THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 262, No. 8, Issue of March 15, pp. 3590-3596, 1987
Printed in U.S.A.

Closely Related α-Tropomyosin mRNAs in Quail Fibroblasts and Skeletal Muscle Cells*

(Received for publication, July 28, 1986)

Patricia L. Hallauer‡, Kenneth E. M. Hastings§, Albert S. Baldwin¶, Sonia Pearson-White, Peter A. Merrifield, and Charles P. Emerson, Jr.‖

From the Department of Biology, University of Virginia, Charlottesville, Virgina 22901

We describe the analysis of two quail cDNA clones representing distinct but closely related α-tropomyosin mRNAs. cDNA clone cC101 corresponds to a 1.2-kilobase RNA which accumulates to high levels during myoblast differentiation and which encodes the major isoform of skeletal muscle α-tropomyosin. cDNA clone cC102 corresponds to a 2-kilobase RNA which is abundant in cultured embryonic skin fibroblasts and which encodes one of two α-tropomyosin-related fibroblast tropomyosins of 35,000 and 34,000 daltons apparent molecular mass (class 1 tropomyosins). The cC102 protein is unique among reported nonstriated-muscle tropomyosins in being identical in amino acid sequence to the major isoform of skeletal muscle α-tropomyosin over an uninterrupted stretch of at least 183 amino acids (residues 75–257). The two protein sequences differ in the COOH-terminal region beginning with residue 258. Because the cC101 and cC102 RNAs share an extensive region (at least 373 nucleotides) of nucleotide sequence identity upstream of the codon for residue 258, they are likely derived from a single gene by alternative RNA splicing, as was recently proposed in the case of related β-tropomyosin mRNAs in human fibroblasts and skeletal muscle (MacLeod, A. R., Houlker, C., Reinach, R. C., Smillie, L. B., Talbot, K., Modi, G., and Walsh, F. S. (1985) *Proc. Natl. Acad. Sci. U.S.A.* 82, 7835-7837). No α-tropomyosin-related RNAs are abundant in undifferentiated myoblasts. This suggests the possibility of a fibroblast-specific function, as opposed to a general nonmuscle-cell function for class 1 tropomyosins and also has implications for the regulation of α-tropomyosin gene expression during embryonic development.

Tropomyosins are a large family of structurally similar proteins found in a wide variety of cell types including striated and smooth muscle (Coté, 1983; Fine and Blitz, 1975; Smillie, 1979). Tropomyosin polypeptides are highly α-helical proteins of characteristic chain lengths, which form rod-like coiled-coil dimers associated with filamentous actin in the thin filaments of the striated muscle sarcomere and in microfilaments in smooth and nonmuscle cells (Smillie, 1979; Squire, 1981; Taylor, 1979; Chamley-Campbell *et al.*, 1977; Lazarides, 1975; Lin *et al.*, 1984; Schloss and Goldman, 1980; Wehland and Weber, 1980). In conjunction with troponin, tropomyosin plays a key role in the Ca^{2+} regulation of striated muscle contraction (Squire, 1981; Taylor, 1979). Because troponin does not appear to be present in other tissues than striated muscle, the function of tropomyosin in smooth muscle and nonmuscle cells is not entirely clear (but see Coté, 1983; Marston and Smith, 1985).

Striated muscle contains at least five tropomyosin polypeptides falling into two electrophoretic classes, α and β. These show differential expression both in the α:β tropomyosin ratio, which varies characteristically from muscle to muscle, and in the existence of distinct isoforms of α- and β-tropomyosin in fast and slow skeletal muscle fibers and in avian cardiac muscle (Billeter *et al.*, Bronson and Schachet, 1982; Hayashi *et al.*, 1977; Izant and Lazarides, 1977; Leger *et al.*, 1976; Montarras *et al.*, 1981; Steinbach *et al.*, 1980). Smooth muscle contains α- and β-tropomyosins which are distinct from those expressed in striated muscle (Hayashi *et al.*, 1977; Helfman *et al.*, 1984; Izant and Lazarides, 1977; Montarras *et al.*, 1981). Tropomyosins of nonmuscle cells also display heterogeneity (Giometti and Anderson, 1984). Four tropomyosins, all distinct from striated muscle tropomyosins, have been identified in a single mammalian fibroblast cell line (Talbot and MacLeod, 1983), and seven tropomyosins have been detected in embryonic avian fibroblast cultures (Hendricks and Weintraub, 1984; Lin *et al.*, 1985). Thus, the tropomyosin protein family consists of at least 10–15 distinct but similar proteins showing a complex pattern of cell- and tissue-specific expression.

Besides the regulation implicit in the existence of multiple cell-type-specific tropomyosins, changes in tropomyosin isoform expression have been observed within individual cell types. Avian fibroblasts transformed by Rous sarcoma virus show specific quantitative alterations in their pattern of tropomyosin isoforms (Hendricks and Weintraub, 1984; Lin *et al.*, 1985). Differentiating skeletal muscle myoblasts activate the synthesis of muscle-specific tropomyosin isoforms during a general coordinate activation of muscle-specific contractile protein synthesis (Montarras *et al.* 1981; Devlin and Emerson, 1979). The diversity of tropomyosin isoform expression patterns suggests important biological functions, but the physiological significance of tropomyosin diversity has not yet been elucidated, and the regulatory mechanisms that control

* This work was supported by National Institutes of Health predoctoral fellowships (to P. L. H. and A. S. B.), Muscular Dystrophy Association postdoctoral fellowships (to K. E. M. H. and P. A. M.), and a National Institutes of Health research grant (to C. P. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J02688.

‡ Present address: Montreal General Hospital Research Institute, 1650 Cedar Ave., Montreal, Canada H3G 1A4.

§ Present address: Montreal Neurological Institute, McGill University, 3801 University St., Montreal, Canada H3A 2B4.

¶ Present address: Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139.

‖ To whom correspondence should be addressed.

the expression of tropomyosin isoforms are unknown.

Recent evidence indicates that mechanisms of alternative RNA splicing play a role in the generation of tropomyosin isoform diversity in *Drosophila* (Basi *et al.*, 1984; Karlik *et al.*, 1984) and in vertebrates (Ruiz-Opazo *et al.*, 1985; MacLeod *et al.*, 1985). In the rat a single α-tropomyosin gene gives rise to both striated muscle and smooth muscle isoforms by alternative RNA splicing (Ruiz-Opazo *et al.*, 1985 and see also Fiszman *et al.*, 1986). Similarly, alternative RNA splicing has been hypothesized to account for the production of skeletal muscle β-tropomyosin and a related fibroblast tropomyosin in humans (MacLeod *et al.*, 1985). Results of this nature have several important implications, including 1) that protein structural features associated with striated muscle-specific tropomyosin functions (such as troponin interactions) may be entirely confined to limited, sharply defined molecular domains; 2) that individual tropomyosin genes are complex in their structure and expression; and 3) that "striated muscle" tropomyosin gene transcription is not limited to striated muscle cells.

In the present paper we report the analysis of α-tropomyosin cDNA clones obtained from embryonic quail skeletal muscle cultures. Our results indicate that closely related mRNAs encode skeletal muscle α-tropomyosin and a major tropomyosin of embryonic skin fibroblasts and that these mRNAs are likely derived by alternative RNA splicing pathways from a single gene. These results contribute new information consistent with alternative splicing of fibroblast and skeletal muscle tropomyosin mRNAs. They also indicate that the fibroblast/skeletal muscle alternative RNA splicing mechanism was established in evolution prior to both the bird/mammal divergence and the α-tropomyosin/β-tropomyosin divergence. Our characterization of quail fibroblast tropomyosins demonstrates the existence of a major class of α-tropomyosin-related proteins of 35,000 and 34,000 daltons apparent molecular mass (class 1 tropomyosins). One of these bears an unprecedented similarity to skeletal muscle tropomyosin, being identical in sequence over a continuous length of at least 183 amino acids, but differing in the COOH-terminal region. Our results also show that no α-tropomyosin-related transcripts accumulate in undifferentiated myoblasts. This suggests the possibility of a fibroblast-specific function of class 1 tropomyosins and indicates that α-tropomyosin gene expression is subject to multiple developmental controls involving mechanisms that regulate both the relative utilization of alternative RNA splicing pathways and the overall levels of mRNA accumulation.

MATERIALS AND METHODS

Cell Cultures—Cell cultures were established from embryos of the Japanese quail, *Coturnix coturnix*. Muscle cultures were established from the breast musculature of day 10 embryos as described by Konigsberg (1979). The cultures were harvested 2 days after the second plating for myoblast material and 5 days after plating for differentiated muscle cell material. Fibroblast primary cultures were established from the back skin of day 9 embryos, by mincing, trypsinizing, and plating 5×10^5 cells/100-mm diameter culture dish. Cultures were harvested 4–6 days after plating. The medium used for muscle and fibroblast cultures was Minimal Essential Medium (*Gibco*) containing 5% chicken embryo extract and 15% horse serum. RNA was extracted by lysis with SDS[1] and phenol extraction (Devlin and Emerson, 1979). To harvest material for protein analysis, cell layers were rinsed three times with saline G and lysed directly into SDS gel sample buffer.

Protein Analysis—Actomyosin was prepared from adult quail leg muscle by two cycles of extraction with 0.6 M KCl and precipitation from 0.1 M KCl. Tropomyosin was purified from human platelets (Cohen and Cohen, 1972; Fine *et al.*, 1973) and from adult quail breast muscle (Hartshorne and Mueller, 1969).

SDS-polyacrylamide gel electrophoresis was done by the method of Laemmli (Laemmli, 1970) using 10% acrylamide, 0.4% bisacrylamide gels. Two-dimensional urea-shift gel electrophoresis was done as described by Carmon *et al.* (1978). Gels (other than those used for immunoblotting) were stained with Coomassie Blue and those containing ^{35}S-labeled proteins were also fluorographed either by the method of Bonner and Laskey (1974) or by the use of ENHANCE (New England Nuclear). Western Immunoblot analysis was done according to the method of Towbin *et al.* (1979) using the mouse monoclonal antibody 3F9 and visualizing immunoreactive material with a peroxidase-coupled second antibody. The 3F9 antibody, an IgG raised against adult quail breast tropomyosin, was produced in Dr. I. R. Konigsberg's lab, University of Virginia. 3F9 recognizes a wide variety of tropomyosins including α- and β-tropomyosins from quail skeletal muscle and from human platelets (see Fig. 6).

DNA Sequence Analysis—Isolation of the tropomyosin cDNA clones cC101 and cC102 has been described (Hastings and Emerson, 1982a, 1982b). These are oligo(dT)-primed cDNAs of differentiated muscle culture poly(A$^+$) RNA inserted by G, C tailing into the *Pst*I site of pBR322. Complete sequences of cC101 and cC102 were obtained by the method of Maxam and Gilbert (1977) by either 5′-labeling at the *Hin*dIII sites (see Fig. 1) or by 3′-labeling at *Pst*I sites at the ends of the cDNA inserts or at *Bgl*I sites in the pBR322 plasmid vector. Note that a previously reported partial sequence (Hastings and Emerson, 1982a) contains an error in the codon for amino acid 174; the correct sequence is shown in Fig. 1.

Analyses Using cDNA Clones—For Northern blot analysis RNA samples were electrophoresed in methyl mercury/agarose gels, transferred to diazophenylthioether paper, and hybridized with cDNA clones ^{32}P-labeled by nick-translation (Alwine *et al.*, 1977; Seed, 1982; Wahl *et al.*, 1979). The final wash following hybridization was in 0.1 × SSC at 65 °C.

Hybridization-translation analysis was done as decribed by (Ricciardi *et al.*, 1979). Hybridization was at 42 °C in 50% formamide, and the final wash was 1 × SSC at 60 °C. Recovered RNA was translated in a nuclease-treated rabbit reticulocyte lysate containing [^{35}S]methionine.

S_1 nuclease protection analysis was done as described by Berk and Sharp (1977). Probes were labeled at *Hin*dIII sites by 3′-fill-in with DNA polymerase Klenow fragment in the present of dATP, dGTP, and [α-^{32}P]dCTP (Maniatis *et al.*, 1982). Size markers were provided by a polynucleotide kinase ^{32}P-labeled *Hin*fI digest of the plasmid pKH47 (Hayashi, 1980).

RESULTS

cDNA Clones Representing Distinct Tropomyosin mRNAs—cC101 and cC102 are tropomyosin cDNA clones isolated from a library representing poly(A$^+$) RNA extracted from differentiated cultures of embryonic quail skeletal muscle cells (Hastings and Emerson, 1982a, 1982b). The complete DNA sequences of cC101 and cC102 were determined and are shown in Fig. 1. These data indicate that the cDNA clones represent two distinct but very closely related mRNA species.

The 456 bp mRNA fragment cloned in cC101 encodes skeletal muscle α-tropomyosin from amino acid 134 to the COOH terminus of the protein (residue 284) and includes the stop codon TAA and 9 bp of 3′-untranslated mRNA sequence. The encoded protein sequence is identical to the corresponding sequence of the major skeletal muscle α-tropomyosin of the chicken, as deduced from chick leg muscle cDNA clones (MacLeod, 1982). There are only five single base differences between the 456-bp sequence of cC101 and the corresponding chicken cDNA sequence. Thus, we can conclude with a high degree of certainty that cC101 is a partial copy of an mRNA encoding the major skeletal muscle of α-tropomyosin of the quail.

The 566-bp mRNA fragment cloned in cC102 encodes a tropomyosin sequence corresponding to amino acid residues 75–261. Between residue 75 and residue 257, the cC102 protein is identical to the chicken major skeletal α-tropomyosin.

[1] The abbreviations used are: SDS, sodium dodecyl sulfate; bp, base pair; kb, kilobase.

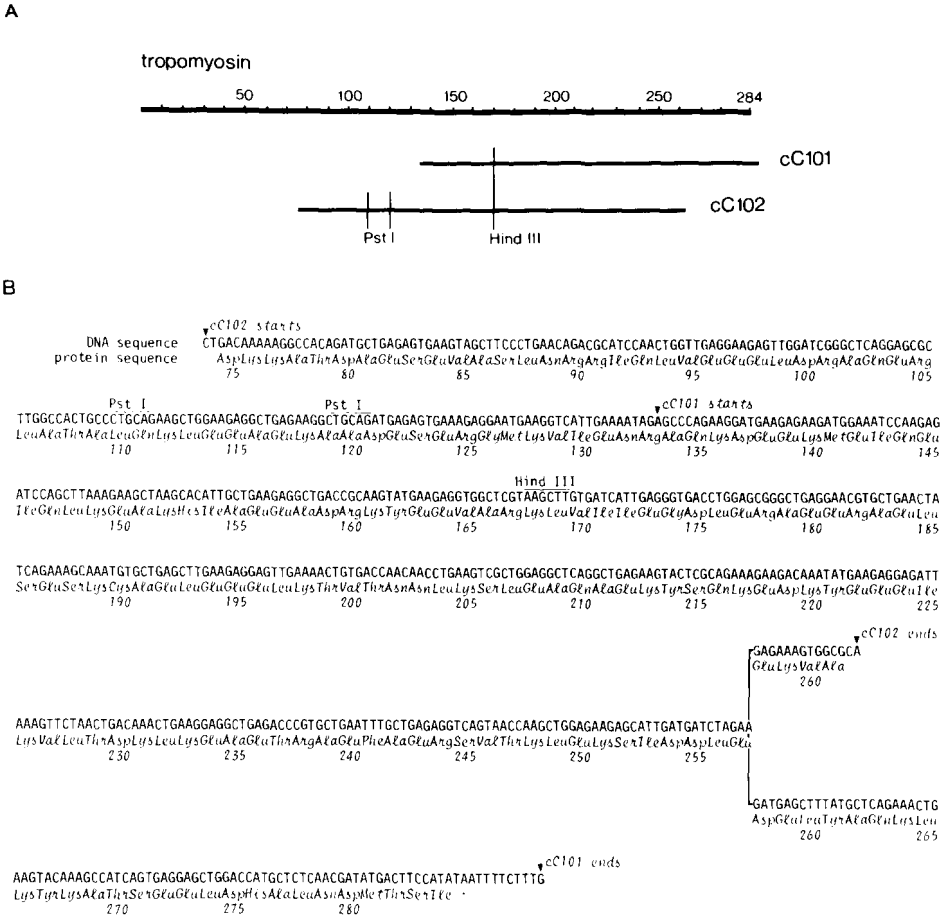

FIG. 1. **DNA sequences of tropomyosin cDNA clones cC101 and cC102.** The overlap relationships of cC101, cC102, and the α-tropomyosin polypeptide are shown in *panel A*. The DNA and corresponding amino acid sequences of cC101 and cC102 are shown in *panel B*. Upstream of codon 258, where both cDNA clones have identical sequences, only a single sequence is shown. The G, C homopolymer tails at the ends of the inserts (about 20 residues) introduced during cloning are not indicated.

However, following amino acid 257 the cC102 protein sequence diverges markedly from any known skeletal muscle tropomyosin sequence. It is not identical to any published tropomyosin sequence, but it resembles corresponding sequences of platelet and smooth muscle tropomyosins in having a lysine at residue 259 and glutamine or histidine at residue 262 (Lewis *et al.*, 1983; Ruiz-Opazo *et al.*, 1985). Thus, cC101 and cC102 represent two distinct mRNA species encoding distinct tropomyosin isoforms. The cC101 mRNA encodes the major skeletal muscle α-tropomyosin, whereas the cC102 mRNA encodes a closely related, previously uncharacterized, protein sequence.

An intriguing feature of these cDNA sequences is that over the 373 bp where cC101 and cC102 overlap and correspond to the same protein sequence (*i.e.* upstream of residue 258), the two nucleic acid sequences are identical, whereas downstream of the codon for residue 257 they are markedly different.

Fibroblast and Muscle Cell Tropomyosin mRNAs—Although muscle fibers are the predominant cell type in differentiated quail muscle cultures, there remains a significant number of undifferentiated myoblasts as well as a small number of fibroblasts (usually less than 5–10% of the total number of cells). In order to assess the possibility that cC102 may have been derived from the fibroblast population, primary fibroblast cultures free of muscle cells were established from the skin of quail embryos. RNA was extracted from the fibroblast cultures and from muscle cultures before and after differentiation and also from several skeletal muscles of adult quail. RNA samples were probed for the presence of RNAs fully complementary to cC102 (and cC101) by S_1 nuclease protection analysis.

S_1 Nuclease Analysis—To prepare probes specific for cC101 and cC102 RNAs both cDNA clones were cut and 3′-end-labeled at the *Hin*dIII site corresponding to codons 168 and 169 (see Fig. 1). Following a second cut in the vector DNA, probe fragments were isolated corresponding to the mRNA region downstream of the *Hin*dIII site. These probes assay mRNA sequence complementarity in the region beginning at codon 169 and continuing in a 3′-direction as far as the cDNA sequence extends. Hybridization to fully complementary mRNAs would result in fully protected cDNA fragments of 360 bases in the case of cC101 and 283 bases in the case of cC102. Cross-hybridization of cC101 RNA with the cC102 probe, or vice versa, would be expected to result in partially protected probe fragments of 270 bases.

When differentiated muscle culture RNA was hybridized with the cC101 probe, the major S_1 nuclease-resistant product (360 bases) corresponded to full-length protection of the probe (Fig. 2*A*, *lane 2*). Undoubtedly, the fully complementary RNA providing full-length protection of the probe in the RNA from which cC101 was originally derived, *i.e.* skeletal muscle α-tropomyosin mRNA. This fully complementary RNA also is abundant in adult muscle RNA (Fig. 2*A*, *lane 3*), but was undetectable in fibroblast RNA (Fig. 2*A*, *lane 4*). However, fibroblast RNA did contain a related RNA species, revealed by the protection of a 270-base cC101 probe fragment (Fig. 2*A*, *lane 4*). This related RNA species consisted of cC102 RNA as shown by the exclusive production of full-length S_1 nuclease-resistant product (283 bases) when the cC102 probe was used (Fig. 2*B*, *lane 4*). RNA fully complementary to the cC102 probe was also detected in differentiated muscle culture RNA (Fig. 2*B*, *lane 2*), as expected, since this was the original source of the cDNA clone. However, the cC102 RNA (represented by the 283-base band in Fig. 2*B*, *lane 2*) in muscle

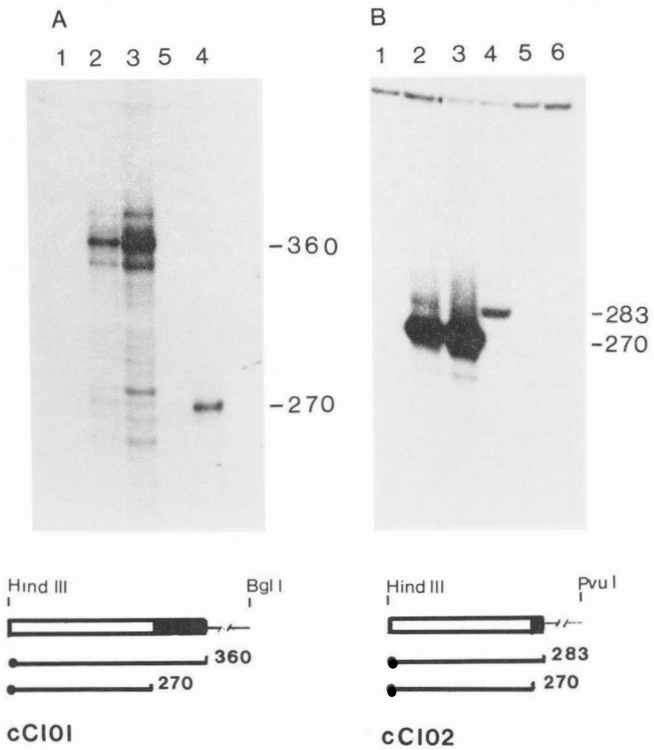

FIG. 2. **Analysis of α-tropomyosin-related RNAs by S_1 nuclease protection using probes derived from cC101 (*A*) and cC102 (*B*).** The RNA samples were from *lane 1*, undifferentiated muscle cultures; *2*, differentiated muscle cultures; *3*, adult breast muscle; *4*, skin fibroblast cultures; *5*, adult liver; and *6*, no added RNA. Product estimated chain lengths (in bases) are indicated. Note the lane numbering in *panel A*. The drawings below illustrate the probes and expected protected fragment sizes (in bases). Probes were 3′-labeled (indicated by *terminal circles*) at the *Hind*III sites corresponding to residues 168/169 and were second cut within pBR322 vector sequences (indicated by *thin interrupted lines*) with the restriction enzymes indicated. cDNA insert sequences are indicated by *thick bars* and the regions of divergent COOH-terminal sequences are shown in *black*. Products expected from full-length protection and from protection up to but not including the regions of divergent sequences are indicated. The presence of small amounts of greater-than-full-length (360 bases) products in *lanes 2* and *3, panel A*, results from complete removal by S_1 nuclease of pBR322 sequences, but incomplete removal of the poly(G) linker at the end of the cDNA sequence in the probe. This could reflect either inherent resistance of the poly(G) sequence to S_1 nuclease digestion or hybridization to a C-rich sequence in the mRNA. Minor less-than-full-length products in *lanes 2* and *3* may indicate the presence of additional minor α-tropomyosin-related RNAs and/or RNA degradation before or during the hybridization reaction.

cultures was less abundant than the cC101 RNA (represented by the 270-base band). Moreover, cC102 RNA was not detectable in adult muscle RNA (although cC101 RNA was; Fig. 2*B*, *lane 3*) and was more abundant in fibroblast RNA than in differentiated muscle culture RNA (Fig. 2*B*, *lanes 2* and *4*). The presence of an abundant fibroblast RNA fully complementary to the cC102-specific probe indicates that cC102 RNA is expressed in fibroblasts and suggests a fibroblast origin for the cC102 cDNA clone.

Myoblast RNA isolated from undifferentiated muscle cultures provides very little protection, either partial or full length, to either the cC101 or cC102 probes (Figs. 2, *A* and *B*, *lane 1*). Liver RNA provides no detectable protection at all (Fig. 2, *A* and *B*, *lane 5*).

Northern Blot Analysis—Tropomyosin RNA expression in muscle and fibroblast cells was also examined by Northern blot hybridization using nick-translated cC102 as the hybridization probe. Fig. 3 shows that cC102 hybridizes with two abundant RNA species. One of the RNA species, approximately 2 kb in length, was abundant in fibroblast RNA (Fig. 3*A*, *lane 1*). Much smaller amounts of the 2-kb RNA were present in differentiated muscle culture RNA (Fig. 3*A*, *lane 3*). A second RNA species, approximately 1.2 kb in length, was abundant in differentiated muscle culture RNA, but not in fibroblast RNA. Neither the 2-kb nor the 1.2-kb RNA species were abundant in myoblast RNA (*lane 2*).

The hybridization and wash conditions used for Fig. 3 are of high stringency. When the Northern blot results of Fig. 3 are compared with the S_1 nuclease analysis of Fig. 2, the relative abundances of the 1.2 and 2-kb RNA species in fibroblast and differentiated muscle culture RNA indicate that the 1.2-kb RNA corresponds to the cC101 cDNA clone and the 2-kb RNA corresponds to the cC102 cDNA clone.

Analysis of Tropomyosin Polypeptides—The nature of the proteins encoded in the abundant α-tropomyosin-related 1.2 and 2-kb RNAs was examined by hybridization-translation analysis. cC102 DNA affixed to nitrocellulose was used to select hybridizing RNA from fibroblast RNA and differentiated muscle culture RNA, and the selected RNAs were translated *in vitro*. As expected on the basis of the cC101 sequence data and the S_1 nuclease results, the hybrid-selected muscle culture RNA directed the synthesis of a 35,000-dalton protein that co-migrated with skeletal muscle α-tropomyosin (Fig. 4, *lane 5*). The hybrid-selected fibroblast RNA directed the synthesis of two proteins of 35,000- and 34,000-dalton apparent molecular mass (Fig. 4, *lane 3*). The production of two proteins suggests the presence of two α-tropomyosin-related mRNAs in fibroblasts. Because Northern blot analysis (Fig. 3) had shown only a single major α-tropomyosin-related RNA band of approximately 2 kb in fibroblast RNA, the two RNA species detected by *in vitro* translation are apparently similar in size. One of the 2-kb mRNAs presumably corresponds to the cC102 cDNA clone.

The absence of a detectable 34,000-dalton band from the hybridization-translation products of muscle culture RNA (Fig. 4, *lane 5*) does not necessarily imply that the 2-kb mRNA species encoding the 34,000-dalton protein is specifically absent in muscle cultures. Because of the low overall levels of 2-kb RNA(s) in muscle culture RNA (see Fig. 3), only low

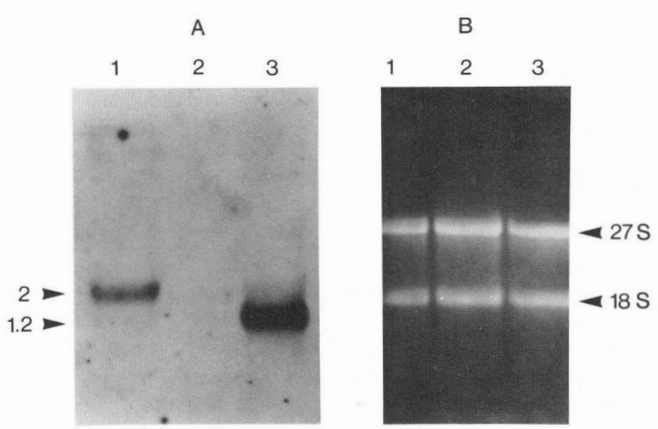

FIG. 3. **Analysis of α-tropomyosin-related RNAs by Northern blot hybridization.** In *panel A* equal quantities (25 μg) of RNA from *lane 1*, fibroblast cultures; *2*, undifferentiated muscle cultures, and *3*, differentiated muscle cultures were analyzed by Northern blot hybridization with cC102 as the probe. Estimated sizes (in kb) of hybridizing bands are indicated. In *panel B* 2.5-μg samples from the same RNA preparation analyzed in *panel A* were electrophoresed in a similar gel and stained with ethidium bromide, to confirm the integrity and the relative quantities of the RNAs examined in *panel A*. 18 S and 27 S ribsomal RNA bands are indicated.

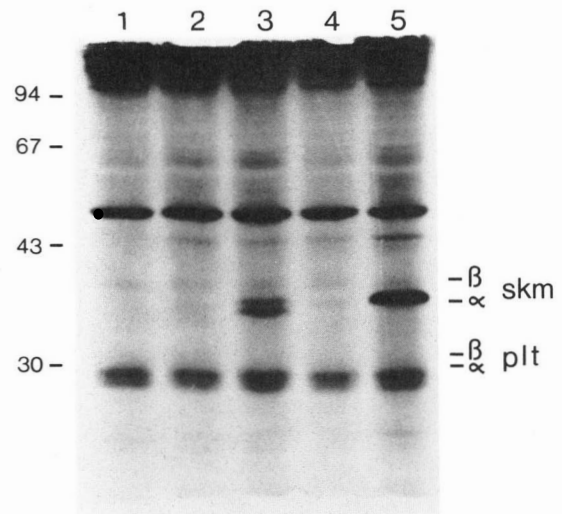

FIG. 4. **Hybridization-translation analysis of α-tropomyosin-related RNAs.** cC101 and, as a control, pBR322 were used to select complementary mRNA from either fibroblast RNA or differentiated muscle culture RNA. The hybrid-selected RNA was translated *in vitro*, and the products were analyzed by SDS-polyacrylamide gel electrophoresis and fluorgraphy. *Lane 1* shows endogenous products of the translation system. *Lanes 2* and *3* show translation products of fibroblast RNAs selected by either pBR322 (*lane 2*) or cC102 (*lane 3*). *Lanes 4* and *5* show translation products of muscle culture RNAs selected by either pBR322 (*lane 4*) or cC102 (*lane 5*). The positions of the molecular mass markers (with molecular masses indicated in thousands of daltons) and quail skeletal muscle (*skm*) and human platelet (*plt*) tropomyosins are indicated.

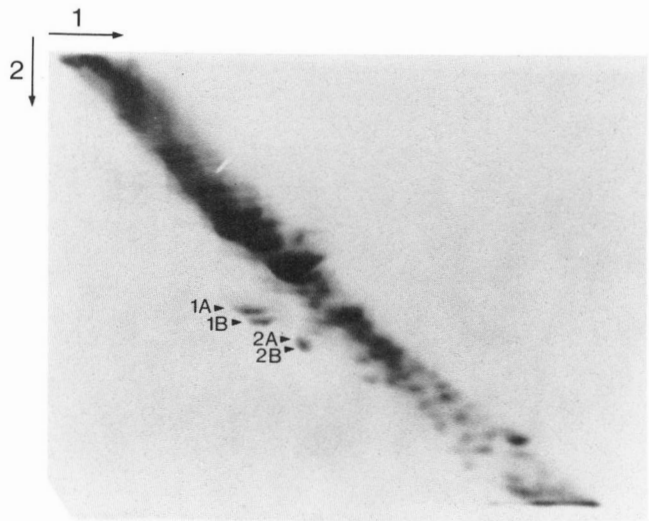

FIG. 5. **Two-dimensional urea-shift electrophoretic analysis of fibroblast tropomyosins.** Fibroblast cultures were dissolved directly in SDS gel sample buffer and analyzed by the two-dimensional technique of Carmon *et al.* (1978). SDS was present in both dimensions of electrophoresis; dimension 1 also contained 5 M urea. Tropomyosins *1A, 1B, 2A* and *2B* are indicated.

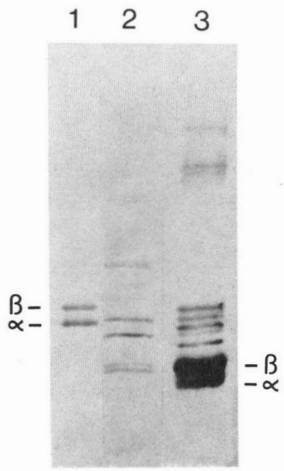

FIG. 6. **Western immunoblot analysis of fibroblast tropomyosins.** Protein samples were dissolved in SDS gel sample buffer, subjected to electrophoresis, electrophoretically transferred to nitrocellulose, and probed with the antitropomyosin monoclonal antibody 3F9. The protein samples analyzed were: *lane 1*, actomyosin prepared from adult quail leg muscle; *lane 2*, whole cell lysate of cultured fibroblasts; and *lane 3*, human platelet tropomyosin. α- and β-isoforms of the skeletal muscle and platelet tropomyosins are indicated.

levels of 2-kb RNA translation products, whether 35,000 or 34,000 daltons, would be expected. The abundant 35,000-dalton protein in Fig. 4, *lane 5*, is presumably skeletal muscle α-tropomyosin produced from the 1.2-kb α-tropomyosin mRNA that is abundant in muscle culture RNA (see Fig. 3).

In order to determine whether the 35,000- and 34,000-dalton hybridization-translation products corresponded to major fibroblast tropomyosins, we characterized tropomyosin proteins in fibroblast cultures.

It is a characteristic feature of tropomyosin that their electrophoretic mobilities in SDS gels are retarded in the presence of urea. This unusual feature is exploited in a two-dimensional gel technique developed by Carmon *et al.* (1978) in which tropomyosins can be identified in complex mixtures of proteins. Two-dimensional urea-shift gel analysis of the total proteins extracted from cultures of embryonic quail skin fibroblasts indicated four possible major tropomyosins, labeled *1A, 1B, 2A,* and *2B* in Fig. 5. Apparent molecular masses based on mobility in the second electrophoretic dimension (without urea) were estimated as 35,000 and 34,000 for tropomyosins *1A* and *1B*, respectively, and 31,000 for tropomyosins *2A* and *2B* whose apparent molecular masses differ by less than 1000 daltons.

In an independent approach we used the monoclonal antibody 3F9 which recognizes an epitope present on many, and perhaps all, tropomyosins (see "Materials and Methods"). 3F9 reacted with four principal (and several minor) bands in a fibroblast whole cell lysate analyzed by immunoblot electrophoresis (Fig. 6, *lane 2*). The largest of the four major 3F9-reactive proteins had a mobility similar to skeletal muscle α-tropomyosin, and hence has an apparent molecular mass of 35,000 daltons. Two 3F9-reactive proteins formed a closely spaced doublet having a mobility similar to the β-component of human platelet tropomyosin, *i.e.* corresponding to a 31,000-dalton apparent molecular mass. The fourth major 3F9-reactive protein had a mobility intermediate between the 35,000- and 31,000-dalton proteins corresponding to a 33,000–34,000-dalton apparent molecular mass.

The molecular mass distribution of major 3F9-reactive fibroblast proteins corresponds with the molecular mass distribution of major fibroblast proteins showing the distinctive tropomyosin urea-shift on two-dimensional electrophoresis. These two independent approaches indicate major tropomyosin proteins of 35,000 and 34,000 daltons (tropomyosins *1A* and *1B*) and a closely spaced doublet of approximately 31,000 daltons (tropomyosins *2A* and *2B*). The concordance in molecular mass estimates suggested that the 35,000- and 34,000-dalton hybridization-translation products of the 2-kb fibroblast RNAs in Fig. 4, *lane 3*, correspond to tropomyosins *1A* and *1B*, and this was confirmed by their co-migration on two-dimensional urea-shift gels (not shown). These results define

two classes of major quail skin fibroblast tropomyosins. One class (class 1 tropomyosins) consists of two proteins of 35,000 and 34,000 daltons apparent molecular mass whose mRNAs are closely related to skeletal muscle α-tropomyosin mRNA. The second class (class 2 tropomyosins) consists of two proteins of very similar apparent molecular masses close to 31,000 daltons.

DISCUSSION

Tropomyosin isoforms differing at the COOH terminus are produced by alternative RNA splicing mechanisms in *Drosophila* (Basi *et al.*, 1984; Karlik *et al.*, 1984) and the rat (Ruiz-Opazo *et al.*, 1985). The juxtaposition of identical and highly divergent sequence blocks of cC101 and cC102 is good evidence for a similar mechanism in birds (see also Fiszman *et al.*, 1986). cC101 and cC102 RNAs are apparently derived by alternative RNA splicing from a single gene with the alternative RNA splice point being, as it is in a *Drosophila* tropomyosin gene (Basi *et al.*, 1984; Karlik *et al.*, 1984), within codon 258. Upstream of codon 258 the cC101 and cC102 RNAs have identical sequences for at least 373 nucleotides and are presumably derived from common exons, whereas downstream of this point different exons are used in the two RNAs. The tropomyosin gene represented by cC101 and cC102 is unambiguously identified as that encoding the major (presumably fast fiber type) isoform of skeletal muscle α-tropomyosin.

One of the quail α-tropomyosin mRNAs, the 1.2-kb cC101 RNA, accumulates to high levels during myoblast differentiation, is present in adult skeletal muscle, and encodes a protein identical to the major isoform of skeletal muscle α-tropomyosin over the entire coding length of the cC101 cDNA, from residue 134 to the COOH-terminal residue 284. The cC101 RNA is undoubtedly responsible for the synthesis of the major isoform of skeletal muscle α-tropomyosin both in differentiating embryonic muscle cells and in adult muscle fibers. It is a muscle-specific RNA that is not present in embryonic skin fibroblast cultures, or in liver.

In contrast with the 1.2-kb cC101 RNA, the alternatively spliced 2-kb cC102 RNA is not present in adult muscle and is more abundant in embryonic skin fibroblast culture RNA than in muscle culture RNA. Its expression in fibroblasts shows that cC102 RNA is not a muscle-specific RNA species. Moreover, the low levels of cC102 RNA in muscle cultures must be derived at least in part from the small proportion of fibroblasts that are always present. The apparent increase in levels of cC102 RNA during myoblast differentiation seen in Fig. 2 may reflect an increasing proportion of fibroblasts as these cells continue to proliferate while myoblasts withdraw from the cell cycle and differentiate. However, we cannot exclude the possibility that differentiated muscle cells in culture may also produce low levels of cC102 RNA.

Our analysis of the cC101 and cC102 cDNA clones suggests that a fibroblast and a skeletal muscle tropomyosin are derived from a single α-tropomyosin gene by alternative RNA splicing affecting the COOH-terminal region beginning with codon 258. Recently MacLeod *et al.* (1985) have presented similar evidence based on cDNA clone sequence analysis for alternative RNA splicing of a human skeletal muscle β-tropomyosin and a human fibroblast tropomyosin. Taken together, these comparable results in different vertebrate classes form a convincing body of evidence for fibroblast/skeletal muscle alternative RNA splicing. Moreover, they indicate that alternative RNA splicing of fibroblast and skeletal muscle tropomyosins was developed in evolution prior to both the bird/mammal divergence and the α-tropomyosin/β-tropomyosin divergence.

Beyond the fundamental similarities of fibroblast/skeletal muscle alternative splicing in quail α-tropomyosin and human β-tropomyosin, there are differences. The alternatively spliced human fibroblast β-tropomyosin mRNA differs from the skeletal muscle mRNA not only in the COOH-terminal exon, but also in an internal sequence block encoding residues 188–213 (MacLeod *et al.*, 1985). In contrast, the quail fibroblast α-tropomyosin RNA does not differ from the skeletal muscle mRNA in this region (see also below). An additional difference is that whereas the human fibroblast β-tropomyosin mRNA is relatively small, 1.1 kb, the quail fibroblast α-tropomyosin mRNA is relatively large, 2 kb. This RNA size difference presumably reflects greatly differing lengths of untranslated sequence at the 5′- and/or 3′-ends.

Translation of the fibroblast RNA hybridizing to the cC102 cDNA clone produced two major fibroblast tropomyosins *1A* and *1B* (class 1 tropomyosins). This result indicates that cC102 RNA encodes either tropomyosin *1A* or *1B* (and suggests that along with cC102 RNA there is present in fibroblasts another 2-kb α-tropomyosin-related mRNA that encodes the other class 1 tropomyosin). (The protection of only one major S_1 nuclease-resistant cC102 probe fragment by fibroblast RNA (Fig. 2) implies either that the two class 1 tropomyosin mRNA species are indistinguishable in the regions represented by the cC102 probe, or that they differ in sequence at or near the labeled end of the probe so that hybridization of the cC102 probe with the second RNA species would result in no labeled S_1 nuclease-resistant product, or one that was undetectably short.) From the cDNA sequence the cC102 protein (either tropomyosin *1A* or *1B*) is identical to the major isoform of skeletal muscle α-tropomyosin from the beginning of the sequence at residue 75 to residue 257, but differs from it in the COOH-terminal region beginning at residue 258.

The cC102 protein sequence is unique among non-striated-muscle tropomyosins in its identity with skeletal muscle α-tropomyosin over an uninterrupted stretch of at least 183 amino acids, residues 75–257. Alternatively spliced non-striated-muscle tropomyosins thus far reported in mammals differ from the corresponding striated muscle isoforms not only in the COOH terminus, but also in discrete internal sequence blocks, residues 41–80 in a rat smooth muscle α-tropomyosin (Ruiz-Opazo *et al.*, 1985), and residues 188–213 in a human fibroblast β-tropomyosin (MacLeod *et al.*, 1985). The blocks of different "striated" and "nonstriated" sequences presumably correspond to alternatively spliced exons in the corresponding genes (MacLeod *et al.*, 1985; Ruiz-Opazo *et al.*, 1985). The presence of skeletal muscle α-tropomyosin sequences in the corresponding regions (where these can be compared) of the cC102 fibroblast tropomyosin suggests either that the quail α-tropomyosin gene lacks such alternative nonstriated exons, or that there is considerable flexibility in the choice of various alternative exon combinations, implying that a relatively large number of distinct transcripts and proteins could be produced from a single tropomyosin gene. Structural analysis of the quail α-tropomyosin gene, currently underway, will resolve this question.

The unprecedented structural similarity of the cC102 protein and skeletal muscle α-tropomyosin has implications for the structural correlates of striated muscle *versus* nonstriated-muscle tropomyosin functions. The chief site of troponin interaction on skeletal muscle tropomyosin is at or near the COOH terminus (Mak and Smillie, 1981; Pearlstone and Smillie, 1982). Here the cC102 protein and other nonstriated-

muscle tropomyosins (Helfman *et al.*, 1984; MacLeod *et al.*, 1985; Ruiz-Opazo *et al.*, 1985; Yamawaki-Kataoka and Helfman, 1985) differ from striated muscle tropomyosins. However, the tropomyosin sequence blocks 41–80 and 188–213 are also implicated in aspects of troponin binding (MacLeod *et al.*, 1985; Ruiz-Opazo *et al.*, 1985). The presence of skeletal muscle tropomyosin sequences in these blocks of the cC102 protein sequence (where the comparison can be made) implies that these particular amino acid sequences must act not only as troponin interaction sites in striated muscle tropomyosin, but also in the troponin-independent functions of the cC102 protein in fibroblasts.

Northern blotting and S_1 nuclease analyses show no appreciable accumulation of either fibroblast or skeletal muscle α-tropomyosin mRNAs in proliferating myoblasts. Consistent with the lack of accumulation of α-tropomyosin-related RNAs, myoblast lysates probed with the antitropomyosin antibody 3F9 showed very low amounts of tropomyosins *1A* and *1B*, although tropomyosins *2A* and *2B* were relatively abundant, as well as a tropomyosin of 37,000 daltons apparent molecular mass which is only a very minor component in fibroblast lysates (data not shown). Thus, myoblast tropomyosin expression differs from that of fibroblasts both in the reduced or absent expression of α-tropomyosin-related isoforms and in the increased expression of an additional isoform which is not transcribed from, or closely related to, the α-tropomyosin gene. This raises the possibility that the function of class 1 tropomyosins may be a fibroblast-specific one and not one of relevance to nonmuscle cells in general or to proliferating cultured cells in general.

The presence of abundant α-tropomyosin gene transcripts in fibroblasts and in differentiated muscle cells, but not in undifferentiated myoblasts, also has implications for α-tropomyosin gene regulation. Two possible developmental programs are consistent with these results: 1) α-tropomyosin gene expression is independently activated during myoblast differentiation and at some unknown point in the fibroblast cell lineage; 2) the gene is expressed in mesodermal precursors to both fibroblast and muscle cell lineages and continues to be expressed in the fibroblast lineage, but is repressed during the developmental establishment of proliferating myoblast cells and is later derepressed during myoblast differentiation. It should be an important experimental goal to determine which of these possible programs is actually operating, not only for what would be learned about tropomyosin gene regulation, but also because this may provide new insight into the molecular events associated with the establishment of embryonic mesodermal cell lineages.

Acknowledgments—We thank I. R. Konigsberg for his role in developing the antibody 3F9; L. B. Smillie, and A. R. MacLeod for providing information before publication; I. Althaus, M. Ober, and D. Robinson for technical assistance; and M. Erdelyi and R. Todd for preparing the manuscript.

REFERENCES

Alwine, J. C., Kemp, D. J., and Stark, G. R. (1977) *Proc. Natl. Acad. Sci. U.SA.* **74,** 5350–5354
Basi, G. S., Boardman, M., and Storti, R. V. (1984) *Mol. Cell. Biol.* **4,** 2828–2839
Berk, A. J., and Sharp, P. A. (1977) *Cell* **12,** 721–732
Billeter, R., Heizmann, C. W., Reist, U., Howald, H., and Jenny, E. (1981) *FEBS Lett.* **132** 133–136
Bonner, W. M., and Laskey, R. A. (1974) *Eur. J. Biochem.* **46,** 83–88
Bronson, D. D., and Schachat, F. H. (1982) *J. Biol. Chem.* **257,** 3937–3944
Carmon, Y., Neuman, S., and Yaffe, D. (1978) *Cell* **14,** 393–401
Chamley-Campbell, J., Campbell, G. R., Gröschel-Stewart, U., and Burnstock, G. (1977) *Cell Tissue Res.* **183,** 153–166
Cohen, I., and Cohen, C. (1972) *J. Mol. Biol.* **68,** 383–387
Coté, G. P. (1983) *Mol. Cell. Biochem.* **57,** 127–146
Devlin, R. B., and Emerson, C. P., Jr. (1979) *Dev. Biol.* **69,** 202–216
Fine, R. E., and Blitz, A. L. (1975) *J. Mol. Biol.* **95,** 447–454
Fine, R. E., Blitz, A. L., Hitchcock, S. E., and Mainer, B. (1973) *Nature (New Biol.)* **245,** 182–186
Fiszman, M. Y., Kardami, E., and Lemonnier, M. (1986) in *Molecular Biology of Muscle Development* (Emerson, C. P., Fischman, D., Nadal-Ginard, B., and Siddiqui, M. A. Q., eds) pp. 457–471, Alan R. Liss, New York
Giometti, C. S., and Anderson, N. L. (1984) *J. Biol. Chem.* **259,** 14113–14120
Hartshorne, D. J., and Mueller, H. (1969) *Biochim. Biophys. Acta* **175,** 301–319
Hastings, K. E. M., and Emerson, C. P., Jr. (1982a) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 1553–1557
Hastings, K. E. M., and Emerson, C. P., Jr. (1982b) in *Muscle Development: Molecular and Cellular Control* (Pearson, M., and Epstein, H., eds) pp. 215–224, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Hayashi, K. (1980) *Gene (Amst.)* **11,** 109–115
Hayashi, J., Ishimada, T., and Hirabayashi, T. (1977) *J. Biochem. (Tokyo)* **81,** 1487–1495
Helfman, D. M., Feramisco, J. R., Ricci, W. M., and Hughes, S. H. (1984) *J. Biol. Chem.* **259,** 14136–14143
Hendricks, M., and Weintraub, H. (1984) *Mol. Cell Biol.* **4,** 1823–1833
Izant, J. G., and Lazarides, E. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 1450–1454
Karlik, C. C., Mahaffey, J. W., Coutu, M. D., and Fyrberg, E. A. (1984) *Cell* **37,** 469–481
Konigsberg, I. R. (1979) *Methods Enzymol.* **58,** 511–527
Laemmli, U. K. (1970) *Nature* **227,** 680–685
Lazarides, E. (1975) *J. Cell Biol.* **65,** 549–561
Leger, J., Bouvert, P., Schwartz, K., and Swynghedauw, R. (1976) *Pfluegers Arch. Eur. J. Physiol.* **362,** 271–277
Lewis, W. G., Coté, G. P., Mak, A. S., and Smillie, L. B. (1983) *FEBS Lett.* **156,** 269–273
Lin, J. J.-C., Matsumura, F., and Yamashiro-Matsumura, S. (1984) *J. Cell Biol.* **98,** 116–127
Lin, J. J.-C., Helfman, D. M., Hughes, S. H., and Chou, C.-S. (1985) *J. Cell Biol.* **100,** 692–703
MacLeod, A. R. (1982) *Eur. J. Biochem.* **126,** 293–297
MacLeod, A. R., Houlker, C., Reinach, F. C., Smillie, L. B., Talbot, K., Modi, G., and Walsh, F. S. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 7835–7839
Mak, A. S., and Smillie, L. B. (1981) *J. Mol. Biol* **149,** 541–550
Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Marston, S. B., and Smith, C. W. J. (1985) *J. Muscle Res. Cell Motil.* **6,** 669–708
Maxam, A. M., and Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 560–564
Montarras, D., Fiszman, M. Y., and Gros, F. (1981) *J. Biol. Chem.* **256,** 4081–4086
Pearlstone, J. R., and Smillie, L. B. (1982) *J. Biol. Chem.* **257,** 10587–10592
Ricciardi, R. P., Miller, J. S., and Roberts, B. E. (1979) *Proc. Natl. Acad. U. S. A.* **76,** 4927–4931
Ruiz-Opazo, N., Weinberger, J., and Nadal-Ginard, B. (1985) *Nature* **315,** 67–70
Schloss, J. A., and Goldman, R. D. (1980) *J. Cell Biol.* **87,** 633–642
Seed, B. (1982) *Nucleic Acids Res.* **104,** 1799–1810
Smillie, L. B. (1979) *Trends Biochem. Sci.* **4,** 151–155
Squire, J. (1981) *The Structural Basis of Muscular Contraction*, Plenum Press, New York
Steinbach, J. H., Schubert, D., and Eldridge, L. (1980) *Exp. Neurol.* **67,** 655–669
Talbot, K., and MacLeod, A. R. (1983) *J. Mol. Biol.* **164,** 159–174
Taylor, E. W. (1979) *CRC Crit. Rev. Biochem.* **6,** 103–163
Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
Wahl, G. M., Stern, M., and Stark, G. R. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 3683–3687
Wehland, J., and Weber, K. (1980) *Exp. Cell Res.* **127,** 397–408
Yamawaki-Kataoka, Y., and Helfman, D. M. (1985) *J. Biol. Chem.* **260,** 14440–14445