT box is found upstream of the putative TATA-like sequence. This is in contrast to most of the characterized eucaryotic genes that do have TATA and CCAAT sequence elements approximately 30 and 80 nucleotides, respectively, upstream of the RNA transcription initiation site (8). Further analysis of the 5'-flanking region of the tropomyosin gene revealed the occurrence of two putative Sp1-binding sites (CCGCCC or GGGCGG). These sequence motifs are contained in many other cellular and viral promoters and are thought to function by interacting with the positively acting transcription factor Sp1 (24, 25). The first occurrence of this sequence is 38 nucleotides upstream from the 5'-most CAP site. The second sequence is located 148 nucleotides from the 5'-most CAP site. In addition, this second putative Sp1-binding site is flanked on each side by an 8-bp direct repeat, CCGAGGGG. The functional significance, if any, of this repeat remains to be established.

Comparison of 5'- and 3'-untranslated sequences of rat and human tropomyosin mRNAs. Recently, the structures of cDNA clones encoding a human fibroblast and skeletal muscle beta-tropomyosin were reported (53). These studies suggested that an alternative RNA-splicing mechanism was responsible for the generation of both the fibroblast and skeletal muscle isoforms from a single gene. A comparison of the derived amino acid sequences of the rat tropomyosins encoded by the gene described in the present study demonstrated that both rat and human genes encode the same protein isotypes. The human and rat proteins are identical with the exception of two positions: amino acid 66 (human = Glu, rat = Asp) and amino acid 208 (human = Met, rat = Ile) of the fibroblast proteins. In addition, among 68 nucleotide differences in the coding sequences between the rat and human cDNAs, 66 represented synonymous changes, suggesting that strong selective pressure has operated on both genes. Although no sequence data are currently available concerning the sequence of the human gene, it was of interest to compare the sequences of the untranslated regions of the rat and human mRNAs. In Fig. 9 we present a comparison of the 5'- and 3'-untranslated sequences. It is apparent from the figure that the sequences are homologous. The functional significance of the homology between the rat and human 5'- and 3'-untranslated sequences is unclear at present. For example, the high degree of homology (greater than 75%) observed between the rat and human skeletal muscle 3'-untranslated sequences suggests that these regions have a role in processing of the primary transcript, translational control, or mRNA stability. Similar comparisons have been observed for other genes and have been the subject of a recent review (99). In addition, whether the sequences conserved between the rat and human tropomyosin genes are confined to the mRNA or extend downstream of the polyadenylation site remains to be determined. Sequences downstream of the polyadenylation site have been reported to be important for 3' processing of poly(A)$^+$ mRNAs (for a review, see reference 6). In the future it will be of considerable interest to compare the rat and human genomic DNA sequences 3' of the poly(A) site. Such comparisons may reveal common sequence motifs that may be involved in tissue-specific RNA processing. Similarly, interspecies comparisons of intron sequences 5' and 3' of the tissue-specific

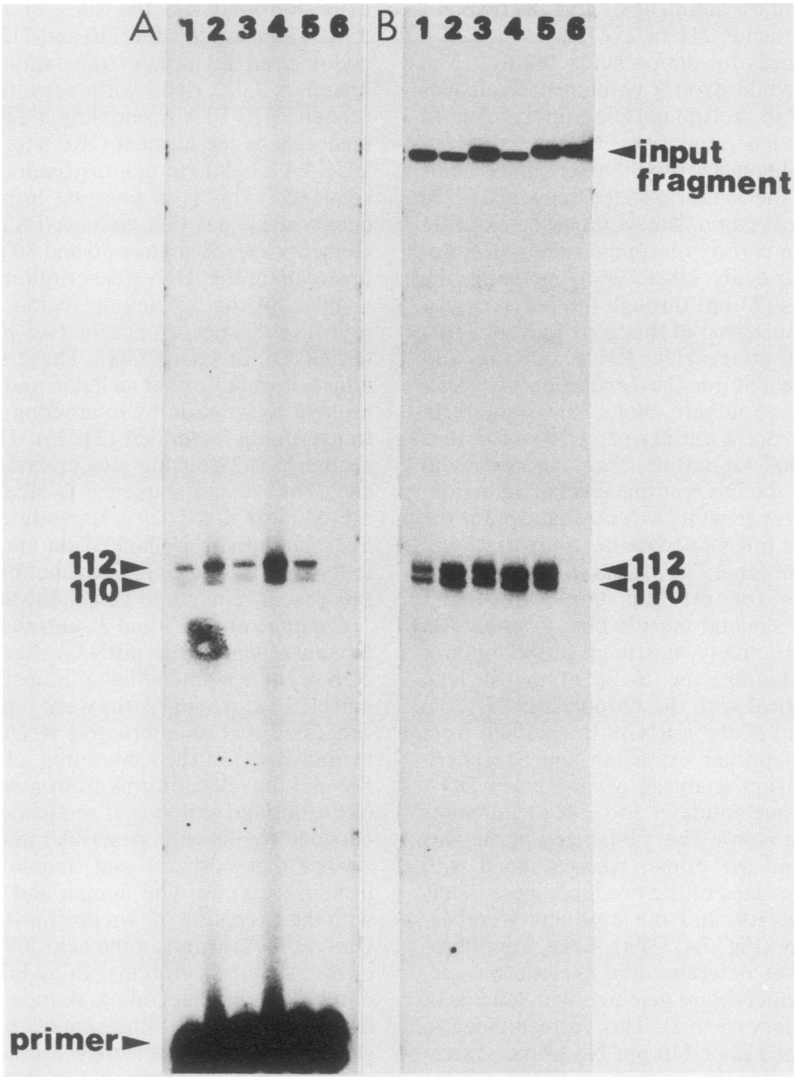

FIG. 7. Primer extension and S1 analysis of the 5′ end of the tropomyosin gene. (A) Primer extension analysis was carried out as described in Materials and Methods. Lanes: 1, primer extension product with fibroblast RNA; 2, primer extension product with stomach RNA; 3, primer extension product with uterus RNA; 4, primer extension product with vas deferens RNA; 5, primer extension with skeletal muscle RNA; 6, primer extension product with tRNA alone. The positions of the 112- and 110-bp primer extension products and the primer are indicated by arrows. (B) S1 analysis was carried out as described in Materials and Methods. Lanes: 1, S1-resistant products with fibroblast RNA; 2, S1-resistant products with stomach RNA; 3, S1-resistant products with uterus RNA; 4, S1-resistant products with vas deferens RNA; 5, S1-resistant products with skeletal muscle RNA; 6, S1-resistant products with no RNA, *Nco*I-*Bam*HI fragment. The position of the 112- and 110-bp S1-protected fragments and the position of the input fragment are indicated by arrows.

exons may help to identify sequences important in the processing of the primary transcript.

DISCUSSION

This paper demonstrates that a single gene encodes both rat embryonic fibroblast tropomyosin 1 and skeletal muscle beta-tropomyosin. The gene contains 11 exons and spans 10 kb. Two mRNAs are generated from this gene: a 1.1-kb mRNA expressed in fibroblasts, as well as stomach, uterus, and vas deferens, and a 1.3-kb mRNA expressed in skeletal muscle. These two mRNAs differ in the length of their 3′-untranslated regions and in the splice patterns at both an internal region and at the 3′ end. Furthermore, both species of mRNA expressed from this gene are spliced from a precursor with an identical 5′ end. A schematic diagram of the gene is shown in Fig. 10. Of the 11 exons that make up the gene, 7 encode sequences common to fibroblast and skeletal muscle mRNAs. Exons 6 and 11 are specific for fibroblast mRNAs, while exons 7 and 10 are used exclusively in skeletal muscle mRNAs. It is worth noting that although the gene is also expressed in smooth muscle (stomach, uterus, and vas deferens), only the fibroblast-type splice products can be detected in these tissues. In no case did we detect mRNAs containing a combination of a fibroblast-specific exon with a skeletal muscle-specific exon, i.e., exon 6 with exon 10 or exon 7 with exon 11. This suggests that the mechanisms responsible for the use of alternative internal and 3′ exons are coupled. The organization of this gene indicates that not only alternate splice sites but alternate polyadenylation sites are selected in a tissue-specific manner. Whether the same cellular factors are involved in the

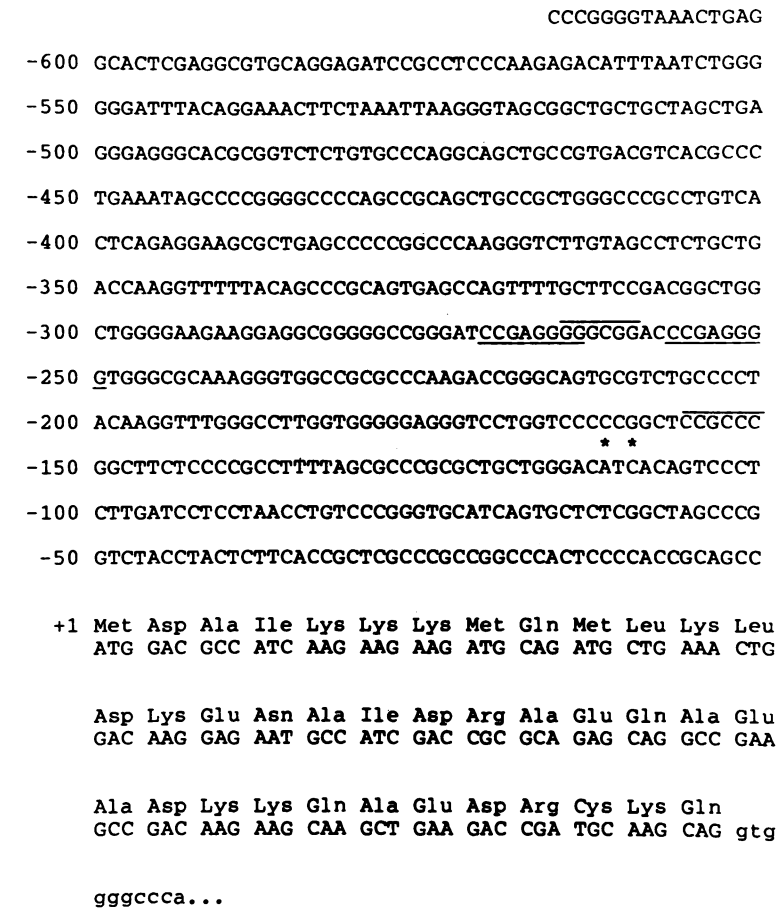

FIG. 8. Nucleotide sequence of 5′ end of the tropomyosin gene. Nucleotide residue +1 denotes the A of the ATG presumed to encode the initiator methionine codon of the tropomyosin protein, and residues preceding it are indicated by negative numbers. In addition, the protein-coding sequence of exon 1 (amino acids 1 to 38) and a portion of the 5′ region of intron 1 (small letters) are indicated. The transcription initiation sites predicted by primer extension and S1 nuclease mapping are indicated by an asterisk above the nucleotides. The positions of the putative Sp1-binding sites (CCGCCC or GGGCGG) are indicated with a line above nucleotide positions −150 and −260. The 8-bp direct repeat CCGAGGGG around the latter Sp1-binding site is underlined.

selection of both polyadenylation sites and splice sites remains to be determined.

Generation of protein diversity from a single gene has been demonstrated in a number of cases. The pathways involved in the expression of such genes in vertebrates can be broadly characterized into two major classes. The first involves rearrangement of DNA segments by recombination which leads to formation of a complete gene encoding different polypeptides. The only known examples of this mechanism are found in the immunoglobulin genes of vertebrates (for a review, see reference 96). The second pathway is characterized by alternative RNA processing, by which regions of a transcript are joined together (spliced) in different combinations resulting in mRNAs encoding distinct protein products. Three types of genomic arrangements have been described for genes whose transcripts are alternatively processed. These include: (i) the use of a single initiation site for transcription but multiple polyadenylation sites associated with different 3′-end splicing, for example, membrane and secreted forms of immunoglobulins (26, 56) and the calcitonin gene (78); (ii) the use of alternative promoters and a single polyadenylation signal but different 5′ or internal splice choices, for example, myosin light chains (67, 73, 75); and (iii) a single promoter and single polyadenylation site but different internal splice choices, for example, troponin T (9, 63). The present report indicates that a fourth type of genomic organization can exist for the generation of protein diversity via alternative RNA processing. The tropomyosin gene contains a single transcription initiation site and multiple polyadenylation sites associated with different 3′-end splicing, in addition to alternate internal splice choices (Fig. 10).

The structural features of the tropomyosin gene reported in this study raise a number of questions concerning the mechanisms involved in the tissue-specific expression of this gene. Although capping of a primary transcript is known to occur very early after transcription begins, the temporal order of polyadenylation and splicing remains to be determined. In the case of the tropomyosin gene in which both alternative splicing and polyadenylation are involved, regulation could occur at the levels of both splice site selection and polyadenylation. The mechanism(s) responsible for generating the mRNAs for the two tropomyosin isoforms must be under regulatory control. In no case did we detect both the 1.1- and 1.3-kb transcripts in the same cell type, nor did we observe mRNA containing combinations of the tissue-specific exons, i.e., fibroblast with skeletal muscle. These results clearly indicate that utilization of tissue-specific

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5'-UNTRANSLATED SEQUENCES OF RAT AND HUMAN mRNAs

RAT ATCACAGTCCCTCTT-GATCCTCCTAACCTGTC-CCGGGTGCATC-AGTGCTCTCGGCTAG
    :: :  :::: ::  : :::::::  :::: : :::: :::: : ::: : :::  : ::
HUM -TC-C--TCCCGCTCCG-TCCTCCTCGCCTGCCACCGG-TGCACCCAGTCCGCTCACCCAG

RAT CCCGGTC--TACCTACTCTTCACCGC-TCGCCCGCCGGCCCACTCCCCACCGCAG-CCATG
    ::: :::  : ::   :: :::::::: : ::: :::::::::: :::::::::: :::::
HUM CCCAGTCCGT-CCGG-TCCTCACCGCCT-GCCGGCCGGCCCACCCCCCACCGCAGGCCATG

3'-UNTRANSLATED SEQUENCES OF RAT AND HUMAN SKELETAL MUSCLE mRNAs

RAT TGAGTT-----CCCA-CTGTGGCCATCGCAGGCCC-CTT-TCTTCT-T--TTTCCATTCT
    ::::       :::: : ::: ::: : ::: : : ::: ::: :: :  ::::::::::
HUM TGAGCCCCACGCCCAGC-GTG-CCACCTCAG-CTCTCTTCTCTCCTCTCCTTTCCATTCT

RAT CTCTGA-GGGGAGGGG-GCAGCCAGGAGGAGCACAAACTGGCCATG-TTGCACAGCCAGT
    :::: : :::::::::: :::: :::::::::::: ::: :: :::   ::::::::::::
HUM CTCT-ATGGGGAGGGGAGCAGGCAGGAGGAGCAGAAATTG-CCAACATTGCACAGCCAGG

RAT CTGAGG-GCAGCCTGAGGGAACACGCCTGCCA-C---CCCTGCCACCCACTCTGGCACTG
    ::: :: ::::::: :::::  : :::  ::: :   :::  ::::::::::::::::::
HUM CTG-GGAGCAGCCT-AGGGAG-A-GCCC-CCATCATGCCCA-CCACCCACTCTGGCACTG

RAT GCTTTC-TCCTGTCATCTATCCACCGTCCACTGACTTCCACCCCTCCTCTACTTAATAAA
    :::: : :::: : : :::::: :: ::::::   :: ::      : :: : ::::::::
HUM GCTT-CATCCTTT-ACCTATCC-CCTTCCACC--CT-CCTT---TGCT-TGCTTAATAAA

RAT CTCTGAACTTGGT (poly A)
     ::::::::::::
HUM TTCTGAACTTGG- (poly A)

3'-UNTRANSLATED SEQUENCES OF RAT AND HUMAN FIBROBLAST mRNAs

RAT TAAGGGTG-GCCCTGCTACCTGCC-GACCAGACTACATAGATGCCTCCTCAGGCCAATAAA
    : ::::   ::::  : :::  :: : :::: :::  : : :::: :: ::  ::::::::
HUM TGAGGGCCAGCCC--C-ACCC-CCAG-CCAGGCTA--TGGTTGCCACCCCAACCCAATAAA

RAT -CGGATGTCT-CTGGC (poly A)
     : ::::: : :: ::
HUM ACTGATGT-TACTAGCCTCTC (poly A)
```

FIG. 9. Comparison of 5'- and 3'-untranslated sequences of rat and human tropomyosin mRNAs. The 5'- and 3'-untranslated sequences of rat and human (53) RNAs were aligned to obtain maximal homology. Identical nucleotides are marked by a colon. The ATG start codon, stop codons, and polyadenylation signal AATAAA are marked by lines below the sequences.

exons does not occur in a random fashion but is under fine control. What cellular factor(s) controls alternative RNA processing of this gene? These factors may include the following: (i) sequences contained within the transcript; (ii) posttranscriptional modification of internal residues, e.g., methylation (11, 23, 84, 98); and (iii) *trans*-acting factors such as proteins and small nuclear RNAs (6, 47, 66, 76). At the nucleotide sequence level, all introns within genes encoding proteins have a well-defined consensus sequence at the 5' and 3' boundaries (7, 65, 86). Thus far, we have identified no obvious nucleotide sequence differences between the common and tissue-specific splice junctions of the tropomyosin gene. The differential RNA processing at the 3' end of the gene likely involves a specific endonucleolytic cleavage and subsequent poly(A) addition. What sequences in the tropomyosin transcript 5' and 3' of the polyadenylation signal AATAAA are important for the tissue-specific 3'-end processing are not known at present. The conservation of sequences observed in the 3'-untranslated regions of the rat and human mRNAs suggests that these sequences contain information that is important in processing (Fig. 9). Whether 3'-end processing is directly associated with the differential splicing of the internal exons or vice versa is unknown. Experiments are currently in progress to determine those sequences required for tissue-specific processing.

Generation of protein diversity by alternative RNA processing has been reported for genes encoding other cytoskeletal proteins including myosin light chain (27, 67, 73, 75), troponin T (9, 17, 63), myosin heavy chain (79), and tropomyosin (3, 43, 44, 53, 80). In addition, alternative RNA processing for the generation of contractile protein diversity appears to be a fundamental mechanism conserved throughout evolution since it has been characterized in various species including drosophila, chickens, rats, and humans. Interestingly, it was recently reported that the gene encoding rat skeletal muscle alpha-tropomyosin is alternatively spliced to generate both a skeletal muscle and a smooth muscle isoform (80). The present report demonstrates that a similar situation exists for the gene encoding rat skeletal muscle beta-tropomyosin. Thus, the two tropomyosin isoforms expressed in skeletal muscle (alpha and beta) are encoded by distinct genes, both of which are alternatively processed in a similar tissue-specific manner. It is worth

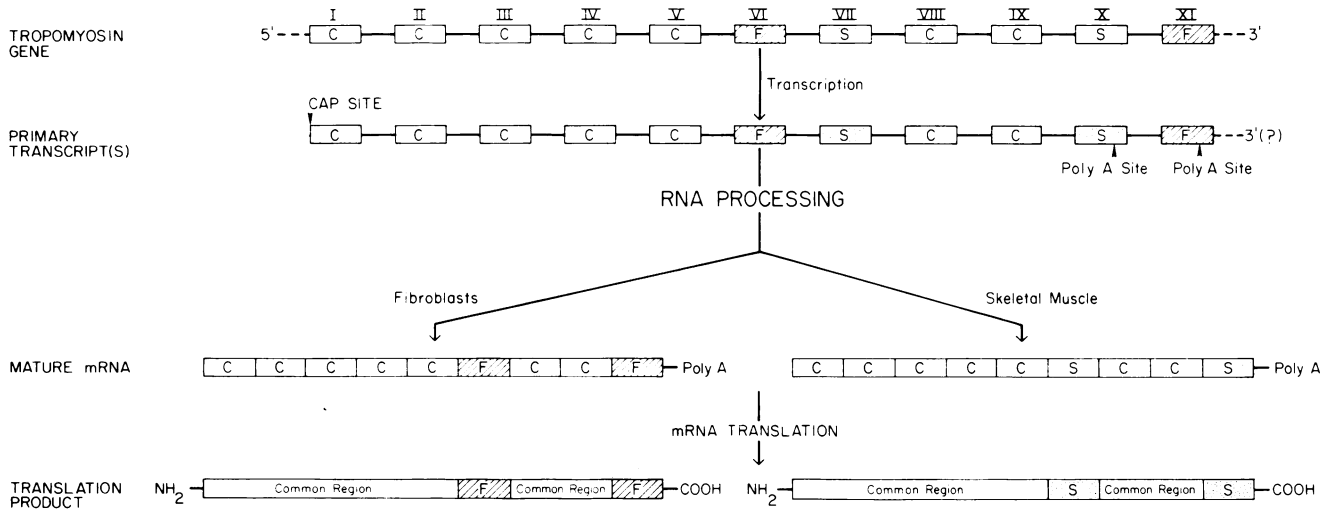

FIG. 10. Diagram of tropomyosin gene and scheme for the generation of rat embryonic fibroblast tropomyosin 1 and skeletal muscle beta-tropomyosin mRNAs by alternative RNA processing. The boxes represent exons and the lines represent introns, but they are not drawn to scale. The 5' and 3' regions of the gene are indicated as dotted lines because the structural and functional borders of the gene remain to be determined. The amino acids encoded by each exon are indicated in Fig. 6. The CAP site and polyadenylation signals are indicated. Exons and coding regions common to all mRNAs generated from this gene are indicated with a C, while fibroblast- (smooth muscle) and skeletal muscle-specific exons and coding regions are indicated with an F and S, respectively.

noting that the gene encoding rat skeletal muscle alpha-tropomyosin is the same gene that encodes rat embryonic fibroblast tropomyosin 2 (apparent molecular weight, 36,500) via alternative RNA processing (Y. Yamawaki-Kataoka and D. M. Helfman, unpublished observations). One difference between the alpha and beta isoforms of rat skeletal muscle is the location of the domains that distinguish the nonmuscle and smooth muscle isoforms from their skeletal muscle counterparts. In skeletal muscle alpha-tropomyosin these isotype-specific regions are located at residues 41 to 76 and 258 to 284 (80), while in skeletal muscle beta-tropomyosin these regions are located at residues 189 to 213 and 258 to 284. Since both genes encoding alpha- and beta-tropomyosins in skeletal muscle are alternatively spliced and polyadenylated in other tissues, it will be of considerable interest to determine whether the processing patterns of each gene are regulated by common or gene-specific factors.

The tissue-specific exons appear to encode distinct functional domains of the tropomyosin molecule. In skeletal muscle, the function of tropomyosin is in association with the troponin complex (troponin I, T, and C) to regulate the calcium-sensitive interaction of actin and myosin (87, 94). Biochemical studies have demonstrated that the site of troponin T binding is close to or at the carboxy-terminal end of skeletal muscle tropomyosin (13–15, 40, 41, 54, 58, 62, 68, 69, 71, 72, 90, 100). Furthermore, there are two regions on the tropomyosin molecule that interact with troponin T; one located in the proximity of Cys-190 and the other at the carboxy-terminal end of the protein (41, 54, 70–72). These two troponin T-binding sites correspond to the two regions where the skeletal muscle beta-tropomyosin and rat embryonic fibroblast tropomyosin 1 differ. On the other hand, smooth muscle and nonmuscle cells do not contain a troponin complex. In these cell types the phosphorylation of myosin by the enzyme myosin light chain kinase appears to be the major calcium-sensitive regulatory mechanism controlling the interaction of actin and myosin (1).

The differences in the regulation of the contractile apparatus of smooth muscle and nonmuscle cells compared with skeletal muscle appear to require structurally as well as functionally distinct forms of tropomyosin. It is worth noting that in the case of the two tropomyosin isoforms reported in this study, in no case did we detect coexpression of the 1.1- and 1.3-kb mRNAs in a single tissue or cell type; nor did we detect mRNAs containing a combination of the fibroblast-specific sequences with the skeletal muscle-specific sequences. These data suggest that expression of specific tropomyosin isoforms is required for the function of these proteins within a given cell type. The functional significance of the internal (amino acids 189 to 213) and carboxy-terminal (amino acids 258 to 284) isoform-specific regions in smooth muscle and nonmuscle cells is not presently understood. Comparison of the same tropomyosin isotypes from chickens (37, 82), rats (102), and humans (53) demonstrates strong conservation among these vertebrate isoforms with respect to the internal (amino acids 189 to 213) and carboxy-terminal (amino acids 258 to 284) regions. This conservation of sequence among evolutionarily distant species may indicate a distinct, as yet uncharacterized, regulatory role for these tropomyosin domains in the actin-linked control system of smooth muscle and nonmuscle cells.

ACKNOWLEDGMENTS

We thank J. D. Watson for his enthusiastic support of this work. We also thank Susan Smith and Paul Thomas for critical reading of this manuscript. We thank Mark Zoller for the synthesis of oligonucleotides and Philip Renna and Madeline Szadkowski for help with the preparation of this manuscript.

This work was supported by Public Health Service grants CA40599 and CA29569 from the National Institutes of Health and a grant from the Muscular Dystrophy Association. E.K. was supported by an EMBO fellowship.

LITERATURE CITED

1. **Adelstein, R. S., M. D. Pato, J. R. Selers, P. De Lanerolle, and M. A. Conti.** 1981. Regulation of actin-myosin interaction by reversible phosphorylation of myosin and myosin kinase. Cold Spring Harbor Symp. Quant. Biol. **46**:921–928.
2. **Aviv, H., and P. Leder.** 1972. Purification of biologically active messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA **69**:1408–1412.
3. **Basi, G. S., M. Boardman, and R. V. Storti.** 1984. Alternative splicing of a *Drosophila* tropomyosin gene generates muscle tropomyosin isoforms with different carboxy-terminal ends. Mol. Cell. Biol. **4**:2828–2836.
4. **Benton, W. D., and R. W. Davis.** 1977. Screening λgt recombinant clones by hybridization to single plaques in situ. Science **196**:180–182.
5. **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.
6. **Birnstiel, M. L., M. Busslinger, and K. Strub.** 1985. Transcription termination and 3′ processing: the end is in site. Cell **41**:349–359.
7. **Breathnach, R., C. Benoist, K. O'Hare, F. Gannon, and P. Chambon.** 1978. Ovalbumin gene: evidence for a leader sequence in mRNA and DNA sequences at the exon-intron boundaries. Proc. Natl. Acad. Sci. USA **75**:4853–4857.
8. **Breathnach, R., and P. Chambon.** 1981. Organization and expression of eukaryotic split genes coding for proteins. Annu. Rev. Biochem. **50**:349–383.
9. **Breitbart, R. E., H. T. Nguyen, R. M. Medford, A. T. Destree, V. Mahdavi, and B. Nadal-Ginardo.** 1985. Intricate combinatorial patterns of exon splicing generate multiple regulated troponin T isoforms from a single gene. Cell **41**:67–82.
10. **Bronson, D. D., and F. H. Schachat.** 1982. Heterogeneity of contractile proteins: differences in tropomyosin in fast, mixed, and slow skeletal muscles of the rabbit. J. Biol. Chem. **257**:3937–3944.
11. **Canaani, D., C. Kahana, S. Lavi, and Y. Groner.** 1979. Identification and mapping of N^6-methyladenosine containing sequences in simian SV40 RNA. Nucleic Acids Res. **6**: 2879–2899.
12. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**: 5294–5299.
13. **Chong, P. C. S., and R. S. Hodges.** 1982. Photochemical cross-linking between rabbit skeletal troponin and alpha-tropomyosin. J. Biol. Chem. **257**:9152–9160.
14. **Cohen, C., D. L. D. Casper, J. P. Johnson, K. Nauss, S. Margosian, and D. A. D. Parry.** 1973. Tropomyosin-troponin assembly. Cold Spring Harbor Symp. Quant. Biol. **37**:287–297.
15. **Cohen, C., D. L. D. Casper, D. A. D. Parry, and R. M. Lucas.** 1971. Tropomyosin crystal dynamics. Cold Spring Harbor Symp. Quant. Biol. **36**:205–216.
16. **Cohen, I., and C. Cohen.** 1972. A tropomyosin-like protein from human platelets. J. Mol. Biol. **68**:383–387.
17. **Cooper, T. A., and C. P. Ordahl.** 1984. A single troponin T gene regulated by different programs in cardiac and skeletal muscle development. Science **226**:979–982.
18. **Cote, G. P., and L. B. Smillie.** 1981. Preparation and some properties of equine platelet tropomyosin. J. Biol. Chem. **256**:11004–11010.
19. **Cummins, P., and S. V. Perry.** 1973. The subunits and biological activity of polymorphic forms of tropomyosin. Biochem. J. **133**:765–777.
20. **Cummins, P., and S. V. Perry.** 1974. Chemical and immunochemical characteristics of tropomyosins from striated and

smooth muscle. Biochem. J. **141**:43–49.
21. **Denhardt, D. T.** 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:641–646.
22. **der Terrossian, E., S. D. Fuller, M. Stewart, and A. G. Weeds.** 1981. Porcine platelet tropomyosin. Purification, characterization and paracrystal formation. J. Mol. Biol. **153**:147–167.
23. **Dimock, K., and C. M. Stoltzfus.** 1977. Sequence specificity of internal methylation in B77 avian sarcoma virus RNA subunits. Biochemistry **16**:471–478.
24. **Dynan, W. S., and R. Tjian.** 1983. Isolation of transcription factors that discriminate between different promoters recognized by RNA polymerase II. Cell **32**:669–680.
25. **Dynan, W. S., and R. Tjian.** 1983. The promoter-specific transcription factor Sp1 binds upstream sequences in the SV40 early promoter. Cell **35**:79–87.
26. **Early, P., J. Rogers, M. Davis, K. Calame, M. Bond, R. Wall, and L. Hood.** 1980. Two RNAs can be produced from a single immunoglobulin mu gene by alternative RNA processing pathways. Cell **20**:313–319.
27. **Falkenthal, S., V. P. Parker, and N. Davidson.** 1985. Developmental variations in the splicing pattern of transcripts from the Drosophila gene encoding myosin alkali light chain results in different carboxyterminal amino acid sequences. Proc. Natl. Acad. Sci. USA **82**:449–453.
28. **Fattoum, A., J. H. Hartwig, and T. P. Stossel.** 1981. Isolation and some structural and functional properties of macrophage tropomyosin. Biochemistry **22**:1187–1193.
29. **Feramisco, J. R., J. E. Smart, K. Burridge, D. M. Helfman, and G. P. Thomas.** 1982. Co-existence of vinculin and vinculin-like protein of higher molecular weight in smooth muscle. J. Biol. Chem. **257**:11024–11031.
30. **Fine, R. E., and A. L. Blitz.** 1975. A chemical comparison of tropomyosins from muscle and non-muscle tissues. J. Mol. Biol. **95**:447–454.
31. **Fine, R. E., A. L. Blitz, S. E. Hitchcock, and B. Kaminer.** 1973. Tropomyosin in brain and growing neurons. Nature (London) **245**:182–186.
32. **Ghosh, P. K., V. B. Reddy, J. Swinscoe, P. Lebowitz, and S. M. Weissman.** 1978. Heterogeneity and 5'-terminal structures of the late RNAs of simian virus SV40. J. Mol. Biol. **126**:813–846.
33. **Giometti, C. S., and N. L. Anderson.** 1981. A variant of human non-muscle tropomyosin found in fibroblasts by using two-dimensional electrophoresis. J. Biol. Chem. **256**:11840–11846.
34. **Grunstein, M., and D. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA **72**:3961–3965.
35. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
36. **Helfman, D. M., J. R. Feramisco, J. C. Fiddes, G. P. Thomas, and S. H. Hughes.** 1983. Identification of clones that encode chicken tropomyosin by direct immunological screening of a cDNA expression library. Proc. Natl. Acad. Sci. USA **80**: 31–35.
37. **Helfman, D. M., J. R. Feramisco, W. M. Ricci, and S. H. Hughes.** 1984. Isolation and sequence of a cDNA clone that contains the entire coding region for chicken smooth muscle alpha-tropomyosin. J. Biol. Chem. **259**:14136–14143.
38. **Helfman, D. M., Y. Yamawaki-Kataoka, W. M. Ricci, S. H. Hughes, and J. R. Feramisco.** 1984. Isolation and characterization of cDNA clones encoding smooth muscle and nonmuscle tropomyosins, p. 479–491. *In* G. Borisy, D. Cleveland, and D. Murphy (ed.), Molecular biology of the cytoskeleton. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39. **Hendricks, M., and H. Weintraub.** 1984. Multiple tropomyosin polypeptides in chicken embryo fibroblasts: differential repression of transcription by Rous sarcoma virus transformation. Mol. Cell. Biol. **4**:1823–1833.
40. **Higashi, S., and T. Ooi.** 1968. Crystals of tropomyosin and native tropomyosin. J. Mol. Biol. **34**:699–701.
41. **Hitchcock, S. E., H. E. Huxley, and A. G. Szent-Gyorgyi.** 1973. Calcium sensitive binding of troponin to actin-tropomyosins: a two-site model for troponin action. J. Mol. Biol. **80**:825–836.
42. **Ishimodo-Takagi, T.** 1978. Immunological purification of sea urchin egg tropomyosin. J. Biochem. **83**:1757–1762.
43. **Karlik, C. C., and E. A. Fyrberg.** 1985. An insertion within a variably spliced Drosophila tropomyosin gene blocks accumulation of only one encoded isoform. Cell **41**:57–66.
44. **Karlik, C. C., and E. A. Fyrberg.** 1986. Two *Drosophila melanogaster* tropomyosin genes: structural and functional aspects. Mol. Cell. Biol. **6**:1985–1993.
45. **Kato, T., and Y. Tonomura.** 1975. Physarum tropomyosin-troponin complex: isolation and properties. J. Biochem. **78**:583–588.
46. **Lau, S. Y. M., C. Sanders, and L. B. Smillie.** 1985. Amino acid sequence of chicken gizzard gamma-tropomyosin. J. Biol. Chem. **260**:7257–7263.
47. **Lerner, M. R., J. A. Boyle, S. M. Mount, S. L. Wolin, and J. A. Steitz.** 1980. Are snRNPs involved in splicing? Nature (London) **283**:220–224.
48. **Lewis, W. G., G. P. Cote, A. S. Mak, and L. B. Smillie.** 1983. Amino acid sequence of equine platelet tropomyosin. Correlation with interaction properties. FEBS Lett. **156**:269–273.
49. **Lewis, W. G., and L. B. Smillie.** 1980. The amino acid sequence of rabbit cardiac tropomyosin. J. Biol. Chem. **255**:6854–6859.
50. **Lin, J. J. C., D. M. Helfman, S. H. Hughes, and C. S. Chou.** 1985. Tropomyosin isoforms in chicken embryo fibroblasts: purification, characterization and changes in Rous sarcoma virus-transformed cells. J. Cell Biol. **100**:692–703.
51. **Lin, J. J. C., F. Matsumura, and S. Yamashiro-Matsumura.** 1984. Tropomyosin-enriched and alpha-actinin-enriched microfilaments isolated from chicken embryo fibroblasts by monoclonal antibodies. J. Cell Biol. **98**:116–127.
52. **MacLeod, A. R.** 1982. Distinct alpha-tropomyosin mRNA sequences in chicken skeletal muscle. Eur. J. Biochem. **126**:293–297.
53. **MacLeod, A. R., C. Houlker, F. C. Reinach, L. B. Smillie, K. Talbot, G. Modi, and F. S. Walsh.** 1985. A muscle-type tropomyosin in human fibroblasts: evidence for expression by an alternative RNA splicing mechanism. Proc. Natl. Acad. Sci. USA **82**:7835–7839.
54. **Mak, A. S., and L. B. Smillie.** 1981. Structural interpretation of the two-site binding of troponin in the muscle thin filament. J. Mol. Biol. **149**:541–550.
55. **Mak, A. S., L. B. Smillie, and G. R. Stewart.** 1980. A comparison of the amino acid sequences of rabbit skeletal muscle alpha- and beta-tropomyosins. J. Biol. Chem. **255**: 3647–3655.
56. **Maki, R., W. Roeder, A. Traunecker, C. Sidman, M. Wabl, W. Raschke, and S. Tonegawa.** 1981. The role of DNA rearrangement and alternative RNA processing in the expression of immunglobulin delta genes. Cell **24**:353–365.
57. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
58. **Margossian, S. S., and C. Cohen.** 1973. Troponin subunit interactions. J. Mol. Biol. **81**:409–413.
59. **Matsumura, F., J. J. C. Lin, S. Yamashiro-Matsumura, G. P. Thomas, and W. C. Topp.** 1983. Differential expression of tropomyosin forms in the microfilaments isolated from normal and transformed rat cultured cells. J. Biol. Chem. **258**: 13954–13964.
60. **Matsumura, F., S. Yamashiro-Matsumura, and J. J. C. Lin.** 1983. Isolation and characterization of tropomyosin-containing microfilaments from cultured cells. J. Biol. Chem. **258**: 6636–6644.
61. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
62. **McLachlan, A. D., and M. Stewart.** 1976. The troponin binding region of tropomyosin. Evidence for a site near residues 197-217. J. Mol. Biol. **106**:1017–1022.
63. **Medford, R. M., H. T. Nguyen, A. T. Destree, E. Summers, and B. Nidal-Ginard.** 1984. A novel mechanism of alternative RNA splicing for the developmentally regulated generation of troponin T isoforms from a single gene. Cell **38**:409–421.

64. **Montarras, D., M. Y. Fiszman, and F. Gros.** 1981. Characterization of the tropomyosin present in various chick embryo muscle types and in muscle cells differentiated in vitro. J. Biol. Chem. **256**:4081–4086.
65. **Mount, S. M.** 1982. A catalogue of splice junction sequences. Nucleic Acids Res. **10**:459–472.
66. **Mount, S. M., I. Petterson, M. Hinterberger, A. Karmas, and J. A. Steitz.** 1983. The U1 small nuclear RNA-protein complex selectively binds a 5′ splice site in vitro. Cell **33**:509–518.
67. **Nabeshima, Y., Y. Kurijama-Fujii, M. Muramatsu, and K. Ogata.** 1984. Alternative transcription and two modes of splicing result in two myosin light chains from one gene. Nature (London) **308**:333–338.
68. **Nonomura, Y., W. Drabikowski, and S. Ebashi.** 1968. The localization of troponin in tropomyosin paracrystals. J. Biochem. **64**:419–422.
69. **Ohtsuki, I.** 1974. Localization of troponin in thin filament and tropomyosin paracrystal. J. Biochem. **75**:753–765.
70. **Ohtsuki, I.** 1979. Molecular arrangement of the troponin-T in the thin filament. J. Biochem. **86**:491–497.
71. **Pearlstone, J. R., and L. B. Smillie.** 1981. Identification of a second binding region of rabbit skeletal troponin-T for alpha-tropomyosin. FEBS Lett. **128**:119–122.
72. **Pearlstone, J. R., and L. B. Smillie.** 1982. Binding of troponin-T fragments to several types of tropomyosin. J. Biol. Chem. **257**:10587–10592.
73. **Periasamy, M., E. E. Strehler, L. I. Garfinkel, R. M. Gubits, N. Ruiz-Opazo, and B. Nadal-Ginard.** 1984. Fast skeletal muscle myosin light chains 1 and 3 are produced from a single gene by a combination of differential RNA transcription and splicing. J. Biol. Chem. **259**:13595–13604.
74. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
75. **Robert, B., P. Daubas, M.-A. Akimenko, A. Cohen, I. Garner, J.-L. Guenet, and M. Buckingham.** 1984. A single locus in the mouse encodes both myosin light chains 1 and 3, a second locus corresponds to a related pseudogene. Cell **39**:129–140.
76. **Rogers, J., and R. Wall.** 1980. A mechanism of RNA splicing. Proc. Natl. Acad. Sci. USA **77**:1877–1879.
77. **Romero-Herrera, A. E., S. Nasser, and N. G. Lieska.** 1984. Heterogeneity of adult human striated muscle tropomyosin. Muscle Nerve **5**:713–718.
78. **Rosenfeld, M. G., J. J. Mermod, S. G. Amara, L. W. Swanson, P. E. Sawchenki, J. Rivier, W. W. Vale, and R. M. Evans.** 1983. Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. Nature (London) **304**:129–133.
79. **Rozek, C. E., and N. Davidson.** 1983. Drosophila has one myosin heavy-chain gene with three developmentally regulated transcripts. Cell **32**:23–34.
80. **Ruiz-Opazo, N., J. Weinberger, and B. Nadal-Ginard.** 1985. Comparison of alpha-tropomyosin sequences from smooth and striated muscle. Nature (London) **315**:67–70.
81. **Salviati, G., R. Betto, and D. D. Betto.** 1982. Polymorphism of myofibrillar proteins of rabbit skeletal-muscle fibres: an electrophoretic study of single fibres. Biochem. J. **207**:261–272.
82. **Sanders, C., and L. B. Smillie.** 1985. Amino acid sequence of chicken gizzard beta-tropomyosin. J. Biol. Chem. **260**: 7264–7275.
83. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
84. **Schibler, U., D. E. Kelley, and R. P. Perry.** 1977. Comparison of methylated sequences in messenger RNA and heterogeneous nuclear RNA from mouse L cells. J. Mol. Biol. **115**:695–714.
85. **Schloss, J. A., and R. D. Goldman.** 1980. Microfilaments and tropomyosin of cultured mammalian cells: isolation and characterization. J. Cell Biol. **87**:633–642.
86. **Sharp, P. A.** 1981. Speculations on RNA splicing. Cell **23**:643–646.
87. **Smillie, L. B.** 1979. Structure and functions of tropomyosins from muscle and non-muscle sources. Trends Biochem. Sci. **4**:151–155.
88. **Sodek, J., R. S. Hodges, and L. B. Smillie.** 1978. Amino acid sequence of rabbit skeletal muscle alpha-tropomyosin. J. Biol. Chem. **253**:1129–1136.
89. **Southern, E.** 1975. Detection of specific DNA sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
90. **Stewart, M., and A. D. McLachlan.** 1976. Structure of magnesium paracrystals of alpha-tropomyosin. J. Mol. Biol. **103**:251–269.
91. **Stone, D., and L. B. Smillie.** 1978. The amino acid sequence of rabbit skeletal muscle alpha-tropomyosin. J. Biol. Chem. **253**:1137–1148.
92. **Talbot, K., and A. R. Macleod.** 1983. Novel form of non-muscle tropomyosin in human fibroblasts. J. Mol. Biol. **164**:159–174.
93. **Tamkun, J. W., J. E. Schwarzbauer, and R. O. Hynes.** 1984. A single rat fibronectin gene generates three different mRNAs by alternative splicing of a complex exon. Proc. Natl. Acad. Sci. USA **81**:5140–5144.
94. **Taylor, E. W.** 1979. Mechanism of actomyosin ATPase and the problem of muscle contraction. Crit. Rev. Biochem. **6**: 103–163.
95. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77**:5201–5205.
96. **Tonegawa, S.** 1983. Somatic generation of antibody diversity. Nature (London) **302**:575–581.
97. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
98. **Wei, C. M., and B. Moss.** 1977. Nucleotide sequences at the N^6-methyladenosine site of HeLa cell messenger ribonucleic acid. Biochemistry **16**:1672–1676.
99. **Yaffe, D., U. Nudel, Y. Mayer, and S. Neuman.** 1985. Highly conserved sequences in the 3′-untranslated region of mRNAs coding for homologous proteins in distantly related species. Nucleic Acids Res. **13**:3723–3737.
100. **Yamaguchi, M., M. L. Greaser, and R. G. Cassens.** 1974. Interaction of troponin subunits with different forms of tropomyosin. J. Ultrastruct. Res. **48**:33–58.
101. **Yamamoto, K. R., B. M. Alberts, R. Benzinger, L. Lawhorne, and G. Treiber.** 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. Virology **40**:734–744.
102. **Yamawaki-Kataoka, Y., and D. M. Helfman.** 1985. Rat embryonic fibroblast tropomyosin 1: cDNA and complete primary amino acid sequence. J. Biol. Chem. **260**:14440–14445.
103. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19. Gene **33**:103–119.