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Fast Skeletal Muscle-Specific Expression of a Quail Troponin I Gene in Transgenic Mice

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We have produced seven lines of transgenic mice carrying the quail gene encoding the fast skeletal muscle-specific isoform of troponin I (TnI_f). The quail DNA included the entire TnI_f gene, 530 base pairs of 5′-flanking DNA, and 1.5 kilobase pairs of 3′-flanking DNA. In all seven transgenic lines, normally initiated and processed quail TnI_f mRNA was expressed in skeletal muscle, where it accumulated to levels comparable to that in quail muscle. Moreover, in the three lines tested, quail TnI_f mRNA levels were manyfold higher in a fast skeletal muscle (gastrocnemius) than in a slow skeletal muscle (soleus). We conclude that the cellular mechanisms directing muscle fiber type-specific TnI_f gene expression are mediated by *cis*-regulatory elements present on the introduced quail DNA fragment and that they control TnI_f expression by affecting the accumulation of TnI_f mRNA. These elements have been functionally conserved since the evolutionary divergence of birds and mammals, despite the major physiological and morphological differences existing between avian (tonic) and mammalian (twitch) slow muscles. In lines of transgenic mice carrying multiple tandemly repeated copies of the transgene, an aberrant quail TnI_f transcript (differing from normal TnI_f mRNA upstream of exon 2) also accumulated in certain tissues, particularly lung, brain, spleen, and heart tissues. However, this aberrant transcript was not detected in a transgenic line which carries only a single copy of the quail gene.

The expression of genes encoding striated-muscle contractile proteins is subject to a variety of developmental regulatory mechanisms which function at different stages of myogenesis and muscle maturation (5). Troponin I is a myofibrillar protein that plays a role in the Ca^{2+} regulation of striated-muscle contraction. Three troponin I isoforms are known, each specific for fast or slow skeletal or cardiac muscle. Each of the three isoforms is apparently encoded by a separate gene (34). The gene encoding the fast skeletal-muscle isoform of troponin I is also one of the diverse set of genes, including actin and myosin, which are coordinately activated when myoblasts fuse to form myofibers (1, 7–9, 15, 28). The regulatory mechanism directing the transcriptional activation of the TnI_f gene (3), and presumably of other muscle-specific genes, is intrinsic to the myoblast, since activation occurs in differentiating myoblast cultures in the absence of other cells, including nerve (15, 19, 22).

Although TnI_f gene activation during myoblast differentiation is muscle cell autonomous, the nervous system plays a major role in regulating the cell-specific expression of troponin I isoforms during the subsequent maturation of striated muscle. Embryonic avian cardiac muscle expresses skeletal as well as cardiac troponin I isoforms, but cardiac expression of the skeletal isoform(s) is repressed prior to hatching (31). Coexpression of skeletal and cardiac troponin I isoforms is also observed in cultured embryonic cardiac myocytes, in which skeletal troponin I expression can be experimentally repressed by coculture with nerves or by culture in the presence of nerve conditioned medium (32).

In the newborn rat, a large proportion of fibers in the soleus muscle coexpress both fast and slow troponin I proteins. During postnatal maturation, the fraction of coexpressing fibers drops essentially to zero, with the great majority of fibers in soleus muscles of mature animals expressing only the slow isoform (10). Denervation of the soleus in adult animals results in the reactivation of expression of the fast isoform in the majority of these fibers (11), indicating that expression in slow-muscle fibers is repressed by a motor neuron-dependent mechanism during maturation. Moreover, a fundamental role for motor innervation in regulating the fiber type-specific expression of troponin I isoforms has been established directly by cross-reinnervation of fast and slow rabbit skeletal muscles (2, 12).

Neural control mechanisms have also been demonstrated for a number of contractile protein isoform families, including myosin (reviewed in reference 24). However, studies of fast and slow myosin isoforms in developing aneural birds (6) and the identification of clonal populations of cultured embryonic skeletal muscle, which appear to be predetermined with respect to fast or slow myosin isoform expression (25), suggest that myoblast autonomous mechanisms may also play a role in fiber type-specific muscle gene expression. Although the possible involvement of myoblast autonomous mechanisms in the differential expression of fast and slow troponin I isoforms has not been explored, it is clear that a variety of distinct regulatory mechanisms, both endogenously programmed and exogenously controlled, direct the expression of troponin I genes during development and maturation.

To determine whether *cis*-regulatory elements direct the tissue-specific expression of TnI_f, we have produced transgenic mice carrying the cloned quail TnI_f gene (3). These mice were analyzed for expression of the quail gene in multiple tissues, including skeletal muscles differing in fast- and slow-muscle fiber content.

In all of the transgenic lines examined, a normally initiated and processed quail TnI_f mRNA was produced and accumu-

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lated predominantly in skeletal muscle. Furthermore, quail TnI_f mRNA accumulated to much higher levels in muscle composed predominantly of fast fibers than in muscle composed predominantly of slow fibers. These results indicate that *cis*-acting genomic elements, which have been functionally conserved since the evolutionary divergence of birds and mammals, play a role in the normal tissue-specific expression of the gene, including discrimination among striated muscle types.

Unexpectedly, a novel quail TnI_f RNA, identical to normal TnI_f mRNA in exons 2 through 6 but differing upstream of exon 2, was also observed in a variety of tissues from lines that carry multiple copies of the quail gene at the transgenic locus. However, one line, which carries only a single copy of the gene at the transgenic locus, did not display this expression pattern.

MATERIALS AND METHODS

Preparation of DNA for injection. Plasmid gC143 has been described previously (18). The quail genomic DNA fragment contained in this plasmid consists of a 6.4-kilobase-pair (kb) insert comprising the entire TnI_f gene, as well as 530 base pairs (bp) of 5'-flanking sequence and 1,500 bp of 3'-flanking sequence, inserted in the *Bam*HI site of pBR322. For microinjection, the plasmid was digested with *Eco*RI and *Sal*I to produce an approximately 7-kb fragment containing the entire genomic clone, as well as 375 and 275 bp of pBR322 sequence flanking the quail DNA at the 5' and 3' ends, respectively. This fragment was purified by agarose gel electrophoresis onto hydroxyapatite (Bio-Rad Laboratories) (30) and by Bio-Gel P-60 (Bio-Rad) gel filtration chromatography. For microinjection, the purified fragment was diluted to 40 ng/μl in 10 mM Tris hydrochloride (pH 7.5)–0.25 mM EDTA.

Production of transgenic mice. Fertilized pronuclear-stage eggs from matings of B6D2F1 or B6C3F1 mice were isolated from the oviducts and treated with hyaluronidase to remove follicle cells. Approximately 1 to 4 pl of DNA solution was injected into each pronucleus, and the eggs were transplanted into the oviducts of pseudopregnant mice and allowed to develop to term. These manipulations were performed as described previously (13). Mice were analyzed by Southern blot hybridization for incorporation of the gene (see below). Four original transgenic founder mice were identified. In one of the founder mice, 354, the quail TnI_f gene had integrated at a single locus, as determined by transmission of the transgene to approximately 50% of progeny. In the three remaining founder mice, 357, 379, and 392, the transgene was initially present at the multiple sites of insertion as indicated by Southern blotting and pedigree analysis. Southern blot analysis was used to monitor the segregation of these multiple transgenic loci in subsequent generations of breeding with B6D2F1 or B6C3FI mice, and two single-locus lines were derived from each of three founder mice. These lines were designated 357-16, 357-17, 379-13, 379-35, 392-6, and 392-23. All mice analyzed in this study were hemizygous for the quail gene.

DNA analysis. DNA was isolated from an approximately 1-cm piece of mouse tail by overnight digestion in 500 μl of 0.1 M EDTA–0.05 M Tris hydrochloride (pH 8)–0.5% sodium dodecyl sulfate with 500 μg of proteinase K per ml at 55°C. The digests were centrifuged to remove debris, and the supernatants were extracted once with equal volumes of phenol and chloroform and once with chloroform alone and then ethanol precipitated. The DNA was then suspended in 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA and analyzed by Southern blotting (20).

To estimate transgene copy number in the transgenic lines, reference standards, equivalent to 1, 5, 10, 25, and 50 copies of the transgene per mouse genome, were made by mixing between 20 and 1,000 pg of *Eco*RI-digested gC143 plasmid DNA with 10 μg of nontransgenic mouse tail DNA. The reference standards and 10 μg of tail DNA from each of the transgenic lines were boiled in 300 μl (total volume) of 1 N NaOH–2M NaCl, cooled on ice, and transferred to nitrocellulose membrane by using a slot-blotting apparatus (Bethesda Research Laboratories, Inc.).

All hybridizations were to either nick-translated (20) or random prime (by using a kit purchased from Boehringer Mannheim Biochemicals and following the instructions of the manufacturer) [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Corp.)-labeled gC143 DNA and were performed under high-stringency conditions including hybridization in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 42°C and washing in 0.1× SSC–0.1% sodium dodecyl sulfate at 65°C. Filters were exposed to Kodak X-AR film with Cronex Lightning-Plus intensifying screens (E. I. du Pont de Nemours & Co.) at −80°C for autoradiography.

S1 nuclease protection analysis. RNA was prepared from various tissues either by phenol and chloroform extractions or by a guanidinium thiocyanate procedure (20). S1 nuclease protection analysis was carried out essentially as described previously (4). A 1.8-kb *Hin*dIII restriction fragment of a cDNA clone encoding quail TnI_f, cC120 (15), or a 1.7-kb BglI restriction fragment of genomic clone gC143 was isolated on a DEAE membrane (Schleicher & Schuell, Inc.) by following the instructions of the manufacturer. The cC120 fragment includes 260 bp of TnI_f cDNA and approximately 1.5 kb of pBR322 vector DNA. Vector and insert cDNA are joined through approximately 30 G · C base pairs generated during cloning. The 1.7-kb gC143 fragment extended 5' from the exon 1 *BglI* site to a *BglI* site on pBR322. The fragments were 5' end labeled with [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham) and T4 polynucleotide kinase (Pharmacia, Inc.) (20). Labeled DNA (5×10^4 cpm [Cerenkov]) was hybridized to total RNA at 51°C (cC120) or 47°C (gC143) in 10 μl of 80% formamide–40 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES; pH 6.4)–0.4 M NaCl–1 mM EDTA for 15 h. The samples were then digested in 300 μl of a freshly prepared buffer of 0.2 M NaCl, 300 mM sodium acetate (pH 4.6), and 2 mM $ZnSO_4$ with 300 U (cC120) or 100 U (gC143) of S1 nuclease (Pharmacia) for 1 h at 37°C. Samples were phenol extracted, ethanol precipitated, and analyzed by electrophoresis on polyacrylamide gels containing 8 M urea (20). The gels were dried and exposed for autoradiography on Kodak X-Omat film.

Northern (RNA) analysis. RNA samples were electrophoretically separated on 1% agarose–formaldehyde gels (20) and transferred to Nytran membranes as recommended by the manufacturer (Schleicher & Schuell). The filters were hybridized to random prime-labeled cC120 and autoradiographed as above. They were then stripped of probe by repeated washings in 70% deionized formamide–0.3 M NaCl–0.06 M Tris hydrochloride (pH 8)–4 mM EDTA at 70°C and then reprobed with a random prime-labeled human skeletal actin cDNA clone, pHM-αA-1-actin (14).

RESULTS

Genetic analysis of transgenic mice. We have produced seven lines of transgenic mice by pronuclear microinjection.

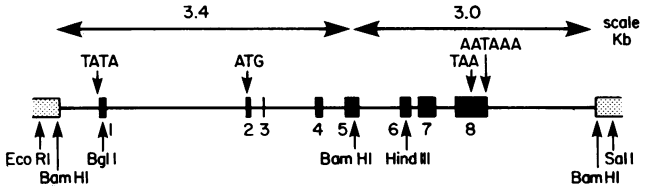

FIG. 1. gC143 DNA. The figure illustrates the quail TnI_f insert in gC143. Exons are shown (▬) and numbered 1 to 8. The TATA box (TATA), protein synthesis initiation (ATG) and termination (TAA) codons, and poly(A) addition signal (AATAAA) are indicated. *Bam*HI sites at the junctions between quail DNA and pBR322 DNA (▭) and in exon 5 are also indicated. The injected DNA included 375 bp of pBR322 vector sequence 5′ to the quail DNA and 275 bp of vector sequence 3′ to the quail DNA. The exon 1 *Bgl*I site and the exon 6 *Hin*dIII site used in labeling for S1 analysis are also indicated. (Other *Hin*dIII sites present in the quail DNA are not shown.)

The injected DNA fragment included the entire coding region of the quail TnI_f gene, flanked by 530 bp of 5′ DNA and 1,500 bp of 3′ DNA. The fragment also contained 375 bp of pBR322 DNA 5′ and 275 bp of pBR322 DNA 3′ to the quail DNA (Fig. 1). All seven lines carry insertions of intact TnI_f genes. Southern blots of *Bam*HI-digested DNA from each of the transgenic mouse lines were probed with gC143, the plasmid from which the injected DNA was derived (Fig. 2). All lines showed the expected major bands of hybridization at 3.4 and 3.0 kb, demonstrating the presence of unrearranged quail TnI_f DNA (Fig. 1). In addition, unique minor bands, presumably deriving from rearrangements affecting some of the incorporated genes and from the generation of transgene-mouse chromosomal DNA junctions, were observed in all lines except 357-17. With the exception of line 357-17, all of the lines also share a band of hybridization at 0.65 kb. This band also hybridizes with pBR322 DNA (data not shown). A 0.65-kb *Bam*HI fragment consisting

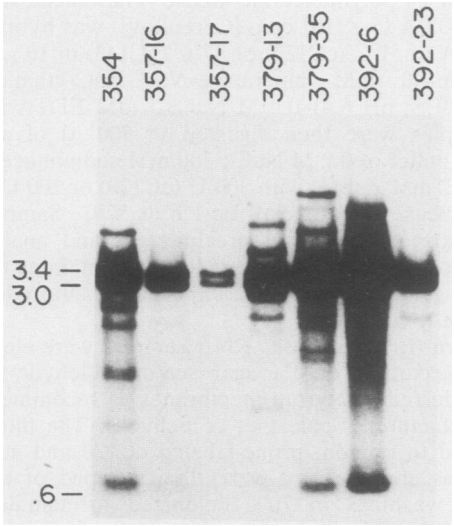

FIG. 2. Southern blot analysis of transgenic mouse lines. Approximately 10 μg of tail DNA from hemizygous mice from the indicated transgenic mouse lines was digested with *Bam*HI and analyzed by Southern blot hybridization with gC143. Fragments of approximately 3.4, 3.0, and 0.6 kb are indicated. Under the stringency conditions used there is no cross-hybridization between mouse and quail TnI_f genes.

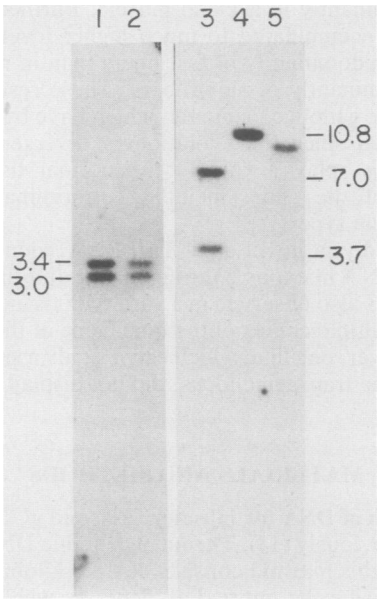

FIG. 3. Southern blot analysis of transgenic line 357-17. DNA (20 μg) from quail (lane 1) or of tail DNA from the 357-17 line (lanes 2 and 5) was digested with *Bam*HI (lanes 1 and 2) or *Bgl*II (lane 5) and analyzed by Southern blotting and hybridization with gC143 DNA. Marker DNA fragments were generated by digestion of 20 pg of gC143 plasmid in the presence of 20 μg of nontransgenic mouse tail DNA with *Eco*RI and *Sal*I (lane 3) or *Eco*RI (lane 4). Marker band sizes are in kilobases, as indicated.

entirely of pBR322 DNA would be generated by the joining of multiple copies of the injected DNA in head-to-tail tandem arrays, a structure typical of transgenic loci (23). The predicted internal junction fragment was also missing from line 357-17 DNA after digestion with *Pst*I or *Kpn*I, although in these digests, two pBR322 hybridizing bands, presumably deriving from transgene-mouse chromosomal DNA junctions, were detected (data not shown). The lack of an internal pBR322 junction fragment suggests that this transgenic locus does not contain head-to-tail tandem copies of the TnI_f gene, but, rather, a single copy. Moreover, restriction digestion of line 357-17 genomic DNA by *Bgl*II, an enzyme that does not have a recognition site in the injected DNA, reveals a single 9-kb band of hybridization (Fig. 3). This single fragment, which must derive from cleavage in mouse DNA on either side of the transgene, is too small to contain two copies of the injected 7-kb fragment.

Quantitative dot blot analysis was also consistent with the conclusion that line 357-17 contains a single copy of the quail gene at the transgenic locus. By this assay, the remaining lines contained between 5 and 30 copies of the quail TnI_f gene (data not shown).

Expression of TnI_f mRNA in transgenic mouse tissues. To determine whether the quail TnI_f gene is expressed in transgenic mice, we extracted RNA from various tissues of mature hemizygous transgenic mice and analyzed it by an S1 nuclease protection assay (Fig. 4). Control RNA samples from quail skeletal muscle (breast) and from nontransgenic mouse skeletal muscle (whole leg) were similarly analyzed. The hybridization probe was derived from a previously described quail TnI_f cDNA clone, cC120 (15). This clone includes sequences derived from exons 1 through 7, beginning 25 nucleotides from the proximal cap site (see below). cC120 was cut and 5′ end labeled at the exon 6 *Hin*dIII site

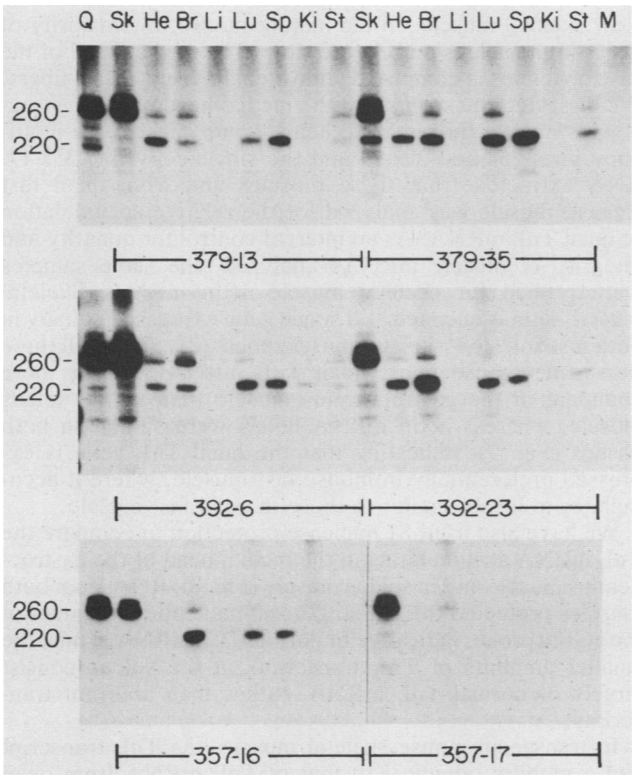

FIG. 4. S1 nuclease analysis of quail TnI_f gene expression in transgenic mouse tissues. S1 nuclease protection analysis of TnI_f expression was performed by using 30 μg of RNA from quail breast muscle (Q), transgenic mouse tissues including skeletal muscle (Sk), heart (He), brain (Br), liver (Li), lung (Lu), spleen (Sp), kidney (Ki), and stomach (St) and from nontransgenic mouse skeletal muscle (M) as indicated. The RNA was hybridized to a 5′-end-labeled, 1,852-bp fragment of cC120 (see Fig. 5) and digested with S1 nuclease. The resistant fragments were resolved on an 8% polyacrylamide–8 M urea gel. Tissues from six of the transgenic lines assayed are presented in the figure. 357-17 lanes were exposed for 4 days, whereas the corresponding quail control and 357-16 lanes were exposed for 24 h. Size estimates (see also Fig. 5) are in nucleotides.

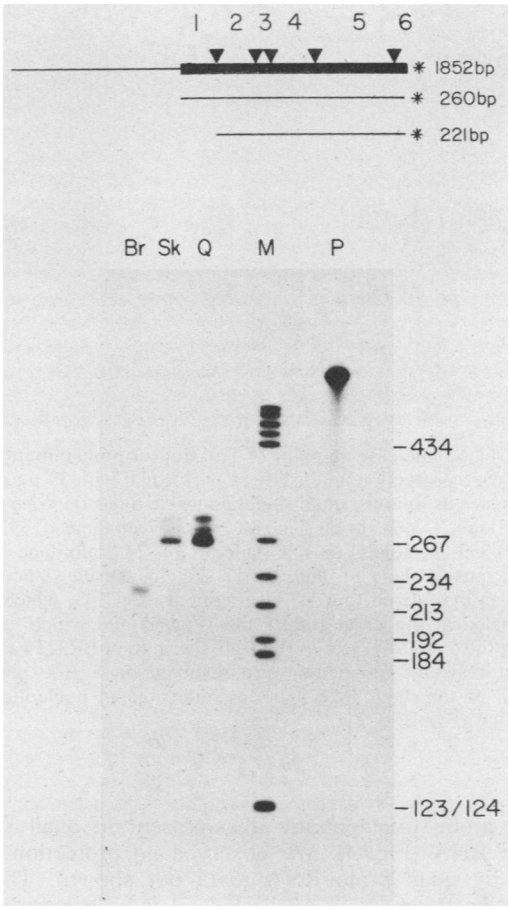

FIG. 5. Determination of S1 nuclease-resistant fragment sizes. RNA samples from quail breast muscle (Q), 354 transgenic skeletal muscle (Sk), and 354 transgenic brain (Br) were hybridized and digested as in Fig. 4 and were resolved on a 8% polyacrylamide–8 M urea gel. Size markers (M) are pBR322 DNA digested with *Hae*III, and band sizes are indicated in nucleotides. The quail TnI_f cDNA probe (P), cC120, used in the S1 assay is diagrammed: ▬, TnI_f cDNA sequence; —, pBR322 vector DNA. The locations of splice points (not to scale) are indicated by arrows, and corresponding exons are numbered as in Fig. 1.

(Fig. 1). This probe tests for complementarity from the 260 nucleotides upstream of the *Hin*dIII site, the full extent of the cDNA clone insert. Protection of a 260-nucleotide fragment of the probe indicates the presence of quail TnI_f mRNA which has been properly spliced between exons 1 and 6 inclusive (Fig. 5). Nontransgenic mouse skeletal muscle RNA shows no protection of this probe (Fig. 4).

In all transgenic lines, skeletal muscle RNA protected a 260-nucleotide fragment of the probe, identical to that protected by quail skeletal muscle RNA (Fig. 4 and 5). (The protection of fragments larger than 260 nucleotides by both quail and transgenic mouse skeletal muscle is presumably due to the presence of relatively S1 nuclease-resistant G tails at the junction of the cDNA insert and the pBR322 vector.) We conclude that normal quail TnI_f mRNA is accumulated in skeletal muscle of all seven transgenic mouse lines. Furthermore, mRNA levels observed in the transgenic skeletal muscle are roughly equivalent to those observed in quail skeletal muscle (Fig. 6).

In line 357-17, RNA protecting a 260-nucleotide fragment of the probe accumulated predominantly in skeletal muscle. A trace of the 260-nucleotide fragment was protected by brain RNA but not by RNA from other tissues (Fig. 4). Thus, a high degree of tissue specificity is exhibited by the TnI_f gene in this line.

A more complex pattern of expression was observed in lines of mice carrying multiple tandemly repeated copies of the gene. Five of these lines have been analyzed extensively. In addition to the 260-nucleotide fragment protected by skeletal-muscle RNA, a shorter probe fragment was protected by RNA from other tissues, with the exception of liver (Fig. 4). RNA providing protection of this fragment is present predominantly in brain, lung, spleen, and heart tissue and to a lesser extent in kidney and stomach tissue. RNA from these tissues also protected generally smaller amounts of the 260-nucleotide probe fragment.

The size of the shorter probe fragment was estimated to be 220 ± 3 nucleotides, a distance from the labeled *Hin*dIII site which corresponds to the junction of exons 1 and 2 (Fig. 5). This suggests that the RNA protecting the 220-nucleotide fragment is identical to normal TnI_f mRNA downstream from and including exon 2, but differs upstream from the normal boundary between exons 1 and 2. It should be noted that relatively low levels of RNA generating a 220-nucleotide

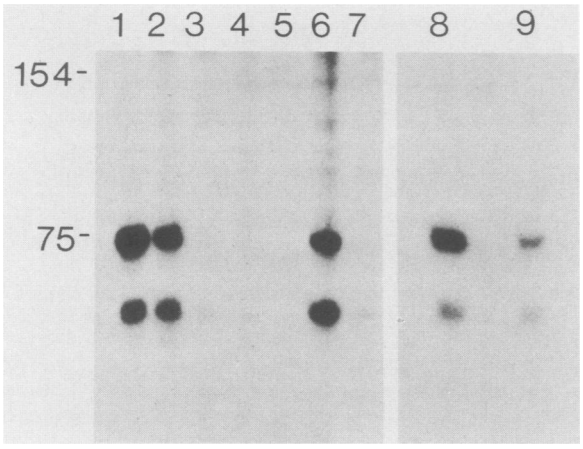

FIG. 6. S1 nuclease mapping of TnI_f transcription initiation sites. S1 nuclease protection analysis was performed with 30 μg of RNA from tissues as follows: quail skeletal muscle (lane 1), 392-6 skeletal muscle (lane 2), 392-6 brain (lane 3), 392-6 spleen (lane 4), 392-6 liver (lane 5), 392-23 skeletal muscle (lane 6), 392-23 brain (lane 7), quail skeletal muscle (lane 8), and 357-17 skeletal muscle (lane 9). The RNA was hybridized to a 1.7-kb fragment of gC143, which was 5′ end labeled at the exon 1 *BglI* site (Fig. 1) and which extended through the 5′ *Bam*HI site to the *BglI* site in the pBR322 vector. S1 nuclease-resistant fragments were resolved on a 10% polyacrylamide–8 M urea gel. Size markers, indicated in nucleotides, are *Hin*fI-digested pBR322.

cC120 probe fragment are also present in quail skeletal muscle RNA (Fig. 4). We observed no protection of the probe by quail brain RNA (data not shown). The 220-nucleotide fragment in quail skeletal muscle may indicate that similarly initiated and/or processed transcripts normally accumulate to low levels in that tissue. Alternatively, the fragment could derive from the presence of small amounts of processing intermediates of the normal primary transcript in which intron 1 has not yet been removed.

In quail skeletal muscle, transcription normally initiates from two functional RNA start sites 43 bp apart (17). To determine whether the TnI_f gene transcripts detected in transgenic mice are similarly initiated, we used an S1 nuclease protection assay with a cloned genomic DNA probe. The hybridization probe extended 1.7 kb 5′ from the *BglI* site in exon 1 of the quail gene (Fig. 1) and was derived from the plasmid gC143 by *BglI* digestion. When it was hybridized with quail skeletal muscle RNA, the predicted resistant fragments of 30 and 73 nucleotides were produced (Fig. 6). Identical fragments were protected by skeletal muscle RNA from both multicopy (392-23) and single-copy (357-17) lines, indicating that normal initiation is occurring in skeletal muscle in transgenic mice. Identical fragments were also protected, at low but readily detectable levels, by brain and spleen RNA, indicating that transcription in tissues other than skeletal muscle can initiate at the same sites used in skeletal muscle. Initiation at the normal TnI_f transcription start sites presumably contributes to the accumulation of the normal TnI_f mRNA detected at tissues other than skeletal muscle in Fig. 4. Whether the abnormal transcript also derives from initiation at these sites or whether it derives from transcription initiating elsewhere remains to be determined.

TnI_f mRNA is preferentially accumulated in fast skeletal muscle. We used Northern analysis to examine the capacity of the quail gene to direct expression in fast as opposed to slow skeletal muscle. In the mature mouse, the majority of fibers in the soleus muscle are slow. The medial head of the gastrocnemius is composed almost exclusively of fast fibers. We dissected the soleus and the medial head of the gastrocnemius muscle from mature hemizygous mice of the multicopy lines 354 and 392-23 and the single-copy line 357-17. RNA extracted from these muscles and from quail fast (breast) muscle was analyzed for the relative accumulation of quail TnI_f mRNA. As an internal control for quantity and integrity of muscle mRNAs analyzed, the same samples were probed for skeletal muscle actin mRNA. Skeletal muscle actin is encoded as a single gene expressed equally in both fast and slow skeletal-muscle fibers (21, 33). In all three transgenic mouse lines, quail TnI_f mRNA was far more abundant in the gastrocnemius muscle than in the soleus muscle, whereas actin mRNA levels were similar in both tissues (Fig. 7), indicating that the quail TnI_f gene is expressed preferentially in mouse fast muscle, where it accumulates to levels similar to those in quail fast muscle.

We have also used S1 nuclease protection to compare the TnI_f mRNA accumulating in the medial head of the gastrocnemius muscle and in soleus muscle (Fig. 8). RNA from both muscles protected the full-length 260-nucleotide fragment of the cC120 probe, indicative of normal TnI_f mRNA. Thus, the smaller amounts of TnI_f transcripts in the soleus consist largely of normal TnI_f mRNA, rather than aberrant transcripts.

In transgenic mouse skeletal muscle, the TnI_f transcript had a mobility identical to that of TnI_f mRNA from quail muscle in Northern analysis, and this, together with the protection observed by S1 nuclease analysis, is consistent with the normal transcription of the quail TnI_f gene, including initiation, processing, and polyadenylation, in mouse fast skeletal muscle.

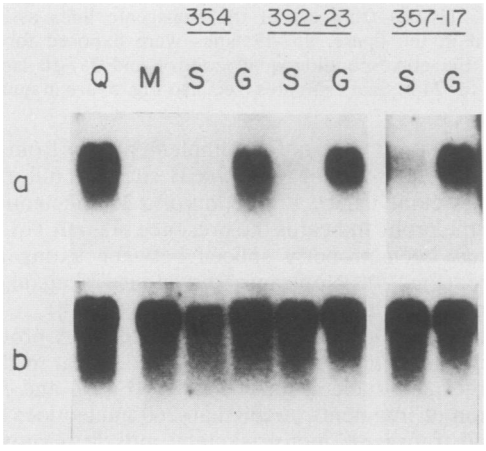

FIG. 7. Northern analysis of TnI_f mRNA accumulation in fast and slow skeletal muscles of transgenic mice. (a) RNA (40 μg) extracted from quail breast muscle (Q), from the soleus muscles (S), and from the medial heads of the gastrocnemius muscles (G) of mice from transgenic lines 357-17, 392-23, and 354 and from the gastrocnemius muscles of nontransgenic mice (M), as indicated, was electrophoresed through a 1% agarose-formaldehyde gel, transferred to Nytran, and hybridized to the quail TnI_f cDNA clone cC120. 357-17 lanes are overexposed (approximately fourfold) relative to the rest of the panel. (b) After the blot was stripped of detectable signal, it was reprobed with a human skeletal α-actin cDNA.

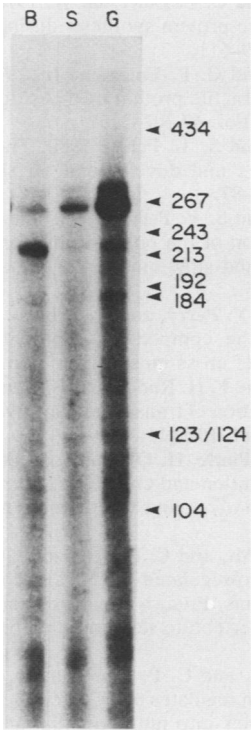

FIG. 8. S1 nuclease protection analysis of TnI_f expression in soleus and gastrocnemius RNA. RNA (4 μg) from 293-23 brain (B), soleus (S), and medial head of the gastrocnemius (G) was hybridized to the 5'-end-labeled 1,852-bp fragment of cC120 (described in Fig. 4 and 5) and digested with S1 nuclease. The resistant fragments were resolved as in Fig. 4. Size markers (in nucleotides) are *Hae*III-digested pBR322.

DISCUSSION

Konieczny and Emerson (18) have analyzed the capacity of the quail TnI_f gene to undergo transcriptional activation after transfection into differentiating mouse myoblast cell cultures. Analysis of deletion mutants in this system identified two functionally important regions. One of these, located within the 500 bp preceding the gene, influences quantitative levels of TnI_f mRNA accumulation in differentiated myoblasts. The second element, located within intron 1, is essential for activation during myoblast differentiation. Our results are consistent with these observations, since the 6.4-kb transgene contains these elements. The present investigation has extended this analysis to the in vivo regulation of the TnI_f gene with respect to both skeletal-muscle-specific expression and discrimination between skeletal-muscle fiber types in mature mice. These two aspects of tissue-specific expression apparently depend on distinct regulatory mechanisms, since the former is based on the endogenously programmed activation of gene expression during muscle differentiation and the latter depends, at least in part, on exogenous instruction from motor neurons.

Fiber type-specific expression of TnI_f. We have analyzed three lines of transgenic mice with respect to the fiber type-specific expression of TnI_f. We find that in all three lines, quail TnI_f mRNA accumulates to much higher levels in RNA from the gastrocnemius muscle than in RNA from soleus muscle. Endogenous mouse TnI_f mRNA shows a comparable preferential expression in gastrocnemius muscle (R. Koppe, personal communication). In the gastrocnemius, mRNA derived from the quail TnI_f gene accumulates to levels similar to those in quail fast muscle. Because the gastrocnemius and soleus muscles of mice are composed predominantly of fast and slow fibers, respectively, this result indicates that the transgene is subject to appropriate fast- versus slow-fiber type-specific regulation. Moreover, this fast versus slow regulatory mechanism mediates its effect by controlling mRNA levels, perhaps through effects on gene transcription. We conclude that fiber type-specific expression is based on *cis* elements which are present within the quail DNA fragment used in the present studies. These elements apparently can respond to signals, including motor innervation, which regulate TnI_f fiber type-specific expression in skeletal muscle. Our results indicate that the regulatory mechanisms in which these *cis* elements participate existed in the common ancestor of birds and mammals and have been functionally conserved in both evolutionary lineages.

A variety of studies (reviewed in reference 16) indicate that the slow postural muscles (such as the anterior latissimus dorsi) of birds exhibit features characteristic of a tonic class of muscle fibers. Tonic fibers are characterized by polyaxonal innervation and commonly respond to neural stimulation with graded local depolarization and gradual sustained contracture. With the exception of the extraocular and tensor tympani muscles, similar tonic fibers are unknown in mammals. In mammalian slow postural muscle fibers, such as in the soleus, the fibers are innervated at a single terminal and the neural impulse elicits a propagated action potential and a single-twitch contraction. These mammalian slow postural muscle fibers are often referred to as slow twitch fibers to draw attention to the differences between them and the tonic fibers that make up the slow postural muscles of birds (and other vertebrate classes). Despite these morphological and physiological divergences, our results suggest that homologous gene-regulatory mechanisms direct isoform-specific gene expression in bird and mammal slow postural muscle.

Skeletal muscle-specific expression of TnI_f. Our results clearly indicate that the 6.4-kb TnI_f fragment contains information necessary for normal tissue-specific expression in terms of fast versus slow skeletal muscle. Our results with respect to skeletal muscle-specific expression are more complex.

Shani has produced mice transgenic for genes encoding skeletal α-actin (27) and skeletal myosin light chain 2 (26) and has concluded that *cis* elements regulating muscle-specific gene expression are present in or near these genes. Similarly, in the single-copy line 357-17, the TnI_f transgene exhibits considerable specificity for skeletal muscle, with ectopic expression limited to trace amounts in the brain. This strongly suggests that the 6.4-kb TnI_f DNA fragment contains elements required to direct normal skeletal muscle-specific expression. In apparent conflict with this, we find that all of the multicopy lines express the gene in a variety of tissues other than skeletal muscle, albeit largely in the form of structurally aberrant molecules (identical to normal TnI_f mRNA in exons 2 through 6, inclusive, but differing upstream of exon 2). This expression in the multicopy lines suggests that the DNA in question lacks key regulatory elements that normally function to repress the gene in those tissues. However, for this hypothesis to be credible, the highly muscle-specific expression pattern seen in the single-copy line must be viewed as an artifact, possibly deriving from the specific site of chromosomal insertion in this line. Alternatively, the tandem arrangement of genes in the mul-

ticopy lines could artifactually permit or induce transcription in tissues in which it would not otherwise occur. Elements present in the injected DNA, which in the single-copy case are located 3′ (or 5′) to the gene, would, in a head-to-tail array, also be joined 5′ (or 3′) to the gene. Experimental juxtaposition of DNA fragments can result in novel tissue specificities in transgenic mice (29), and such a mechanism may account for our result. Regardless of the mechanism underlying widespread expression in multicopy lines, it is clear that the TnI_f gene is differentially expressed in fast and slow muscle, even under conditions where other aspects of normal gene regulation are compromised.

The unexpected expression of TnI_f RNA in tissues other than skeletal muscle offers intriguing possibilities for future analysis. Because the translation initiation codon is located in exon 2 (3), the aberrant transcripts may be capable of directing the synthesis of the TnI_f protein in tissues other than skeletal muscle, including heterologous striated-muscle tissues (heart), smooth-muscle tissues (stomach), and nonmuscle tissues (brain and lung). This could provide novel experimental possibilities for studies of the role of troponin I isoforms (or functionally related proteins) in various muscle and nonmuscle cells. Finally, it will be interesting to determine the mechanism responsible for novel expression seen in the majority of transgenic lines. Such a mechanism may have implications for our understanding of both the evolutionary establishment of differentially regulated multigene families and the range of regulatory phenomena to which experimentally introduced genes may be subject.

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