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Complex Regulation of the Muscle-Specific Contractile Protein (Troponin I) Gene

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A cloned quail troponin I contractile protein gene, stably transfected into a mouse myogenic cell line, exhibits appropriate developmental activation and quantitative expression during myoblast differentiation. Deletion mutagenesis analyses reveal that the troponin I gene has two distinct *cis* regulatory elements required for its developmental expression, as measured by mRNA accumulation and nuclear runoff transcription assays. One element in the 5′ flanking region is required for maximum quantitative expression, and a second larger regulatory element (1.5 kilobases) within the first intron is responsible for differentiation-specific transcription. The upstream region is highly sensitive to negative repression by interaction with pBR322 sequences. The larger intragenic region retains some activity when moved to the 5′ and 3′ flanking regions and when inverted but is maximally active in its native intragenic site. The concerted activities of these two regulatory regions produce a 100- to 200-fold transcriptional activation during myoblast differentiation. The conserved 5′ exon-intron organization of troponin I and other contractile protein genes suggests a possible mechanism by which intragenic control elements coordinate contractile protein gene regulation during skeletal myogenesis.

Transcription of eucaryotic structural genes requires promoter elements such as the TATA box, the CCAAT box, and the GGGCGG box, which are essential for accurate and efficient transcription initiation (reviewed in references 9 and 12). In addition, the activity of eucaryotic genes often is modulated by enhancer elements located at some distance from the promoter elements. Promoter elements and enhancers also may play a role in the developmental regulation of tissue-specific genes that become activated and transcribed at specific rates during the differentiation of specialized cell types (7, 17, 20, 56, 59). The complexity of control elements required to carry out the more precise developmental regulation of tissue-specific genes and to coordinate the transcriptional regulation of sets of functionally related genes within a specific tissue has yet to be defined.

Skeletal muscle provides an opportunity to investigate mechanisms that control and coordinate the developmental activation of tissue-specific genes. The multipotential embryonic cell line C3H10T1/2 can be converted into stable lineages of skeletal muscle precursor cells, fat precursor cells, and cartilage precursor cells, providing a system for the examination of the genetic regulation of muscle cell lineage determination and the establishment of the control system responsible for coordinate contractile protein gene activation during differentiation (26, 27, 57). Our earlier studies demonstrated that although the developmental processes of muscle cell lineage determination and muscle differentiation can be dissociated, myoblast lineage determination is necessary for troponin I (TnI) muscle-specific gene expression (26–28). Transfected TnI genes become activated during myoblast differentiation and are expressed at rates similar to those of endogenous muscle genes regardless of whether transfected genes are introduced initially into multipotential 10T1/2 cells or into determined myoblast cells committed to muscle differentiation. In an earlier study we established that the muscle-specific expression of the TnI gene involves *cis*-acting regulatory elements located within 530 base pairs (bp) of its 5′ end and 1.5 kilobases (kb) of its 3′ end (28).

To investigate the molecular mechanisms that regulate and coordinate contractile protein genes during skeletal muscle differentiation, we have undertaken to investigate the *cis* regulatory elements responsible for the transcriptional activation of a quail fast skeletal TnI gene during myoblast differentiation. In this study we show that a cloned quail TnI gene, including 5′ and 3′ flanking sequences and all of its intragenic sequences, is regulated equivalently to an endogenous TnI gene in quail myogenic cells when it is stably transfected at a low gene copy number (one to five copies per cell) into the 10T1/2-derived mouse myogenic cell line 23A2 (27, 28). This quail TnI gene was then subjected to systematic deletion mutagenesis and sequence rearrangements to localize and characterize the control elements responsible for its appropriate developmental regulation. The transcriptional regulation of transfected wild-type (and reference genes) and mutated genes was assayed by nuclear runoff transcription (18) and by S1 hybridization (3) to measure mRNA accumulation. The effects of mutations on TnI gene expression in myoblasts and myofibers were quantitated by analysis of the expression of a cotransfected reference gene or expression of endogenous mouse contractile protein genes or both. With this approach, a low number (one to five copies) of TnI genes could be stably incorporated into the genomes of transfected cells, and specific position effects of the transfected TnI genes in the mouse genome could be minimized by pooling 500 independently derived colonies for RNA analysis in each experiment.

Our experiments demonstrate an unexpected complexity in the regulatory elements required for TnI gene activation during myoblast differentiation. The proper developmental regulation of the quail TnI gene requires at least two regulatory elements that function in a concerted fashion to produce the normal 100- to 200-fold activation in TnI gene expression as proliferating myoblasts differentiate to form myofibers. One regulatory region, located in the 5′ flanking sequence, is required for proper quantitative expression and

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can be drastically repressed by plasmid sequences. Another regulatory region, located in the first intron, appears complex since it is large (1.5 kb); although it can be moved and inverted in orientation, it functions optimally in its native intragenic position. Our findings on this TnI gene, when compared with those on other muscle genes, suggest that intragenic regulatory elements as well as elements present in the 5′ flanking sequences may be responsible for the coordinated regulation of the contractile protein gene set.

MATERIALS AND METHODS

Materials. Restriction endonucleases, exonuclease III, DNA polymerase I (Klenow), T4 DNA ligase, S1 nuclease, T4 polynucleotide kinase, and calf intestinal alkaline phosphatase were purchased from New England Biolabs, Inc. (Beverly, Mass.), Pharmacia Fine Chemicals (Piscataway, N.J.), or Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Cell culture supplies were purchased from GIBCO Laboratories (Grand Island, N.Y.), and radioactive nucleotides [α-^{32}P]dCTP, [α-^{32}P] UTP, and [γ-^{32}P]ATP were purchased from New England Nuclear Corp. (Boston, Mass.) or Amersham Corp. (Arlington Heights, Ill.).

Wild-type plasmid. Plasmid gC143 (Fig. 1) consists of the quail TnI gene, containing eight exons, plus 530 bp of 5′-flanking DNA and approximately 1.5 kb of 3′-flanking DNA cloned into the *Bam*HI site of pBR322. The *Bam*HI sites flanking the insert were destroyed by Klenow polymerase fill-in (32) to produce clone gC149. The complete sequence of this clone (previously referred to as pTnISB) has been reported (1).

5′ deletion constructions. The subclone gC144 contains the 5′ *Sal*I-*Bam*HI fragment of gC149 and was used in the initial deletion experiments. gC144 was linearized by *Sal*I digestion, treated with exonuclease III for various amounts of time and blunt ended with S1 nuclease. *Sal*I linkers were ligated to the blunt-ended plasmids, and *Escherichia coli* DH5 was transformed by standard protocols. Recombinants containing deletions were identified by restriction endonuclease digestion, and inserts were subcloned into pEMBL18 (10) to facilitate dideoxy sequencing (51) of the deletion boundaries. In addition, inserts were exchanged with the wild-type *Sal*I-*Bam*HI fragment in gC149 for use in the transfection studies described below.

3′ deletion constructions. 3′ deletions were produced by restriction endonuclease digestion. Numbers preceded by + or − refer to map positions. Plasmid gC143 was digested with *Sal*I-*Eco*RI (−530 through +5870, wild type), *Bam*HI (−530 through +2842), *Bam*HI-*Sac*I (partial digest; −530 through +2680), *Bam*HI-*Bcl*I (−530 through +2470), *Bam*HI-*Sac*I (partial digest; −530 through +1825), *Bam*HI-*Stu*I (−530 through +1260), *Bam*HI-*Kpn*I (−530 through +920), and *Bam*HI-*Sac*I (−530 through +240). Each correct linear fragment was gel purified and quantitated before transfection.

Internal deletion constructions. Internal deletions were generated with the subclone gC144 and restriction endonuclease digestion. ΔA (+240 through +1825) and ΔB (+1825 through +2680) were produced by ligating partial *Sac*I digests. ΔA/B (+240 through +2680) was produced as above with a complete *Sac*I digest. These subclones were substituted into gC149 producing complete TnI genes containing the specified internal deletions. ΔA1, ΔA2, and ΔA3 were produced as above by using *Sac*I (partial)-*Kpn*I, *Kpn*I-*Stu*I, and *Stu*I-*Sac*I (partial) digests, respectively, except that the fragments were blunt ended with Klenow polymerase before ligation. A similar strategy was used to produce the ΔA1/A2 and ΔA2/A3 constructs.

The position of the internal regulatory region was changed by first subcloning the +240 through +1825 sequences into the *Sac*I site of the vector pUC19 in both orientations. The ΔA construct was digested with *Sal*I-*Eco*RI, pBR322 was digested with *Sal*I-*Hin*dIII, and the pUC19 subclones were digested with *Eco*RI-*Hin*dIII. A three-part ligation produced a ΔA gene containing the +240 through +1825 sequences positioned in both orientations in the pBR322 *Eco*RI site, which is 3′ to the TnI poly(A) addition site. A similar strategy was employed for positioning these sequences at the 5′ end of the −530 through +1 promoter sequences. In this instance, ΔA was digested with *Sal*I-*Eco*RI, pBR322 was digested with *Eco*RI, and the pUC19 subclone was digested with *Eco*RI-*Sal*I. A three-part ligation produced a ΔA gene containing the +240 through +1825 sequences positioned in both orientations in the pBR322 *Sal*I site, which is present 5′ to the −530 through +1 sequences of the TnI gene. Finally, the +240 through +1825 sequences were reinserted into the ΔA/B gene by using the unique *Sac*I sites present for each fragment. In this instance, the internal sequences were placed in a 3′-to-5′ orientation. Each of the above constructs was utilized in transfection experiments as described below.

Cell culture and DNA transfection. The myogenic mouse cell line 23A2 was used in all experiments. The growth and differentiation properties of this cell line have been extensively studied and were reported previously (27). DNA transfections were performed by the calcium phosphate precipitation method of Wigler et al. (60) as modified by Konieczny and Emerson (28). Samples of 30 ng of pKO-NEO plasmid DNA (8, 13, 55), 250 to 500 ng of test and reference plasmid DNA (in equal molar amounts), and 30 μg of 23A2 genomic DNA were used per plate of 23A2 cells. At 24 h after transfection, each plate was subcultured into five gelatinized 100-mm plates containing medium supplemented with 400 μg of geneticin (G418, GIBCO) per ml. Approximately 300 to 500 individual G418-resistant colonies were pooled, and 23A2 myoblast and 23A2 myofiber RNA or nuclei were isolated as described previously (18, 28).

Gene expression analysis. Southern blot hybridization, RNA and nucleus purification, S1 protection analysis (3), and nuclear runoffs (18) were performed exactly as described previously (28), with the following exceptions. A cDNA S1 probe was constructed that contains a homologous quail TnI cDNA (19) containing sequences corresponding to exons 1 through 5 of the quail TnI gene. The unique *Bam*HI restriction site present in this clone corresponds to the *Bam*HI site found in the fifth exon of the genomic clone. This site was end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (32) and used in an S1 hybridization assay. The mRNA produced by the wild-type TnI gene protects a 200-nucleotide portion of the cDNA probe. ΔA, ΔB, and ΔA/B genes produce 149-, 109-, and 109-nucleotide fragments, respectively, and ΔA1, ΔA2, and ΔA3 genes produce 200-, 200-, and 149-nucleotide fragments, respectively. For each experiment, individual S1 protected fragments were excised from the acrylamide-urea gel and quantitated by scintillation counting (28). All samples were normalized to control reference plasmid signals as well as to control background levels in each experimental lane (see below). In some experiments, values were reconfirmed by scanning Kodak XAR films that were exposed without intensifying screens and maintained within the linear range of the film.

Nuclear runoff assays were performed as described previously (28). Briefly, nuclear transcription reactions were

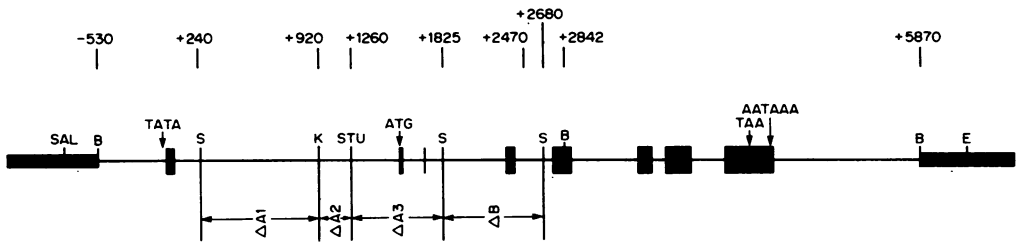

FIG. 1. Structure of the quail fast TnI gene showing exon (■) and intron (——) organization. Thick lines (▬) represent pBR322 plasmid sequences. TATA and AATAAA are the Goldberg-Hogness TATA box and poly(A) addition consensus sequences, respectively. Initiation (ATG) and termination (TAA) codons as well as a few restriction endonuclease sites (SAL, *Sal*I; B, *Bam*HI; S, *Sac*I; K, *Kpn*1; STU, *Stu*I; E, *Eco*RI) are indicated. The numbers above the figure represent the nucleotide positions of individual deletions as described in the text. ΔA1, ΔA2, ΔA3, and ΔB correspond to areas of the TnI gene that have been deleted for individual experiments.

incubated at 27°C for 35 min, and ^{32}P-labeled transcripts were hybridized to isolated DNA fragments (TnI, skeletal α-actin, pKO-NEO) that were applied directly to nitrocellulose by using a slot blot apparatus (Schleicher & Schuell Co., Keene, N.H.) or hybridized to restricted fragments that were electrophoresed on agarose gels and then transferred to nitrocellulose (28). Although the α-actin cDNA clone (36) will hybridize to cytoplasmic and cardiac actin mRNAs, we have found that under stringent hybridization conditions (28) the predominant signal is muscle specific. The relative transcriptional level for the S1 analysis, calculated as described by Myers et. al. (40), is equal to $(M/R_m)/(W/R_w)$, where M is the mutant test signal, W is the wild-type test signal, and R is the reference signal per each transfection and assay. Similar values were obtained for the nuclear runoff data with signals generated by the transfected pKO-NEO gene or the endogenous mouse α-actin gene or both (36). See below for specific details.

RESULTS

Myofiber-specific expression of 5′ promoter deletion constructions. A 6.4-kb quail fast TnI contractile protein gene (Fig. 1), including 530 bp of 5′ flanking sequence and 1.5 kb of 3′ flanking sequence, is developmentally regulated when transfected into the mouse myogenic cell line 23A2 (28). To examine the role of 5′ flanking sequences in the myofiber-specific activation of the quail TnI gene, a series of 5′ deletion mutants was produced. Individual 5′ deletions were cotransfected into 23A2 myoblasts with the selectable marker gene pKO-NEO (13). Initially, each 5′ deletion construct was transfected with and without pBR322 sequences (see Materials and Methods for details) to test for possible pBR322 position effects on TnI gene expression. G418-resistant transfected lines were pooled, and the expression of quail TnI was monitored in cultures of proliferating myoblasts and cultures of differentiated myofibers by S1 analysis and nuclear runoff assays. The results of these experiments revealed dramatic inhibitory effects of pBR322 when positioned near the TnI transcription start site. For example, clone −343 did not accumulate detectable levels of TnI message in myofiber cells when linked to pBR322 but expressed high levels of TnI when the pBR322 sequences were absent (Fig. 2A). The expression of TnI genes containing other deletions in this region also was inhibited by pBR322 sequences. The inhibition of expression by plasmid sequences was at the transcriptional level, as shown by nuclear runoff assays of myofiber nuclei (Fig. 2B). These data prompted us to repeat a series of transfection experiments with 5′ deletion constructions in which all pBR322 sequences were removed. In addition, we cotransfected 23A2 myoblasts with a TnI reference gene (see Materials and Methods for details) to normalize for transfection efficiency and myofiber differentiation in each experiment. All 5′ deletion constructions (lacking pBR322 sequences) expressed high levels of TnI message (Fig. 3). TnI genes containing only 95 nucleotides of 5′ flanking DNA still

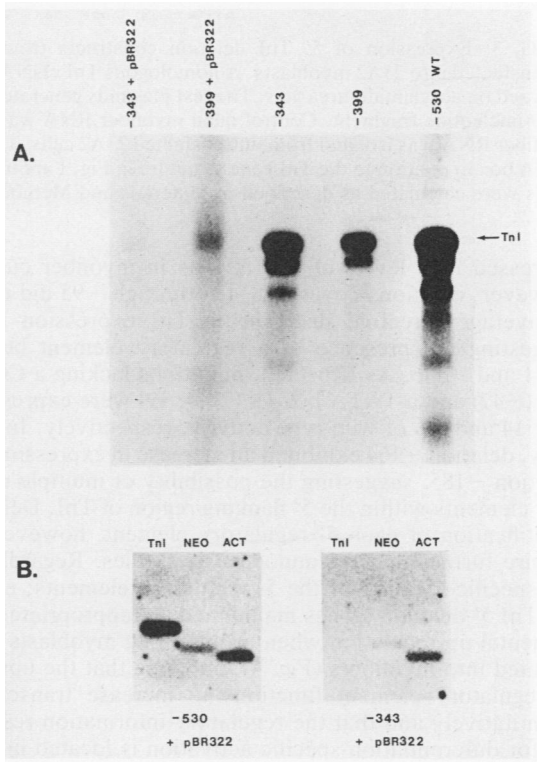

FIG. 2. (A) S1 analysis of myofiber RNA isolated from 23A2 transfected cells. 5′ TnI deletion constructs were transfected with (+) or without pBR322 sequences attached; myofiber RNA was isolated and hybridized to a TnI cDNA end-labeled probe, generating a 200-nucleotide S1-protected fragment. The presence of pBR322 sequences greatly inhibits the stable accumulation of TnI mRNA. (B) Transcription in isolated myofiber nuclei of 5′ TnI deletion genes transfected with pBR322 sequences attached. ^{32}P-labeled nuclear transcript products were isolated and hybridized to a nitrocellulose blot containing TnI, pKO-NEO (NEO), and mouse skeletal α-actin (ACT) gene sequences separated by agarose gel electrophoresis. The −343 deletion construct does not exhibit detectable TnI transcription in myofiber nuclei when transfected with pBR322 sequences attached.

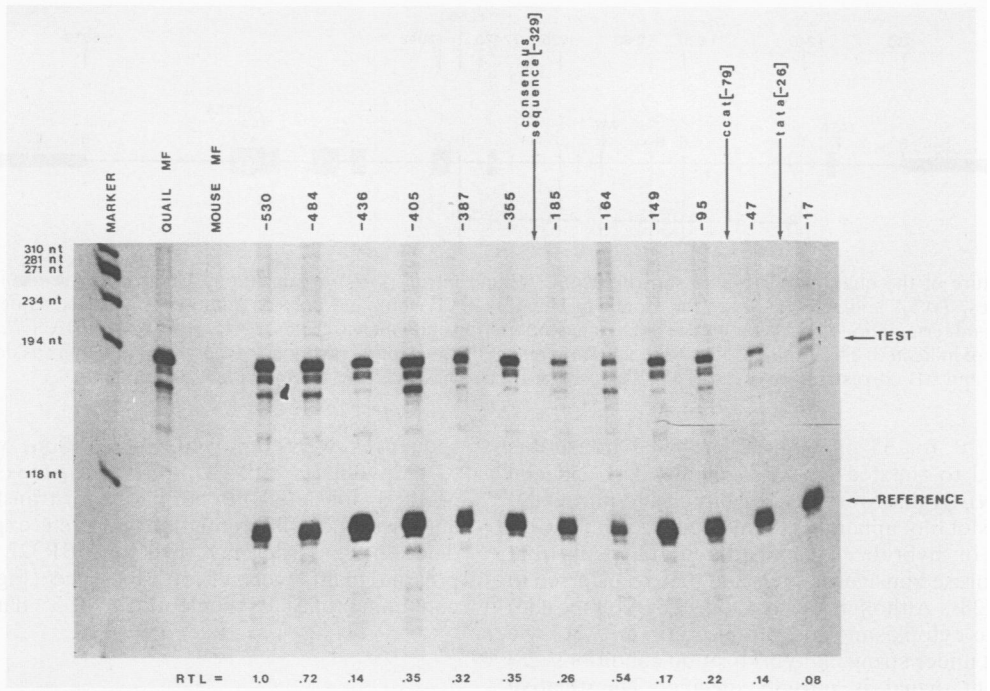

FIG. 3. Expression of 5′ TnI deletion constructs (transfected without pBR322 sequences attached) and a reference TnI plasmid cotransfected into 23A2 myoblasts. A homologous TnI cDNA probe was hybridized to myofiber mRNA, and the S1 protected fragments were analyzed on acrylamide-urea gels. The test plasmids generated a protected 200-nucleotide fragment, whereas the reference plasmid generated a 109-nucleotide fragment. Control quail myofiber RNA was isolated from differentiated quail skeletal muscle cultures, and control mouse myofiber RNA was isolated from untransfected 23A2 cells. The positions of a contractile protein gene consensus sequence, CCAAT box, and TATA box in relation to the TnI gene structure in Fig. 1 are indicated. Size marker DNA is from a ϕX174 *Hae*III digest. Relative transcription levels were calculated as described in Materials and Methods and are given for each test construct.

expressed high levels of TnI mRNA in myofiber cultures. However, deletions covering −436 through −95 did exhibit an average threefold decrease in TnI expression levels, suggesting the presence of a regulatory element between −484 and −436. As expected, mutations lacking a CCAAT box (−47) and a TATA box (−17) (9, 12) were expressed at only 14 and 8% of wild-type activity, respectively. Interestingly, deletion −164 exhibited an increase in expression over deletion −185, suggesting the possibility of multiple regulatory elements within the 5′ flanking region of TnI. Definitive identification of each 5′ regulatory element, however, will require further in vitro mutagenesis studies. Regardless of the specific location of the 5′ regulatory elements; each of the TnI 5′ deletion clones maintained the appropriate developmental up-regulation when proliferating myoblasts differentiated into myofibers (Fig. 4), implying that the upstream 5′ regulatory element functions to increase transcription quantitatively and that the regulatory information responsible for differentiation-specific activation is located in the 3′ TnI flanking sequences or within the TnI gene itself.

Myofiber-specific transcription of 3′ deletion constructions. To examine whether the 3′ end of the TnI gene has differentiation-specific control sequences, a series of 3′ deletion constructions was produced by restriction endonuclease digestion. Linear truncated TnI genes (lacking pBR322 sequences) were transfected into 23A2 myoblasts, and stable transformant lines were pooled. Intact nuclei were isolated from proliferating myoblasts and differentiated myofibers, and nascent TnI RNA transcripts were analyzed by nuclear runoff transcription assays. Linear TnI constructs containing −530 through +5870 (wild type) and −530 through +2842 were not expressed in myoblast nuclei (28; data not shown) but did exhibit similar myofiber-specific transcription rates in differentiated cells (Fig. 5). Identical results were obtained for TnI constructs containing −530 through +2470 (data not shown). Conversely, TnI constructs containing −530 through +1260 produced weak TnI transcription signals in myofiber nuclei. No TnI transcription was detected in TnI constructs containing −530 through +240. We conclude that essential regulatory information associated with the quail TnI gene resides within the +240 through +2470 region. In addition, the 3′ end of the TnI gene (+2470 through +5870), although likely important for the stability of the message (reviewed in references 4 and 49), does not play an essential role in the developmentally regulated activation of the TnI gene.

Internal deletions effect the expression of the TnI gene. To localize the intragenic regulatory regions of the quail TnI gene, a series of internal TnI deletions was produced. Initially, three large areas were removed from the TnI gene. These spanned the regions +240 through +1825 (ΔA), +1825 through +2680 (ΔB), and +240 through +2680 (ΔA/B) (Fig. 1). These three internal deletions, plus a reference wild-type TnI gene, were used to cotransfect 23A2 myoblasts, and mRNA extracted from myoblasts and myofibers was analyzed by S1 hybridization. Although the reference gene was expressed to high levels in myofiber cultures, the ΔA and ΔA/B constructs were expressed at only 6 and 7% of the wild-type level, respectively (Fig. 6, Table 1). In contrast, ΔB exhibited normal developmental regulation as evidenced by the 100- to 200-fold increase in message levels measured as proliferating myoblasts differentiated into myofibers (Fig. 6). ΔA produced only a three- to fourfold increase in TnI expression as 23A2 myoblasts differentiated into myofibers, in support of our hypothesis

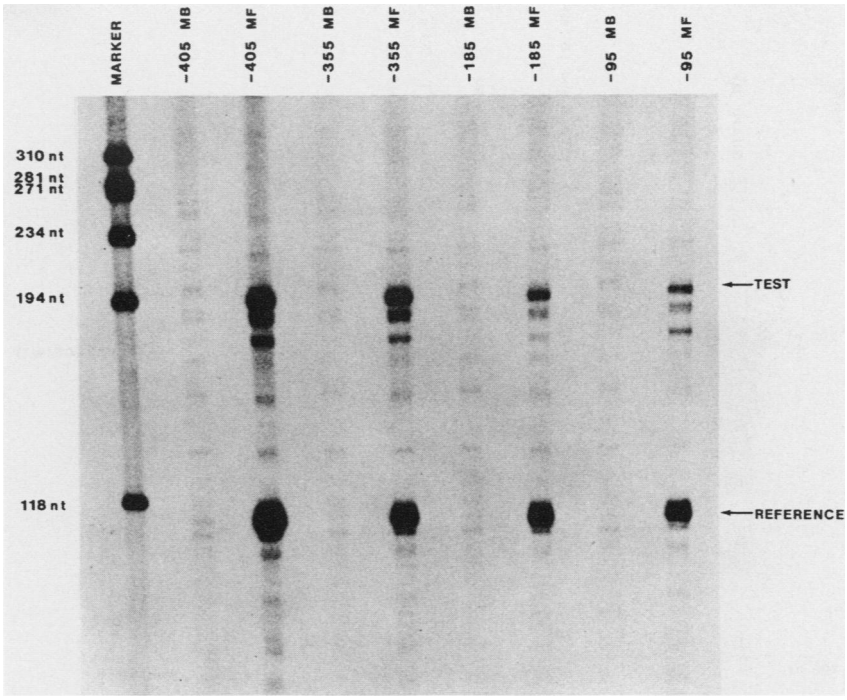

FIG. 4. S1 analysis of mRNA isolated from TnI-transfected undifferentiated myoblasts and differentiated myofiber cultures. 23A2 cells were cotransfected with different 5′ deletion test plasmids and a TnI reference plasmid as indicated. S1 hybridization was performed as described in the legend to Fig. 3. All 5′ deletion constructs and the reference TnI gene exhibit a myofiber-specific expression pattern.

that an essential regulatory region is present internal to the TnI gene. This internal region is required for both the proper developmental regulation and the proper quantitative level of TnI expression.

To delineate further the regulatory regions present within the +240 through +1825 region of the TnI gene, three smaller internal deletions were constructed: ΔA1 covering +240 through +920, ΔA2 spanning +920 through +1260, and ΔA3 deleting +1260 through +1825 (Fig. 1). These deletions were cotransfected with a reference gene (ΔB) into 23A2 myoblasts and examined by S1 hybridization for their developmental up-regulation in myofibers. TnI genes containing the deletions ΔA1 and ΔA1/A2 were expressed at 1 and 2%, respectively, of the level that the wild-type genes exhibited in 23A2 myofiber cultures (Fig. 7, Table 1). TnI genes containing the deletion ΔA2 or ΔA3 alone were expressed at higher levels in myofibers, but still at only 6% the level observed with the reference gene. These values correlate well with our previous data obtained with the ΔA deletion construct. Our results indicate that the regulatory domain associated with this internal TnI region is large, covering about 1.5 kb. This domain may include multiple elements, one within the ΔA1 region and a second or third regulatory element (or both) within (or shared between) the ΔA2 and ΔA3 regions. Alternatively, the entire ΔA region may function as a single regulatory element.

Internal deletion constructions exhibit a reduced transcriptional activity in myofiber nuclei. TnI genes containing unique internal deletions spanning +240 through +1825 accumulated only 1 to 7% of the level of wild-type TnI messages. There are a number of explanations for not detecting mature transcripts from these genes. Removal of internal intron sequences may affect the splicing or stability of the TnI message in these cells, although this seems improbable since each of our internal deletions maintained the normal 5′ and 3′ splice junctions (39) as well as the consensus lariat

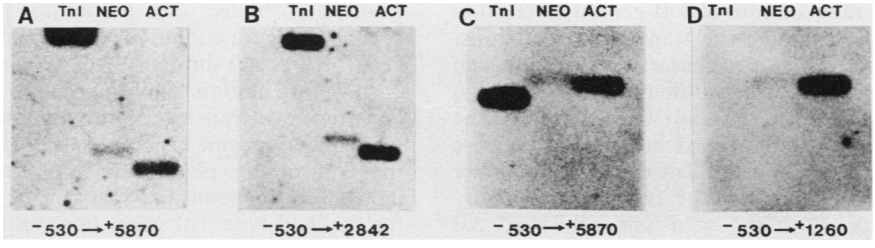

FIG. 5. Transcription analysis of 3′ TnI deletion constructs transfected into 23A2 myoblasts. Myofiber nuclei were isolated from transfected cultures, and ^{32}P-labeled transcripts were hybridized to specific DNA fragments separated on agarose gels and immobilized on nitrocellulose blots. TnI (either a 3.4-kb fragment [A, B] or a 770-bp fragment [C, D], pKO-NEO (NEO), and mouse skeletal α-actin (ACT) DNA sequences were used for the hybridization as described previously (28). All constructs contained 530 bp of 5′ flanking sequences as well as the indicated amounts of TnI genomic sequences (Fig. 1). Clones containing less than 1,260 nucleotides of 3′ sequences are not expressed in myofiber nuclei (see the text for details).

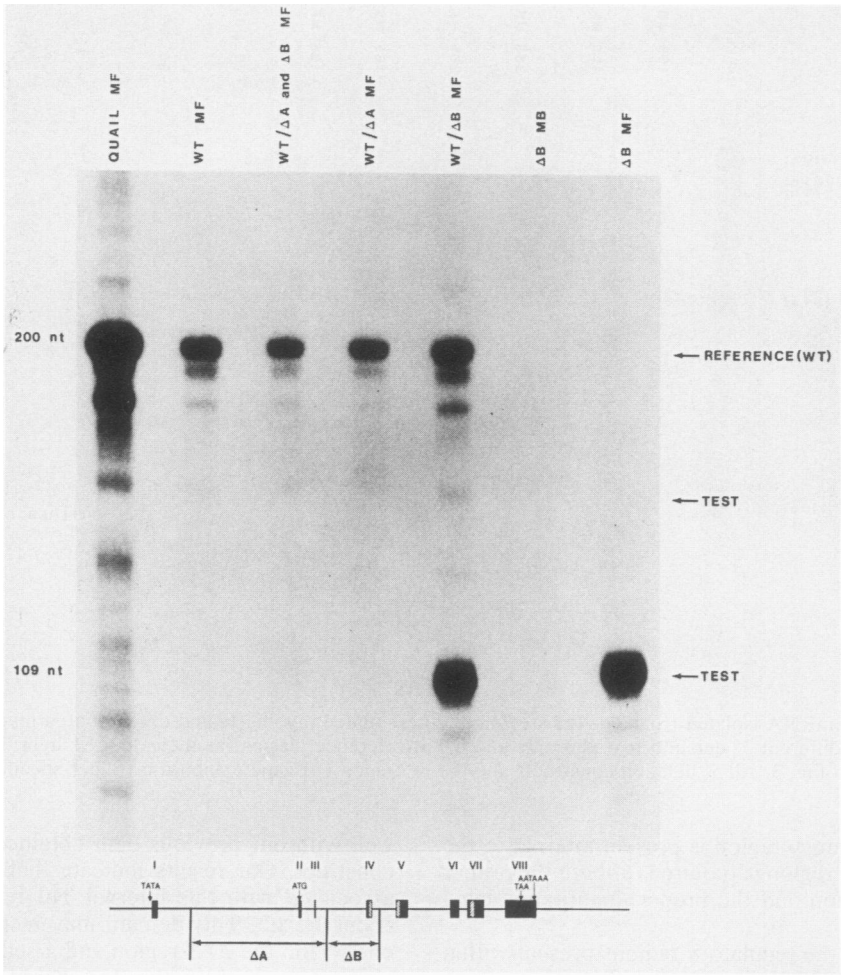

FIG. 6. S1 analysis of TnI internal deletions and reference genes cotransfected into 23A2 myoblasts. Myofiber RNA was isolated from differentiated cells transfected with the indicated genes and hybridized to a TnI cDNA probe as described in the legend to Fig. 3. The reference gene used in these experiments was gC149 (wild type), which generates a 200-nucleotide protected fragment. Test constructs ΔA/B, ΔA, and ΔB contain the deletions indicated on the map below the figure and are described in detail in the text. ΔA generates a 149-nucleotide fragment, and ΔA/B and ΔB generate a 109-nucleotide fragment. Only ΔB is regulated and expressed in myofibers to a level similar to that of the reference wild-type plasmid.

formation sequences (25, 50). Furthermore, all internal deletion constructions, including ΔB, are expressed at very low but equivalent levels in the mouse transformed cell line $TK^{-}L$ (data not shown), indicating that the difference observed in accumulated message levels for each gene is not due to differences in the stability of the mutant message produced. Alternatively, the low level of message accumulation could reflect a reduced rate of TnI gene transcription in myofiber nuclei. To examine the transcription of transfected TnI genes directly, it was necessary to transfect each individual deletion construct without the reference TnI plasmid. 23A2 myoblasts were transfected with the wild-type gene, ΔA, ΔB, or ΔA/B. Stable transfected cell lines were pooled and assayed for the average TnI gene copy number by Southern blot hybridization and for TnI transcription by nuclear runoff assays. Both the wild-type gene and ΔB exhibited similar myofiber-specific transcriptional activation. In contrast, the ΔA and ΔA/B genes were 16-fold less active in myofiber-specific transcription when rates were adjusted for gene copy number and for the relative expression of the neomycin genes or the endogenous α-actin genes (Fig. 8, Table 1). The reduced transcription rates of ΔA and ΔA/B correlate well with reduced mRNA levels and provide an independent basis for concluding that internal regions of the TnI gene are required for TnI transcription in differentiated myofiber nuclei.

An enhancer-like element is associated with the internal regulatory region of the TnI gene. To further characterize the function of the +240 through +1825 TnI gene region, we examined whether the orientation and placement of this sequence region confers developmental regulation on a TnI gene. The +240 through +1825 region was reinserted in a 3′-to-5′ orientation into the ΔA/B construct. When this construct was transfected into myoblasts, a sixfold increase in myofiber-specific expression was observed compared with the control ΔA plasmid (Fig. 9, lane 5; Table 1). This increase corresponds to 38% of the expression level obtained for the wild-type TnI gene, demonstrating that the internal regulatory region can function in an opposite orientation if it is maintained internal to the gene itself.

To examine whether the ΔA region (+240 through +1825) can function at different locations, ΔA was reinserted into a ΔA gene either 800 bp upstream of the transcription start site or 1,800 bp downstream from the poly(A) addition site in

TABLE 1. Quantitative expression of transfected TnI constructs

TnI construct[a]	S1 analysis[b]	Nuclear runoff analysis[c]
Wild type	1.00	1.00
ΔB	1.00	1.00
ΔA	0.06	0.07
ΔA/B	0.07	0.06
Wild type	1.00	
ΔA1	0.01	
ΔA2	0.06	
ΔA3	0.06	
Wild type	1.00	
ΔA	0.06	
Internal ΔA/B, 3′ to 5′	0.38	
5′ ΔA, 5′ to 3′	0.12	
5′ ΔA, 3′ to 5′	0.08	
3′ ΔA, 5′ to 3′	0.19	
3′ ΔA, 3′ to 5′	0.12	

[a] TnI constructs transfected into 23A2 myoblasts. Internal ΔA/B, 3′ to 5′, refers to positioning the +240 through +1825 region of the wild-type TnI gene into the ΔA/B construct in a 3′-to-5′ orientation; 5′ ΔA, 5′ to 3′, refers to positioning the +240 through +1825 region of the wild-type gene at the 5′ end of the ΔA gene in a 5′-to-3′ orientation. Similar nomenclature is used for all other experimental constructions.

[b] Relative (myofiber-specific) transcription levels, $M/R_m/W/R_w$, where M is the mutant test signal, W is the wild-type test signal, and R is the reference signal per each transfection and assay. The relative transcription levels for 5′ deletions were: −530, 1.00; −484, 0.72; −436, 0.14; −405, 0.35; −387, 0.32; −355, 0.35; −185, 0.26; −164, 0.54; −149, 0.17; −95, 0.22; −47, 0.14; −17, 0.08.

[c] Relative (myofiber-specific) transcription rates were obtained by normalizing values to the transfected gene copy number as well as to the expression rates of control pKO-NEO and actin genes.

5′-to-3′ and 3′-to-5′ orientations (see Materials and Methods for details). Although each clone exhibited a developmentally regulated increase in TnI expression levels, this increase was only two- to threefold over the expression found for the control ΔA gene (Fig. 9, lanes 1 through 4; Table 1). Clearly, these values do not approach the expression level of the wild-type gene. The fact that a limited myofiber-specific increase is observed when the +240 through +1825 region is placed as far away as 4 kb from its original location suggests that the regulatory elements present within these sequences may share some properties of tissue- and development-specific enhancerlike elements. However, this region does not appear to act as a true enhancer since the quantitative increase in expression is very small when compared with that in similar studies utilizing immunoglobulin enhancers (14, 17). Although the mechanism by which the internal regulatory region potentiates myofiber-specific transcription of the TnI gene is unknown, it is clear that the +240 through +1825 domain functions more efficiently when positioned internal to the TnI gene, regardless of orientation.

DISCUSSION

The quail fast skeletal TnI gene is one member of a set of more than 20 contractile protein genes that are activated coordinately during skeletal myogenesis (11, 19). The mechanisms controlling each of these muscle genes may be highly specific, since they are rapidly activated to a high level only in muscle lineages. This seemingly complex regulation for TnI and other muscle genes involves the activation of a muscle-specific *cis-trans* regulation system or systems. These systems may involve multiple *cis*-acting regulatory elements and multiple tissue-specific factors that may be unique to each contractile protein gene. Alternatively, contractile protein genes may be activated by a common *cis-trans* regulatory system shared by all contractile protein genes and activated during myoblast differentiation.

Our current data suggest that the regulation of the TnI gene is complex, involving multiple regulatory elements present in the 5′ flanking DNA and internal to the TnI gene in the region of the first intron. We conclude that these two regions function as positive regulatory elements and together are necessary for the appropriate timing, tissue specificity, and quantitative levels of TnI gene expression associated with muscle cell differentiation. The small 5′ regulatory element, tentatively localized between −484 and −436, plays a role in the quantitative level of expression associated with the wild-type gene, whereas the larger (1.5-kb) internal region is responsible for the tissue specificity and developmentally regulated expression of TnI in skeletal muscle cells. Since none of the deletions tested so far increases the activity of the TnI gene, we have no evidence for negative control elements. Preliminary data from heterologous gene systems indicate that these regions alone will not support the normal regulatory activity associated with TnI. Rather, it is the concerted action of each of these regions that produces the complete developmental expression of this contractile protein gene. We are currently investigating whether individual or multiple myofiber-specific *trans* factors must also interact with each regulatory element to produce this appropriate expression. A detailed comparison of the TnI *cis-trans* control elements with those of other contractile protein genes should identify common and different elements that regulate and coordinate the muscle gene set.

We have shown that the region −484 through −95 is essential to the normal expression of TnI, since individual deletion constructs covering this region produce an average threefold drop in the level of expression when compared with the wild-type TnI gene. Surprisingly, all 5′ deletion clones still maintained an appropriate up-regulation as the cells differentiated into myofibers except when combined with pBR322 sequences, which drastically inhibit transcription. The nature of this inhibitory effect is unknown, but it has been observed in a number of transgenic mouse experiments (5, 53). One possibility for the inhibition of expression is that the high G+C content of plasmid sequences may produce structural alterations that inhibit interactions of the promoter with regulatory factors on RNA polymerase. In any case, our current study emphasizes the importance of examining the influence of pBR322 sequences on the activity of transfected genes as well.

The major TnI control element is intragenic, located primarily within the first intron of the TnI gene. Removal of this internal regulatory region results in a 16-fold decrease in its transcription rate as well as in the amount of stable TnI message in myofibers. This internal region can function in either a 5′-to-3′ or 3′-to-5′ orientation to produce the myofiber-specific expression pattern of the wild-type gene if it is maintained in its natural location. In addition, the TnI internal region exhibits some limited properties that are normally associated with enhancer elements (9). As with the 5′ deletion clones, however, the complete removal of the first intron does not entirely abolish the ability of the TnI gene to be developmentally regulated, since clones lacking these sequences still produce a three- to fourfold increase in TnI mRNA in myofiber cells. However, our transcription data suggest that this increase is small when compred with the normal 100- to 200-fold increase associated with the wild-type gene and likely reflects the fact that myofibers are

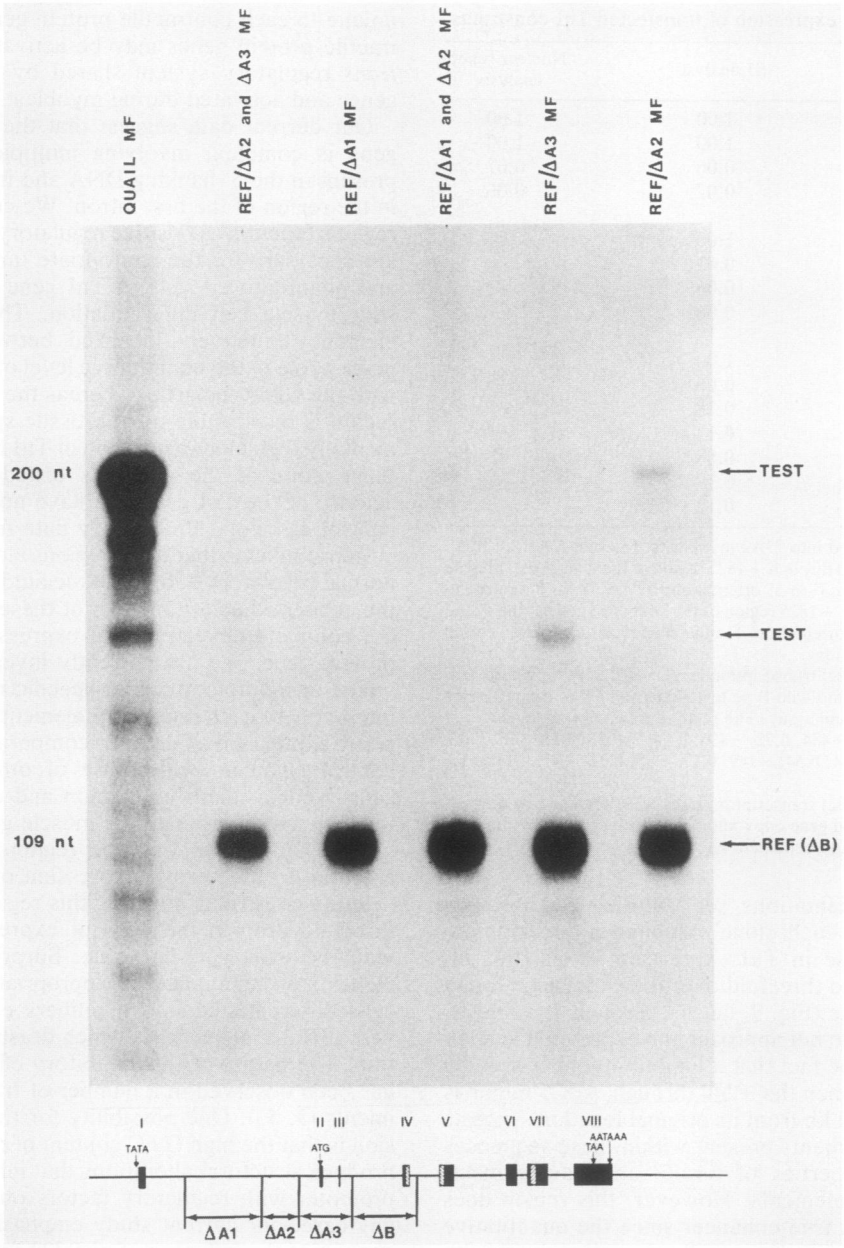

FIG. 7. S1 analysis of TnI internal deletions transfected with a reference TnI plasmid into 23A2 myoblasts. Myofiber RNA was isolated from each transfected group and hybridized to a TnI cDNA clone as described in the legend to Fig. 3. The reference gene (ΔB) protects a 109-nucleotide fragment, whereas the test genes ΔA1, ΔA2, and ΔA1/A2 protect a 200-nucleotide fragment and ΔA3 and ΔA2/A3 protect a 149-nucleotide fragment. The position of the various deletions are indicated in the map below the figure. These positions correspond to the positions indicated in Fig. 1 and in the text. For each test construct, only a small amount of TnI mRNA was detected in myofibers compared with the reference signal.

postmitotic, so that mRNA produced from a weakly transcribed gene accumulates in the postmitotic myofiber to a slightly higher level than in a rapidly dividing myoblast.

Our studies of the TnI gene provide a basis for future comparison of *cis-trans* muscle gene control systems that regulate and coordinate other contractile protein genes activated during myoblast differentiation. In this study, our strategy was to dissect the control sequences of the TnI gene starting with a gene and a transfection assay that duplicates the temporal, lineage specificity, and quantitative regulation of TnI in its natural location in the quail chromosome. This assay involves the stable transfection of a low copy number of intact genes. Previous studies have provided evidence for *cis* regulatory sequences in 5′ flanking regions of other contractile protein genes. However, in most studies, regulatory elements have been assayed using transient transfections and hybrid gene fusions of promoter and 5′ flanking sequences to the chloramphenicol acetyltransferase (CAT) reporter gene. When the 5′ flanking DNA of rat (33) and chicken (2, 16, 46) skeletal α-actin, human cardiac α-actin (35, 37), mouse M-creatine kinase (23), and rat myosin light chain 2 (44) were tested, only a 6- to 20-fold increase in the amount of CAT protein was observed in transfected myofibers compared with transfected myoblasts and

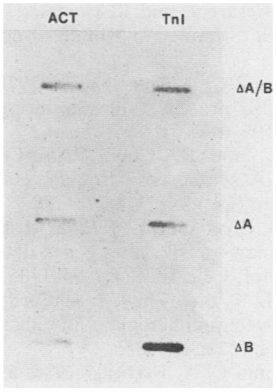

FIG. 8. Transcription analysis of TnI internal deletions ΔA, ΔB, and ΔA/B transfected into 23A2 myoblasts. Myofiber nuclei were isolated from each transfected culture, and ^{32}P-labeled transcripts were hybridized to separate nitrocellulose slot blots containing mouse skeletal α-actin (ACT) and quail TnI DNA sequences. Southern analysis on these clones demonstrated that there were four times the number of ΔA and ΔA/B gene constructs stably integrated in these cells when compared with the ΔB construct (data not shown). Based on gene copy number and the expression of the endogenous actin gene, only the ΔB construct exhibits myofiber-specific transcription rates that are comparable to the wild-type TnI gene (Fig. 2B and 5).

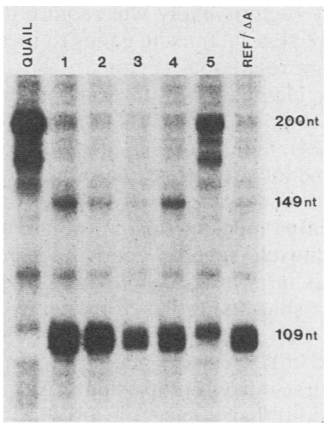

FIG. 9. S1 analysis of myofiber RNA isolated from 23A2 transfected cells. TnI constructs containing the internal +240 through +1825 region positioned either 800 bp upstream of the transcription start site or 1,800 bp downstream from the poly(A) addition site in 5′-to-3′ or 3′-to-5′ orientations, or positioned internal to the gene but in a 3′-to-5′ orientation, were cotransfected into 23A2 myoblasts with reference plasmids. Lanes: QUAIL, control RNA isolated from quail skeletal muscle cultures; 1, 3′ ΔA, 5′ to 3′ (reference ΔB); 2, 3′ ΔA, 3′ to 5′ (reference ΔB); 3, 5′ ΔA, 5′ to 3′ (reference ΔB); 4, 5′ ΔA, 3′ to 5′ (reference ΔB); 5, internal ΔA/B, 3′ to 5′ (reference gC149 [wild type]); REF/ΔA, ΔA control (reference ΔB). Reference ΔB and internal ΔA/B, 3′ to 5′, genes produce a 109-nucleotide protected fragment, ΔA constructs produce a 149-nucleotide protected fragment, and the reference gC149 gene produces a 200-nucleotide fragment (see text for details). Note that lanes 1 through 4 exhibit a modest increase in the 149-nucleotide signal when compared with the control ΔA construct and reference genes. A more pronounced increase in expression is observed with the construct internal ΔA/B, 3′ to 5′ (compare the 109-nucleotide signal of lane 5 with the 149-nucleotide signal of the control ΔA).

transfected nonmyogenic cells. Deletion studies for two of these genes have tentatively localized regulatory sequences at approximately −200 (chicken skeletal α-actin (2) and at −443 through −395 and −177 through −118 (human cardiac α-actin) (36) since removal of these regions decreases the expression of the CAT vector in transient transfections. Similar to our results, these decreases in promoter activity were quite small in 5′ deletion constructs. The −443 through −395 region of the human cardiac α-actin gene and the −200 through −144 region of the chicken skeletal α-actin gene, when deleted, decrease CAT expression only twofold. Further deletions of these 5′ regions (to approximately −100) result in a 10-fold decrease in CAT activity, but the −100 actin constructs no longer contain a CCAAT box that may be required for the general expression of eucaryotic promoters (9, 12). As shown in this study, the inclusion of plasmid sequences in these deletion constructs also may contribute to this apparent decrease in promoter activity and complicate interpretation of these data.

Muscle gene promoters exhibit limited expression when compared with endogenous contractile protein genes in transient gene transfection assays with CAT gene fusions. In contrast, when the entire rat myosin light chain 2 gene (52) and two-thirds of the rat skeletal α-actin gene (53) are introduced into transgenic mice, they each exhibit the normal 100- to 200-fold up-regulation associated with normal muscle differentiation. A similar result has been reported for the entire mouse M-creatine kinase (23) and human cardiac α-actin (21) genes when transfected into cultured myogenic cells. Additional sequences (we suggest intragenic regulatory elements) not present in the 5′ flanking regions of these genes apparently are required for their complete developmental regulation. Analysis of such sequences may be essential to understand the mechanisms that coordinate muscle gene activity.

The coordinate activation of the contractile protein genes suggest that these genes share homologous *cis* control sequences that regulate transcription during muscle differentiation. We previously identified a consensus sequence (at −329 for the TnI gene) among five unrelated contractile protein genes in their 5′ flanking sequences (26). Our current mutagenesis data, however, do not support the idea that this sequence region is involved in the transcriptional regulation of the quail TnI gene. Its removal has only a minor quantitative effect on expression of the transfected TnI gene. Whether muscle genes have homologous sequences in their

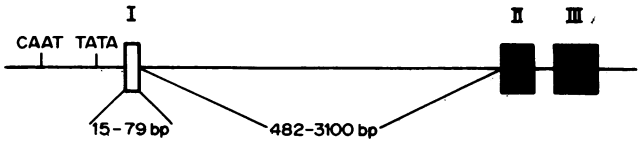

FIG. 10. Common 5′ organization of known contractile protein genes. Each gene listed below contains an untranslated first exon followed by a relatively large first intron. The genes presented here represent only a partial listing of all known contractile protein gene structures. The conservation of an untranslated first exon followed by a large first intron suggests that this general structure may be important in the coordinate regulation of this gene set (see the text for details). Genes (references): rat skeletal α-actin (62), mouse skeletal α-actin (22), chicken cardiac α-actin (6), mouse M-creatine kinase (23), chicken myosin light chain 2 (61), *Drosophila* myosin light chain 2 (47), rat myosin light chain 2 (45), chicken myosin light chain 3 (41), rat myosin light chain 3 (48), rat embryonic myosin heavy chain (31), rabbit cardiac α-myosin heavy chain (58), rat cardiac α-myosin and β-myosin heavy chain (30), chicken fast TnI (43), quail fast TnI (1), *Drosophila* tropomyosin (24), rat troponin T (42). In the heavy-chain genes, exons I and II are untranslated. □, Untranslated exon; ■, protein-coding exon.

cis regulatory regions likely will require detailed mutagenesis analyses of several muscle genes to localize and compare their important regulatory regions.

Regulatory elements within first introns also have been reported for the mouse immunoglobulin (17), mouse thymidine kinase (34), and human growth hormone (38, 54) genes. This intragenic location of regulatory elements suggests that the transcriptional activation of these genes may be controlled by related mechanisms. Although it is not yet known whether all muscle-specific genes have internal regulatory elements, it is intriguing to note that at least 17 contractile protein genes share a similar 5′ intron-exon structure. Specifically, the first exon of each of these genes encodes 5′ nontranslated mRNA sequences only, followed by a relatively large first intron comprising 482 to 3,100 nucleotides (Fig. 10). Additional contractile protein genes also fit this pattern (15). The existence of this similar gene structure is remarkable, considering that muscle contractile protein genes are unrelated evolutionarily. Our data suggest that there may be evolutionary advantages for maintaining regulatory elements within the first intron, since the TnI elements function most efficiently in this specific internal location and since the quantitative levels of muscle gene transcription are critical to the formation of functional muscle (29). These structural and functional considerations suggest that future studies should focus on examining differentiation-specific regulatory functions of introns in other muscle genes.

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