MOLECULAR AND CELLULAR BIOLOGY, Nov. 1993, p. 7019–7028 Vol. 13, No. 11
0270-7306/93/117019-10$02.00/0
Copyright © 1993, American Society for Microbiology

cis-Acting Sequences of the Rat Troponin I Slow Gene Confer Tissue- and Development-Specific Transcription in Cultured Muscle Cells as well as Fiber Type Specificity in Transgenic Mice

SHARMILA BANERJEE-BASU AND ANDRES BUONANNO*

Unit on Molecular Neurobiology, Laboratory of Developmental Neurobiology, Building 49, Room 5A-38, National Institute of Child Health and Human Development, 9000 Rockville Pike, Bethesda, Maryland 20892

Received 13 May 1993/Returned for modification 23 June 1993/Accepted 24 August 1993

Transcription of the genes coding for troponin I slow (TnI_{slow}) and other contractile proteins is activated during skeletal muscle differentiation, and their expression is later restricted to specific fiber types during maturation. We have isolated and characterized the rat TnI_{slow} gene in order to begin elucidating its regulation during myogenesis. Transcriptional regulatory regions were delineated by using constructs, containing TnI_{slow} gene sequences driving the expression of the chloramphenicol acetyltransferase (CAT) reporter gene, that were transiently transfected into undifferentiated and differentiated C2C12 cells. TnI_{slow} 5′-flanking sequences directed transcription specifically in differentiated cells. However, transcription rates were approximately 10-fold higher in myotubes transfected with constructs containing the 5′-flanking sequences plus the intragenic region residing upstream of the translation initiation site (introns 1 and 2), indicative of interactions between elements residing upstream and in the introns of the gene. Deletion analysis of the 5′ region of the TnI_{slow} gene showed that the 200 bp upstream of the transcription initiation site is sufficient to confer differentiation-specific transcription in C2C12 myocytes. MyoD consensus binding sites were found both in the upstream 200-bp region and in a region residing in the second intron that is highly homologous to the quail TnI_{fast} enhancer. Transactivation experiments using transfected NIH 3T3 fibroblasts with TnI-CAT constructs containing intragenic and/or upstream sequences and with the myogenic factors MyoD, myogenin, and MRF4 showed different potentials of these factors to induce transcription. Transgenic mice harboring the rat TnI-CAT fusion gene expressed the reporter specifically in the skeletal muscle. Furthermore, CAT levels were approximately 50-fold higher in the soleus than in the extensor digitorum longus, gastrocnemius, or tibialis muscle, indicating that the regulatory elements that restrict TnI transcription to slow-twitch myofibers reside in the sequences we have analyzed.

Myogenesis involves an ordered progression of events leading to the fusion of mononucleated myoblasts into multinucleated myofibers, with the concomitant activation of functionally related muscle-specific genes coding for proteins that form the contractile apparatus (8, 42). The distinct isoforms of these contractile proteins, which assemble to generate the myofibrils, underlie the slow and fast contractile properties of muscle fibers (40). The genes coding for the different contractile protein isoforms are generally expressed selectively in either the fast- or the slow-twitch muscle; examples include myosin light chain (MLC), myosin heavy chain, and troponins C, I, and T (reviewed in reference 2). The *cis*-acting elements needed for muscle-specific transcription of these genes have been extensively characterized (6, 17, 21, 31, 38, 39, 43, 44). Regulatory regions of these genes share many common sequence motifs that constitute binding sites for both muscle-specific and ubiquitous nuclear factors (42).

In recent years, several regulatory factors involved in muscle-specific gene activation have been identified (37, 49). The best-characterized myogenic transcription factors are the helix-loop-helix proteins of the MyoD family, which includes MyoD (14), myogenin (20, 52), myf5 (5), and MRF4 (4, 32, 41). Each of these factors is expressed exclusively in the skeletal muscle and can activate myogenesis when introduced into a variety of nonmuscle cells. They heterodimerize with the widely expressed basic helix-loop-helix (bHLH) proteins encoded by E2a genes (35) and bind to the sequence CANNTG (referred to as an E box), which is present in the transcriptional regulatory regions of many muscle-specific genes. In addition to the MyoD family, other factors such as serum response factor (3), muscle-CAT binding factor (31), and myocyte specific enhancer factor 2 (MEF-2) (53) are known to participate in the transcriptional regulation of muscle-specific genes. Transcriptional activation during muscle differentiation appears to involve complex, and often cooperative, interactions between these myogenic transcription factors (19).

While the transcriptional basis of muscle differentiation is well explored, much less is known about the regulatory mechanisms dictating diversity among muscle fibers and pattern formation in the developing muscle. A strict sequential pattern of contractile protein isoform expression has been observed in developing muscles (16, 46), and a number of factors including cell lineage, hormonal inputs, and innervation have been implicated in modulating the properties of adult muscle fibers. Recently, Donoghue et al. (18) have shown that transgenic mice carrying an MLC-CAT chimeric gene construct expressed the reporter in a fast fiber-type-specific manner, indicating that transcription is a major mechanism dictating fiber-type-specific expression. It is apparent that elucidating the molecular mechanisms of selec-

* Corresponding author.

tive muscle gene expression, especially transcription, may be a key to understanding how diversity among muscle fiber types is generated and maintained.

We have focused our attention on the transcriptional regulation of the slow isoform of troponin I (TnI_{slow}). TnI is a component of a large protein complex which also includes troponins C and T. The troponin complex is involved in Ca^{2+}-mediated regulation of acto-myosin ATPase during skeletal muscle contraction (56). Three different isoforms of TnI are known: TnI_{slow} and TnI_{fast} are expressed in slow- and fast-twitch adult skeletal muscle fibers, respectively, while $TnI_{cardiac}$ is confined to the cardiac muscle (47, 51). The genes encoding TnI isoforms are activated in terminally differentiated muscle cells (7), but diverse molecular signals restrict their expression to specific fiber types. Characterization of *cis*-acting elements and *trans*-acting factors mediating TnI_{slow} gene expression offers potential insight into the molecular mechanisms of fiber type generation. Here, we report the cloning of the rat TnI_{slow} gene and begin to delineate the regulatory regions necessary for slow fiber-type-specific transcription in skeletal muscle. We show that the rat TnI_{slow} gene has two distinct *cis*-regulatory regions: one in the 5′-flanking sequence and the other in an intragenic region. The upstream regulatory element is sufficient to activate transcription in terminally differentiated muscle cells, and the intragenic element is required for maximal activation. Finally, we demonstrate that mice harboring the CAT gene directed by the upstream and intragenic regulatory elements of the TnI_{slow} gene confer specifically slow fiber type transcription in transgenic mice.

MATERIALS AND METHODS

Cloning the 5′ end of TnI_{slow} transcripts. The 5′ end of the TnI_{slow} mRNA was selectively amplified by the RACE procedure as described by Frohman et al. (22). Briefly, 1 μg of poly(A)$^+$ RNA isolated from rat hind limb muscles was reverse transcribed with Moloney murine leukemia virus reverse transcriptase in the presence of 20 μCi of [^{32}P]dCTP by using the oligonucleotide primer TnI-RT (5′-CGTAGG GATGCGCTCCG-3′), corresponding to a sequence within the rat TnI_{slow} cDNA (28). Excess TnI-RT primer was separated from the first-strand cDNA on a Sephadex G-75 column, and fractions corresponding to the first radioactive peak were collected. The 3′ end of the first-strand cDNA was further modified by adding a homopolymeric tail, by using dATP and terminal deoxynucleotidyltransferase, to provide an anchor for the subsequent polymerase chain reaction (PCR) amplification. The tailed first-strand cDNA was specifically amplified by PCR using a nested oligonucleotide primer, TnI-AMP (5′-GAGGTAACGCACCTTCTC-3′), corresponding to a region more 5′ on the coding strand, and two oligonucleotide primers corresponding to the anchor region (22). The amplified double-stranded cDNA was gel purified and cloned into the Bluescript SK (Stratagene) vector for sequencing.

Primer extension analysis. Primer extension was performed with an antisense oligonucleotide primer TnI-2RC (5′-TCCGGCATGGTGGTAACAAG-3′), corresponding to a sequence encompassing the initiator methionine codon of TnI_{slow} mRNA (28). One hundred thousand counts per minute of ^{32}P-end-labeled TnI-2RC was added to 5 μg of rat soleus or liver total RNA in a final volume of 10 μl containing 50 mM Tris (pH 8.3), 50 mM KCl, and 10 mM $MgCl_2$. The primer was annealed to the RNA by incubating at 62°C for 20 min and slowly cooling to room temperature. The reaction volume was adjusted to 20 μl with final concentrations of 50 mM Tris (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 2.8 mM sodium pyrophosphate, 1 mM each deoxynucleoside triphosphate and 0.5 mM spermidine. Extension was performed at 42°C for 45 min by using 1 U of avian myeloblastosis virus reverse transcriptase (Promega). Following ethanol precipitation, the products were fractionated on an 8% polyacrylamide denaturing sequencing gel. A dideoxy sequencing ladder generated with ^{32}P-end-labeled TnI-2RC as a primer on a RACE cDNA clone, as well as phosphorylated φX174 *Hin*fI fragments (Bethesda Research Laboratories), was used as molecular weight markers to precisely determine the size of the primer-extended product.

Isolation of rat TnI_{slow} genomic clones. Approximately 10^6 recombinants from a rat genomic library made in the vector Lambda Dash (Stratagene) were screened with a ^{32}P-end-labeled oligonucleotide probe, TnI-5, designed from the sequence obtained by the RACE strategy. The sequence of the oligonucleotide TnI-5 (5′-CTAGGCTGGCCCGAGCCT CACCACAGACGGCAGATTA-3′) corresponds to the 5′ end of the rat TnI_{slow} cDNA sequence. Phage lifts on duplicate filters were hybridized for 36 h at 56°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 10% dextran sulfate, 100 μg of salmon sperm DNA, and 10^6 cpm of the phosphorylated probe per ml of hybridization solution. The filters were then washed twice at room temperature for 30 min each in 2× SSC–0.1% sodium dodecyl sulfate (SDS) and then subjected to a high-temperature wash at 56°C for 45 min in the same wash solution. Positive plaques were picked and carried through three successive rounds of purification to obtain pure clones.

Mapping, subcloning, and DNA sequencing. Positive lambda phage clones Tn1, Tn5, Tn7, and Tn9 were mapped by using oligonucleotide probes corresponding to the 5′ and 3′ ends of the TnI_{slow} cDNA (28), respectively. The phage clone Tn7, which hybridized with both 5′- and 3′-specific probes, was selected for subcloning and sequence analysis. *Bam*HI restriction fragments from Tn7 were subcloned into the Bluescript II (Stratagene) vector to generate the clones TN7.Bm1, TN7.Bm2, and TN7.Bm3, which were used for mapping and sequencing, by the dideoxy chain termination method for denatured double-stranded plasmids (29). DNASTAR DNA sequencing software (DNASTAR, Inc., Madison, Wis.) was used for sequence analysis.

RNA isolation and Northern analysis. Isolation of total RNA from rat hind limb muscles or C2C12 cells was performed by the guanidium thiocyanate procedure as described by Chirgwin et al. (11). For Northern (RNA) analysis, total RNA was fractionated on 1.5% formaldehyde-agarose gels, transferred to a nylon membrane, and hybridized with the rat TnI_{slow} or mouse TnI_{fast} cDNA probes. For detection of TnI_{slow} mRNA, hybridization was performed at 42°C for 16 h in 40% formamide, 5× SSC, 5× Denhardt's solution, 1% SDS, 100 μg of salmon sperm DNA, and 10^6 cpm of the phosphorylated probe per ml of hybridization solution. Following hybridization, the blot was washed at moderate stringency (55°C, 0.2× SSC–1% SDS) and exposed to Kodak X-ray film. Subsequently, the blot was stripped and hybridized with the TnI_{fast} cDNA probe. Hybridization with the fast probe was done under similar conditions except that 50% formamide was used and the blot was washed at higher stringency (65°C, 0.1× SSC–1% SDS). The signals on the blots were also quantitated with a PhosphorImager (Molecular Dynamics).

Troponin reporter gene constructs. The TnI-CAT chimeric gene constructs were made by inserting sequences from the

rat TnI_{slow} gene into the vector pCATbasic (Promega) containing the CAT reporter gene. Standard molecular techniques were used for construction of the plasmids. A brief description of these constructs is given below (see Fig. 4 for details).

A *Sal*I-*Xba*I fragment (containing the ~2.2 kb upstream and 2.3 kb downstream sequences relative to the transcriptional start site) from Tn7.Bm1 was inserted into the *Sal*I-*Xba*I site of pCATbasic to generate the clone TnSX.CAT. TnSX.CAT was cleaved at the *Xba*I site and blunt ended, and a 1.7-kb blunt-ended *Bam*HI-*Eco*RI fragment from Tn7.Bm2 was inserted to generate the clone TnSXBE.CAT. In order to generate the splice acceptor site, TnSXBE.CAT was further modified by inserting a 300-bp PCR-amplified fragment from Tn7.Bm2 corresponding to the last 300 bp of the second intron to generate the clone Tn6500.CAT. Tn4400.CAT was constructed by inserting the same 300-bp PCR fragment at the *Xba*I site of TnSX.CAT. Tn2700.CAT was constructed by insertion of a 550-bp PCR fragment (corresponding to nucleotides +50 to −500 relative to the transcriptional start site) into *Kpn*I-*Xba*I-cleaved, blunt-ended Tn4400.CAT. Tn2300.CAT was generated by deletion of a 2.2-kb *Sal*I-*Kpn*I fragment from the 5′ upstream region of Tn4400.CAT, followed by blunt ending and religation. Tn500.CAT, Tn300.CAT, Tn200.CAT, and Tn45.CAT were constructed by PCR amplification of the corresponding regions using specific oligonucleotide primers and ligation of the PCR fragments into pCATbasic. All the clones obtained with fragments generated by PCR were verified by sequencing. Large-scale preparations of plasmid DNA were made by using Qiagen columns (Qiagen Inc.).

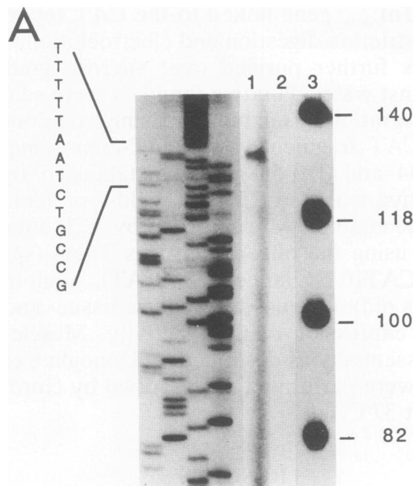
Cell culture, transfections, and CAT assays. C2C12 myoblasts were maintained in growth medium (Dulbecco's modified Eagle's medium containing 20% fetal calf serum) and induced to differentiate by transferring cells to differentiation medium (Dulbecco's modified Eagle's medium containing 2% horse serum). NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium containing 10% calf serum. Transient transfections were performed by DNA-calcium phosphate precipitation as described by Graham and Van der Eb (25). C2C12 myoblasts were transfected with 15 μg of test CAT construct and 5 μg of reference plasmid pSVβgal, used for normalization. Cells transfected for 12 to 14 h were subjected to a 2-min glycerol shock and transferred to either growth medium (for myoblasts) or differentiation medium (for myotubes). Myoblasts were incubated for an additional 24 h in growth medium prior to harvesting; myotubes were harvested 48 h following the switch to the differentiation medium. NIH 3T3 transfections were performed essentially as described above, and cells were harvested 36 h following glycerol shock. Cell extracts were prepared in 0.25 M Tris (pH 8.0) according to standard procedures, and extracts normalized to equal β-galactosidase activity were assayed for CAT activity by thin-layer chromatography. CAT assays were performed as described by Gorman et al. (23) for 1 h at 37°C, and β-galactosidase assays were performed as described by Nielsen et al. (36). Multiple transfections were performed for each reporter construct with at least two separate plasmid preparations.

Generation of transgenic mouse lines. Founder transgenic mice were generated by DNX, The National Transgenic Development Facility. The DNA fragment used for injection into the pronuclei of fertilized mouse eggs (C57BL/6 × SJL)F_2 was prepared as follows. Plasmid Tn6500.CAT (Fig. 3) was digested with *Nde*I to release a 7.3-kb fragment containing ~5.5 kb of sequences upstream of the first coding exon of the TnI_{slow} gene linked to the CAT reporter gene. Following restriction digestion and electroelution, the DNA fragment was further purified over sucrose gradient and dialyzed against water. Putative founders were screened for transgene integration by tail blotting using a random-primed, ^{32}P-labeled CAT fragment. Two CAT-expressing founder lines (TnI-434 and TnI-548) were established by mating tail-blot-positive males to C57BL/6 wild-type females. Offspring of these matings were screened by PCR amplification of tail DNA using the oligonucleotides Tn-44 (specific for TnI_{slow}) and CAT10.NC (specific for CAT). Adult transgenic mice (8 weeks old) were used to analyze tissue- and muscle-type-specific expression of CAT activity. Muscle extracts were made essentially as described by Donoghue et al. (18). CAT assays were performed as described by Gorman et al. (23) for 1 h at 37°C.

RESULTS

Determination of the transcription initiation site of the TnI_{slow} gene. Genomic sequences corresponding to the 5′ untranslated region of several skeletal muscle genes are interrupted by large introns, as is the case with the TnI_{fast} gene (27). Considering the extensive homology of TnI_{slow} and TnI_{fast} coding sequences, we suspected the existence of intervening sequences in the upstream region of the TnI_{slow} gene. Since we were interested in obtaining genomic clones encompassing the transcription start site and regulatory regions of the TnI_{slow} gene, we felt it was advantageous to screen genomic libraries with probes that extended fully to the 5′ end of the TnI message. Therefore, we began by mapping the 5′ end of TnI_{slow} mRNA by primer extension analysis. Using an antisense oligonucleotide primer that extended upstream from the third codon of the rat TnI_{slow} cDNA sequence (28), we detected a major extension product of 131 nucleotides with RNA isolated from the rat soleus muscle (Fig. 1A). The size of the primer-extended product indicated that the 5′ end of TnI_{slow} mRNA lies 79 nucleotides upstream of the cDNA sequence reported by Koppe et al. (28). No extension product was obtained with rat liver RNA, which does not express the TnI genes. To further characterize the TnI_{slow} message, we generated cDNA clones corresponding to the full-length 5′ untranslated region of the mRNA by the RACE strategy (see Materials and Methods). In independent experiments, a single abundant ~300-bp fragment (visualized on agarose gels) was obtained following PCR amplifications by using an antisense primer that extended upstream from codon 45. The amplified fragments obtained in two separate experiments were subcloned, and six independent cDNA clones were isolated and sequenced. The cDNAs had identical sequences that extended to the same 5′ residue, and they differed only in the length of the poly(T) track introduced during the RACE procedure. The nucleotide sequence of this region is shown in Fig. 1B. The 5′-most nucleotide of the RACE cDNA clones mapped exactly to the primer-extended product (Fig. 1A), and we designate this residue +1. Extending in the 3′-to-5′ direction, the first 72 bp of sequence we obtained matched perfectly with the rat TnI_{slow} cDNA sequence reported by Koppe et al. (28) and extended an additional 79 bp to the 5′ end. In addition, we found that the 5′ noncoding cDNA is identical to the rat TnI_{slow} genomic sequence, except that the latter is interrupted by two introns that are flanked by canonical splice donor-acceptor sites (see below).

Characterization of the rat TnI_{slow} gene. A synthetic 37-mer oligonucleotide probe designed from the 5′-most region



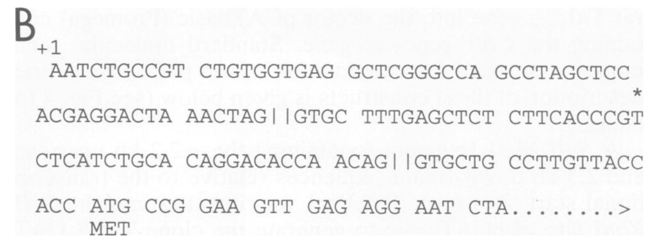


FIG. 1. Mapping the transcription start site of the rat TnI_{slow} gene. (A) Rat soleus muscle (lane 1) and liver (lane 2) total RNAs were annealed with a γ-^{32}P-end-labeled antisense oligonucleotide primer and extended with avian myeloblastosis virus reverse transcriptase to yield a 131-nucleotide-long product. The size of the primer-extended product was determined from a dideoxy sequence ladder generated from a RACE cDNA clone primed with the same γ-^{32}P-end-labeled oligonucleotide (left) and phosphorylated ϕX174 *Hin*fI fragments (lane 3). The sequence of the coding strand is shown. (B) The complete nucleotide sequence of the 5′ noncoding region of the rat TnI_{slow} mRNA was derived from cDNA RACE products. The sequence shown here was obtained from six independent clones representing the 5′ end of TnI_{slow} mRNA. The 5′-most nucleotide of this sequence is designated +1 and corresponds exactly to the same residue as that obtained by primer extension. The boundaries between the exons are marked (||). The first nucleotide of the rat TnI_{slow} cDNA sequence previously reported by Koppe et al. (28) is indicated (asterisk at position 80), and sequences extending downstream of the initiator methionine (MET) are shown as codons.

of the TnI_{slow} cDNA sequence (Fig. 1B) was used to screen a rat genomic library. Four clones were obtained after 10^6 plaques were screened. Southern blot analysis of the genomic clones using oligonucleotide probes corresponding to the 5′ and 3′ ends of the cDNA revealed that the rat TnI_{slow} gene is ~10.5 kb long and extends over three BamHI fragments (2a). A lambda clone, Tn7, containing the entire TnI_{slow} gene was selected for subcloning and structure analysis. The 5′ portion of the TnI_{slow} gene, including 485 bp upstream of the transcription start site, was sequenced (Fig. 2). Comparison of the genomic and cDNA sequences revealed that the rat TnI_{slow} gene contains two 5′ noncoding exons which are flanked by consensus splice donor-acceptor sites. The TnI_{slow} gene is interrupted by large intervening sequences with the initiator methionine positioned 3.7 kb downstream of the transcription start site. The complete structure of the rat TnI_{slow} gene will be reported elsewhere (2a).

Developmental regulation of the TnI_{slow} gene. The mouse myogenic cell line C2C12 provides an excellent system to study regulation of muscle gene expression. C2C12 myoblasts do not express detectable levels of myofibrillar proteins, but when induced to differentiate by withdrawal of growth factors, they express a battery of myofibrillar genes (7). In order to determine whether the TnI genes are regulated during C2C12 differentiation, the expression of the endogenous genes was monitored by Northern blot analysis. Total RNA isolated from C2C12 myoblasts and myotubes at various times after induction of differentiation was probed with a rat TnI_{slow} and a mouse TnI_{fast} cDNA. The levels of both transcripts were practically undetectable in undifferentiated C2C12 cells and dramatically increased following a switch to low-mitogen-containing medium (Fig. 3). Quantitation of the relative levels of TnI_{slow} mRNA showed that these transcripts begin to accumulate as early as 14 h after mitogen withdrawal and the levels increased ~40-fold in 48 h (data not shown). TnI_{fast} transcripts were observed to accumulate 24 h after a medium change, and by 48 h levels were ~36-fold higher than those in myoblasts.

Analysis of the rat TnI_{slow} promoter. In an attempt to define the sequence elements that are involved in the myotube-specific transcription of the TnI_{slow} gene, a series of reporter constructs were tested in transient transfection assays. Since several skeletal muscle genes, including muscle creatine kinase (45) and TnI_{fast} (30), contain transcriptional regulatory elements residing in upstream and intragenic regions, TnI-CAT constructs containing various lengths of both regions of the rat TnI_{slow} gene were generated. The C2C12 cell line was used to test the activities of these TnI-CAT fusion constructs. Each reporter construct was transiently transfected into C2C12 myoblasts that were either maintained in an undifferentiated state or induced to differentiate into myotubes. In order to control for differences in transfection efficiencies, the β-galactosidase-expressing plasmid, pSVβgal, was cotransfected with each construct. Subsequently, cell extracts were normalized to equivalent amounts of β-galactosidase activity and assayed for CAT (see Materials and Methods). In these experiments, the promoterless plasmid pCATbasic (Promega) served as a negative control, and pCATcontrol, containing the promoter and enhancer of simian virus 40, was used as a positive control. The results of transfection experiments are presented in Fig. 4; CAT activities of TnI_{slow} reporter constructs are expressed relative to that of pCATcontrol.

To begin identifying regulatory regions of the rat TnI_{slow} gene, the reporter construct Tn6500.CAT, containing 2.7 kb of the 5′ region upstream of the transcriptional start site and 3.8 kb of intragenic sequences (two noncoding exons and two introns) driving the CAT reporter gene, was tested in C2C12 myocytes. Transfected myoblasts expressed practically undetectable levels of CAT activity; however, upon differentiation, there was an ~80-fold increase in reporter activity (Fig. 3). We also measured the CAT activity of a construct containing the 750 bp of the upstream sequence of the nicotinic acetylcholine receptor γ subunit gene, shown previously to be expressed specifically in differentiated myotubes (9). The level of expression of Tn6500.CAT is approximately twofold higher than that of the nicotinic acetylcholine receptor γ construct (data not shown). Deletion of 1.7 kb of the second intron in construct Tn4400.CAT had no effect on the level of CAT expression (Fig. 4),

```
GGTACCAGAG ACACAAATAC TTAGGTCTTT GGTTTTTTGA GGGTTTTTGG AGCATGGTCT   -426
GGGGTTGCAT GTCTACATGT CCTGGCTGGA GAGGAAGTGT CAGCCTGCTA GCTCCCTCCC   -366
TGATCTTTAA TGGTTCCTCA GACCTCCCAG TGCCCTGATT CCCTTCATCC CCCTTCCTGT   -306
                                                            E3
TCACATCTCT GGTCAATTCC ATATCCTGTC TAGTCTTCCC GTCTCTGTCC TCCCCCAGGT   -246
GAGTGCTGTT TTCCCCAAGT CAAAGGCTCT GTGTGTGCAG GGTTTGTGTG TGTGCTCCTG   -186
ATGTATACAC ACTGGTCTCT AAGCCTGTGA GGTACACACG GCAGCACGTA TCTGCCCTGC   -126
                                                       E2
GAGGTGCGCA TGGTGGGAGG GGGTGGGAAG GAGGGGCAGC TGGAGGGGGC AGTGGCTGTT    -66
   MEF2          E1
CTATTTTTAC TGGCCAGTTG CCGGAGGCCA CGGTTTTCAT AGCCTGCCCT CAGCTCTGCC     -6
    +1
CCCACAATCT GCCGTCTGTG GTGAGGCTCG GGCCAGCCTA GCTCCACGAG GACTAAACTA     55
Ggtaagtgtc tacaacactg accatggacc tgggcagctg agtagagagg tgaggttttg    115
tttagagggg aaatgaagtt tgggagacag agatgtttct tatttggatt tgtgtgggca    175
ggtttgttgg cagatactct aagactccta gccttttgac ccaccaaatg gcatttctcc    235
tgtacgatgc tgtgggcagt tggacctgga agggaagact gtccaacatg ttcctcagaa    295
acccaaagca gtggactatg ctctcacgag ggactgcagc caggggcagg gtggaccgag    355
ctcctggtgg gaggggtggc cctggggacc aaggcatcac atttgggaga agcctggggg    415
agaagcacca gtggtcatgc aggactctga ggagacagtc cagagggcct gctgaccgaa    475
gagaccatct cgtttctttg tcctttctct ctgacagGTG CTTTGAGCTC TCTTCACCCG    535
TCTCATCTGC ACAGGACACC AACAGgtata gacagaccac catgcctccc ccactgctcc    595
tgggctctga gtcggagaag gagttgtagg ggaggtggat gaccacagtg tctggggtca    655
gagtacaatt gtctgtgagc atgtaagcta gcaagacatc tacagcatga tgatcggagg    715
aggtttgggc taaaggcagc ccctacccca agtcctggac cctaagcggt tctaatgctg    775
gacttctggc cacaagcttg ggagatcagc ttgttagcac cctgtttggg aggatggggt    835
tgcccttgaa ctaagttcca aggtcagagg tggggtgcaa gggtagagca tgacgcatac    895
gccctatcag gggttggtt ccttctgct gtgtctcaca caggatttac acactccata    955
ctcaagcaag tgggcttggc gtggaaaact gacagtcttt cctctccaag gcctcagggc   1015
caggaaggtg agtcttgagt caggcctgag tctgctccct tccttccagc tctgtgcttt   1075
ctcttatctc tcgctttcca cttgtggtcc agatggaatt cggccctcca gcccctctgc   1135
tatctatatc ctggcttcta atcaggcccc tgcttctctt ccatagagcc tgtcaagggg   1195
tcctgcctgg tgggtgtcag agcacaggtc agggcctcat gcttgatgtg ggacccctgg   1255
gcctggcatt tgtcagtgag gcagttaggg actgcctgga gcttggcagg agcccaggct   1315
gcccagcagt gggccccaca ctggtgacag gggggctctc tcagggatag aggaaggaca   1375
gccatggcta agtgcccacc agggcgaagc ataatctagg gagacagatg caagacctat   1435
cggctaagac tcgcattccc agaggccaga cagacttggg aggggaggat ctttgtcagc   1495
tagtgtctcc ttttggactt ccccccagtg tcttcggaaa ggcagagctc ttgagtctgt   1555
gtattaaacc taatttcagc taagcatctt gggaatgcat gaatggttcc cacatctctt   1615
                                 IRE
ccccagtaac ccctggggac agctgagact ccagggtcac ctcccaccta gccccattct   1675
ctgcatgaac agaggcttag gtgcaaggct ctgtaacagg ctgggatcc              1724
```

FIG. 2. Nucleotide sequence corresponding to the 5′ untranslated region of the rat TnI_{slow} gene. The nucleotide sequence of a *Kpn*I-*Bam*HI restriction fragment of the rat TnI_{slow} gene is shown. Sequences extending upstream of the transcription start site (+1) are marked with negative numbers, and the exons and the introns extending downstream are positively numbered. The 5′ noncoding exons are boldfaced, the 5′-flanking region is uppercased, and the introns are lowercased. An AT-rich sequence that could function as a TATA box is present at −35. Additional transcription regulatory sequence motifs obtained from a computer search are underlined: three E boxes (CANNTG), a MEF-2-like sequence, and a region within the second intron bearing homology to the quail TnI_{fast} enhancer (IRE) (see the text for details).

indicating that this region does not contain elements that regulate transcription in the differentiated C2C12 myotubes. Complete removal of intragenic sequences in Tn2700.CAT resulted in a threefold reduction in reporter activity in differentiated myotubes, suggesting the presence of positive regulatory elements within the first and second introns. Plasmid Tn2300.CAT, containing 485 bp of the upstream sequence and the short intragenic region, was constructed to delineate the 5′ boundary of TnI_{slow} regulatory sequences. Unexpectedly, this construct was found to be about 2.5-fold more active than the larger constructs Tn4400.CAT and Tn6500.CAT, indicating that there may be a negative ele-

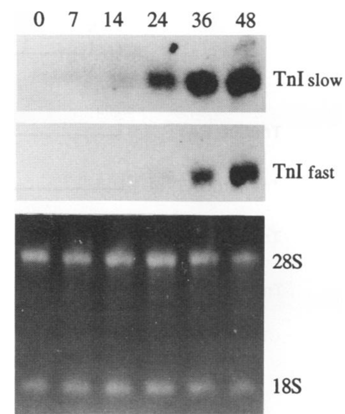


FIG. 3. Expression of TnI isoforms is induced during differentiation of C2C12 myoblasts. The levels of TnI mRNAs were analyzed during differentiation of C2C12 cells by using random-primed rat TnI_{slow} and mouse TnI_{fast} cDNA probes. The number of hours following a change of medium to induce differentiation is indicated at the top of each lane. Each lane on the Northern blot contains 5 μg of total RNA. Ethidium bromide staining of the gel (bottom panel) is shown to indicate that equivalent amounts of RNA were loaded in each lane.

ment located between −485 and −2700. The construct Tn500.CAT, containing only 485 bp of the 5′-flanking region, directed myotube-specific transcription. However, the level of expression with Tn500.CAT was about 10-fold lower than that obtained with the construct Tn2300.CAT, which spans the identical 5′ upstream sequences but includes the intragenic region extending from +36 to +1729. On the basis of these results, it is tempting to speculate that transcriptional *cis*-acting elements are present in the intragenic region; however, it is possible that these sequences selectively stabilize the reporter transcripts in differentiated C2C12 cells. Most of the TnI-CAT constructs tested exhibited practically undetectable levels of CAT activity in C2C12 myoblasts or in the fibroblast cell line NIH 3T3 (Fig. 4). Only construct Tn2300.CAT, which was the most active in C2C12 myotubes, showed slightly higher activity in myoblasts. This low level of activity could result from the premature differentiation of myoblasts frequently observed with C2C12 cells. Taken together, these results indicate the existence of two distinct regulatory regions, one in the 5′ upstream sequence and another within the intragenic regions of the TnI_{slow} gene. The upstream regulatory region is sufficient to mediate stage-specific transcription to a reporter gene in C2C12 cells, and the intragenic region is required for high-level expression.

To further delineate the regulatory region residing in the upstream TnI sequences, we generated 5′ deletions in Tn500.CAT and tested these deletion constructs for their abilities to confer development-specific activation in C2C12 cells. Deletions down to position −300 (Tn300.CAT) and −200 (Tn200.CAT) had little effect on transcriptional activation in C2C12 myotubes. Further deletion of the upstream sequences to −45 (Tn45.CAT) resulted in constitutive promoter activity, expressing the reporter gene in myoblasts, differentiated myotubes, and NIH 3T3 fibroblasts. Sequences suppressing transcription in nonmuscle cells have been possibly removed in this construct. Similar observations, of genes being nonspecifically expressed after removal of sequences residing close to the CAP site, have been reported for other genes including the muscle-specific δ-sub-

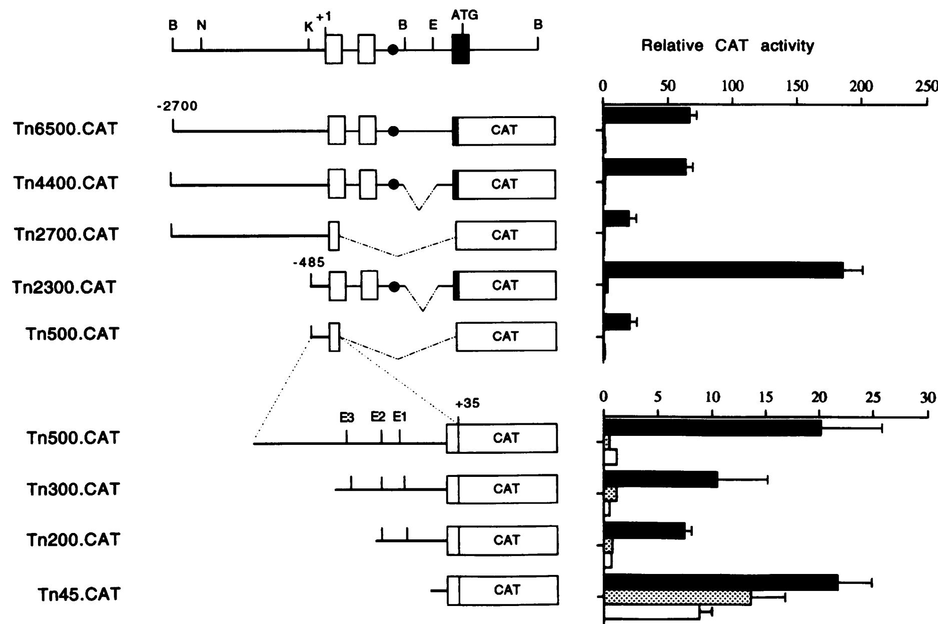


FIG. 4. Identification of regulatory elements in the rat TnI_{slow} gene directing cell-type- and development-specific transcription. A partial restriction endonuclease map of the portion of the rat TnI_{slow} gene used in creating fusion constructs is shown at the top (not drawn to scale). Restriction sites: B, *Bam*HI; N, *Nde*I; K, *Kpn*I; E, *Eco*RI. Open boxes represent the 5′ noncoding exons, the filled box represents the first coding exon, +1 is the transcription start site, and ATG is the initiator methionine. The filled circle represents the IRE homology (see the text). Schematic representations of rat TnI-CAT reporter constructs employed in transient transfection assays are shown below the restriction map. Tn6500.CAT contains 2.7 kb of upstream sequences and the intragenic region including the first two noncoding exons and two introns. All other constructs are deletion mutants of Tn6500.CAT, and intragenic deletions are indicated by dashed lines. Tn4400.CAT has an internal deletion within the second intron between +1730 and +2430. The intragenic region is deleted in construct Tn2700.CAT, which contains nucleotides between −2700 and +35. Tn2300.CAT contains 485 bp of the 5′-flanking sequence and the internal deletion within the second exon. Tn500.CAT through Tn45.CAT are serial 5′ deletion constructs. Graphic representations of CAT activities in transiently transfected C2C12 myotubes (filled bars), C2C12 myoblasts (stippled bars), and NIH 3T3 fibroblasts (open bars) for each construct are shown on the right. The CAT activity levels obtained with each construct have been normalized to the levels obtained with pCATcontrol. The data represent the mean values for four or more independent transfections. Cells were cotransfected with a pSVβgal expression plasmid to correct for transfection efficiencies.

unit gene of the nicotinic acetylcholine receptor (48). Therefore, the minimal region necessary for cell-type- and stage-specific transcription of the TnI_{slow} gene resides between −200 and −45.

The 0.5 kb of the upstream sequence and 1.7 kb of intragenic sequence of the rat TnI_{slow} gene were scanned for previously described *cis*-acting elements that might participate in the transcriptional regulation. A computer-aided search, using the program Findpattern of the Genetics Computer Group (15), revealed the existence of several transcription-regulatory *cis*-acting elements found in other muscle-specific enhancers, as well as cellular and viral enhancers. As shown in Fig. 2, three MyoD binding sites (E boxes) are located in the upstream region of the TnI_{slow} gene at positions −51, −78, and −250 relative to the transcription start site. Two AT-rich sequences are found at positions −25 to −32 and −56 to −66. The proximal sequence at −25 (TTTTCATA) might function as a TATA box, while the distal sequence at −56 (CTATTTTTAC) is similar to the MEF-2 binding site. The MEF-2 site has been identified for enhancers of many muscle-specific genes (24). In addition, a potential CArG motif (CTTTAATG) is positioned at −323. A region within the second intron, spanning nucleotides +1624 to +1669, shares many features of the muscle-specific enhancer (internal regulatory element [IRE]) of the quail TnI_{fast} gene (34) (see Discussion).

Differential *trans* activation of the rat TnI_{slow} promoter by the myogenic factors MyoD, myogenin, and MRF4. The presence of potential E boxes in the upstream regulatory region and within the second intron of the TnI_{slow} gene (see above) prompted us to investigate whether the TnI-CAT constructs could be transactivated in NIH 3T3 fibroblasts by forced expression of MyoD-related transcription factors. To examine the individual roles of the upstream and intragenic regions, three different TnI-CAT constructs representing different regions of the TnI_{slow} gene were tested in transactivation experiments. The constructs tested were Tn6500.CAT (long upstream and intragenic regions), Tn2300.CAT (short upstream and intragenic regions), and Tn500.CAT (only the short upstream region). As shown in Fig. 5, transcription of all three constructs was activated by MyoD and myogenin. The relative level of transactivation observed with these constructs paralleled the pattern observed with C2C12 myotubes, suggesting that the interactions between the upstream and the internal regulatory regions are necessary for full TnI_{slow} gene activation. An interesting pattern of transactivation of the TnI-CAT constructs was obtained with the myogenic factor MRF4; it increased transcription from constructs (Tn2300.CAT and Tn500.CAT), containing 485 bp of the upstream sequence, but it failed to transactivate the construct (Tn6500.CAT) containing sequences extending to −2700. MRF4 also failed to transactivate Tn4400.CAT, which has the same intragenic deletion as Tn2300.CAT but extends to −2700 (data not shown). These results suggest

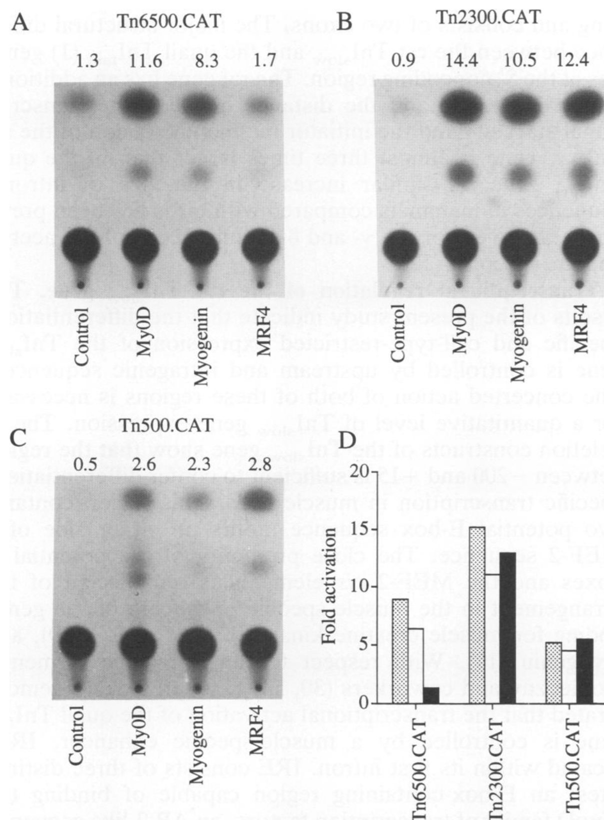


FIG. 5. Transactivation of TnI-CAT reporter constructs in fibroblasts by the muscle regulatory factors MyoD, myogenin, and MRF4. (A to C) Analysis of CAT activity from NIH 3T3 cells following transient transfection with CAT reporter constructs alone (control) or with reporter constructs mixed with 2 μg of either the MyoD, the myogenin, or the MRF4 expression vector. A representative CAT assay for each reporter construct is shown. CAT activity was normalized to equal β-galactosidase activity obtained from cotransfection with plasmid pSVβgal. (D) Transactivation by the myogenic factors relative to control values. Stippled bars, MyoD; open bars, myogenin; filled bars, MRF4.

that sequences residing between −2700 and −485 may interfere with MRF4 transactivation. The inability of MRF4 to transactivate certain muscle-specific enhancers has previously been reported (see Discussion).

Regulatory sequences of the $\mathrm{TnI_{slow}}$ gene confer tissue- and muscle-type-specific expression in transgenic mice. Although differentiation-specific activation of the $\mathrm{TnI_{slow}}$ gene is muscle cell autonomous, several extrinsic factors are known to modulate fibertype specification during skeletal muscle maturation. In order to begin characterizing the *cis*-acting sequences that confer skeletal muscle- and fiber-type-specific expression to the $\mathrm{TnI_{slow}}$ gene, we have generated and characterized transgenic mice carrying germ line integration of a TnI-CAT fusion construct. Transgenic mice were generated by injecting a 7.3-kb *Nde*I fragment excised from plasmid Tn6500.CAT (see Materials and Methods). Using Southern blot, we determined that two of the founder lines analyzed, TnI-548 (~40 copies) and TnI-424 (~10 copies), had integrated multiple copies of the fragment in a head-to-tail fashion and had germ line transmission (data not shown). To determine whether transcription of the CAT reporter was tissue specific, we made extracts from muscle, brain, heart,

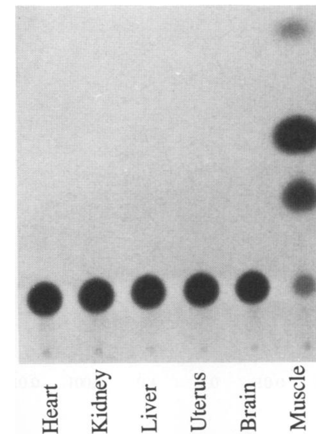


FIG. 6. $\mathrm{TnI_{slow}}$ regulatory sequences direct skeletal muscle specificity transcription in transgenic mice. A representative CAT assay showing distribution of CAT activity in various tissues from adult mice derived from the transgenic line TnI-548. Each CAT assay included equivalent amounts of extract containing 1 μg of protein and incubated for 1 h at 37°C.

liver, kidney, and uterus tissues of 8-week-old transgenic mice and assayed for CAT expression. As shown in Fig. 6, high levels of CAT activity were found in the skeletal muscle but not in any other tissues, including those of cardiac and smooth muscles. These results demonstrate that the TnI regulatory regions restrict transcription of a reporter gene to the skeletal muscle in vivo.

The endogenous $\mathrm{TnI_{slow}}$ gene is expressed exclusively in slow-twitch fibers. As a first step to determine whether the sequences responsible for slow fiber-type-specific expression of the $\mathrm{TnI_{slow}}$ gene are retained in the TnI-CAT transgene, we compared the levels of CAT activity in different hind limb muscles of adult transgenic mice. Initially, CAT assays were performed with extracts prepared from the soleus and the extensor digitorum longus (EDL) at different protein concentrations to quantitate CAT levels in the linear portions of the assay. These two muscles were chosen because they are composed of different myofiber types; the EDL has predominantly type II (fast) fibers, whereas the mouse soleus has approximately equal numbers of type I (slow) and type II myofibers. As shown in Fig. 7A, the levels of CAT activity are approximately 50-fold higher in the soleus than in the EDL muscles of mice derived from line TnI-548. Quantitation of CAT levels in two additional muscles that are predominantly composed of type II fibers, the superficial gastrocnemius and tibialis, showed that transcription was dramatically higher in the solei of the two TnI lines compared (Fig. 7B). The absolute levels of CAT activity varied about fivefold in muscles of these two lines, possibly because of a difference in copy number or integration site. Nevertheless, the ratios of CAT activity in the soleus to the activity in the other three muscles were similar in the two transgenic lines. These results indicate that transcription is the major mechanism confining $\mathrm{TnI_{slow}}$ expression to the slow-twitch muscle and that the regulatory elements reside in the sequences used to generate the transgenic mice.

DISCUSSION

The genes encoding muscle TnI isoforms serve as molecular targets for diverse regulatory signals during development and maturation of the skeletal muscle. Although the

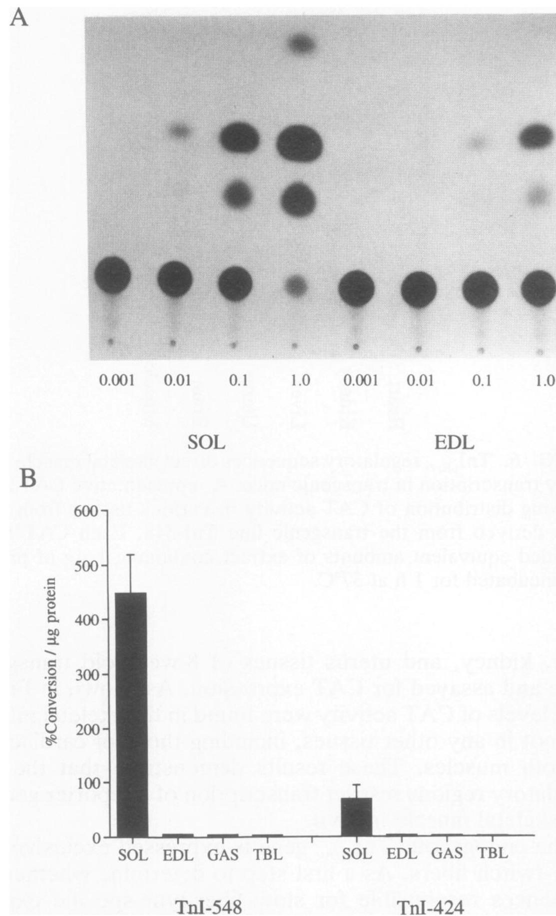


FIG. 7. Fiber-type-specific transcription of the TnI-CAT construct in transgenic mice. (A) Analysis of CAT activity using different amounts of protein from extracts made from the soleus (SOL), containing predominantly slow-twitch fibers (first four lanes), and EDL, containing fast-twitch fibers (last four lanes). The amount of protein used in each assay is shown below each lane. (B) Distribution of CAT activity in various hind limb muscles of adult mice from the transgenic lines TnI-548 and TnI-424. The CAT assays were performed for 1 h at 37°C with protein concentrations that maintained the assay in the linear range (see above). Four mice from each line were analyzed. GAS, gastrocnemius; TBL, tibialis.

transcription of these genes is activated in terminally differentiated myotubes, their expression is restricted to specific fiber types in the mature muscle. We have chosen the slow isoform of the contractile protein TnI as a model system to investigate the transcriptional basis of isoform gene expression.

Cloning of the rat TnI_{slow} gene. In this article, we focus on the characterization of the 5′ portion of the rat TnI_{slow} gene and its upstream region. We isolated genomic clones using a 37-mer oligonucleotide probe corresponding to the 5′ end of rat cDNA. The 5′-end-specific probe was used because a previous report on the structure of the quail TnI_{fast} gene (1) revealed that the exons towards the 5′ end of the gene are rather short and flanked by large intronic sequences. Since the reported sequence of TnI_{slow} cDNA (28) lacked the 5′-most sequence, we used the RACE technique to clone the full-length 5′ noncoding region and obtain sequences to screen the library. Analysis of the rat TnI_{slow} gene structure revealed that the 5′ untranslated portion of this gene is 3.7 kb long and consists of two exons. The major structural difference between the rat TnI_{slow} and the quail TnI_{fast} (1) genes lies at the 5′ noncoding region. The rat gene has an additional noncoding exon, and the distance between the transcriptional start site and the initiator methionine codon of the rat TnI_{slow} gene is almost three times larger than in the quail TnI_{fast} gene. A similar increase in the size of intronic sequences in mammals compared with birds has been previously observed for the γ- and δ-subunit genes of the acetylcholine receptor (12).

Transcriptional regulation of the rat TnI_{slow} gene. The results of the present study indicate that the differentiation-specific and cell-type-restricted expression of the TnI_{slow} gene is controlled by upstream and intragenic sequences. The concerted action of both of these regions is necessary for a quantitative level of TnI_{slow} gene expression. The 5′ deletion constructs of the TnI_{slow} gene show that the region between −200 and +15 is sufficient to confer differentiation-specific transcription in muscle cells. This region contains two potential E-box sequence motifs on either side of a MEF-2 sequence. The close proximity of the potential E boxes and the MEF-2 *cis* elements is reminiscent of the arrangement in the muscle-specific enhancers of the genes coding for muscle creatine kinase (13), MLC 1/3 (50), and myogenin (19). With respect to the intragenic elements, Konieczny and coworkers (30, 54) have previously demonstrated that the transcriptional activation of the quail TnI_{fast} gene is controlled by a muscle-specific enhancer, IRE, located within its first intron. IRE consists of three distinct sites: an E-box-containing region capable of binding the MyoD family of transcription factors, an AP-2-like sequence (site I), and a C-rich region (site II). Sequence comparison of the rat TnI_{slow} and the quail TnI_{fast} genes revealed that the former contains a 50-bp region within the second intron which is highly homologous to the quail IRE (Fig. 1B). In the rat TnI_{slow} gene, 10 of 14 nucleotides are conserved in the E-box site located between +1633 and +1647. In addition, an AP-2-like site is present immediately 5′ to the E-box site, and a C-rich region is located 9 bp downstream of this site. The location of these elements in an intron and the fact that addition of this region to a construct containing 485 bp of upstream sequences increases transcription ~10-fold strongly suggest that the IRE-like region in TnI_{slow} could also function as an enhancer. We are currently investigating how elements located in the upstream and intragenic regions may interact to direct a high level of TnI_{slow} transcription during muscle differentiation.

The muscle regulatory factors of the MyoD family are capable of transactivating cotransfected contractile protein genes in various nonmuscle cell backgrounds. Although these factors have been shown to bind to a common sequence, the mechanism of transactivation is not fully understood. The MyoD-related factors have been shown to exhibit different potentials to transactivate the muscle creatine kinase (10) and TnI_{fast} (55) enhancers, which contain canonical E-box sequences. Whereas MyoD and myogenin transactivated these enhancers, MRF4 was completely inefficient, although MRF4 did bind to the *cis* element. Sequences outside the E box might have interfered with transactivation by MRF4, since a construct containing muscle creatine kinase E boxes multimerized upstream of the herpes simplex virus thymidine kinase promoter was efficiently transactivated by MRF4. Chakraborty and Olson (10) have generated myogenin/MRF4 fusions to demonstrate that the MRF4 sequences residing outside the DNA-binding domain could account for these differences in transactivation potential. In

the present study, we show that MyoD, myogenin, and MRF4 activate transcription of the TnI_{slow} promoter with different efficiencies. All three factors transactivated the shorter TnI-CAT constructs, whereas MRF4 failed to activate the larger TnI_{slow} construct containing the region between −2700 and −485. It is tempting to speculate on the basis of these observations that inhibitory protein-protein interactions between MRF4 and a factor binding to the upstream region repress transactivation.

Fiber type specificity of the TnI-CAT transgene. Cultured muscle cells have been extensively used to study transcriptional regulation of skeletal muscle genes during differentiation. Our current understanding of myogenesis relies heavily on the wealth of information obtained with the tissue culture systems. However, the fact that cultured myocytes do not manifest specific adult fiber type properties has considerably hindered the progress towards understanding determinants of fiber type specification. Transgenic mice provide an alternative system for analyzing in vivo regulation of myogenesis as well as mechanisms underlying fiber type generation. We have begun this analysis by generating transgenic mice carrying germ line integration of a fragment containing a TnI-CAT fusion gene.

The TnI_{slow} gene is expressed in skeletal and cardiac muscles during fetal and neonatal development (34). After birth, expression in cardiac tissues progressively diminishes, and by day 21 expression of TnI_{slow} is restricted to slow-type fibers. Although differentiation-specific activation of the TnI_{slow} gene is muscle cell autonomous, several factors such as hormones and innervation are known to modulate the fiber type specification during skeletal muscle maturation which occurs late in development. Current research seeks to understand the nature of the molecular signals that specify fiber-type-specific expression. To this extent, Hallauer et al. (26) initially reported that transgenic mice carrying the entire quail TnI_{fast} transcription unit expressed the quail mRNA specifically in fast fibers. Since the entire TnI_{fast} gene and flanking sequences were used in generating these mice, it was uncertain whether transcriptional or posttranscriptional mechanisms directed fiber-type-specific expression. Recently, Donoghue et al. (18) have shown very elegantly that transgenic mice carrying an MLC-CAT construct, containing a 1.5-kb promoter fragment and a 0.9-kb enhancer of the MLC 1/3 fast gene, expressed the CAT reporter preferentially in type II fibers. These experiments indicate that fiber-type-specific transcription of the MLC 1/3 fast gene is directed by *cis*-regulatory regions in the construct. We have characterized transgenic mice carrying TnI-CAT fusion constructs which direct transcription exclusively to the skeletal muscle. When different hind limb muscles were analyzed, the reporter gene was preferentially expressed in the soleus compared with either EDL, superficial gastrocnemius, or tibialis. Since the soleus muscle in the mouse is composed of approximately equal numbers of type I and type II fibers whereas the other muscles are predominantly composed of type II fibers, these results strongly suggest that the TnI_{slow} sequences confine transcription to slow-type fibers. To our knowledge, this report constitutes the first characterization of *cis*-regulatory sequences in transgenic mice that confine transcription to slow-type fibers. In both the MLC-CAT and the TnI-CAT transgenic mice, a small fragment of the 5′ noncoding region is transcribed with the reporter gene. An extremely unlikely possibility that merits consideration, however, is that these 5′ noncoding sequences could participate in stabilizing transcripts in specific fiber types. Future experiments will be directed towards identifying the elements that developmentally regulate the TnI_{slow} gene and confine its expression to the slow-twitch muscle.

ACKNOWLEDGMENTS

We are grateful for the generous gifts of cDNAs from K. Hastings (rat TnI), A. Lassar and H. Weintraub (MyoD), E. Olson and W. Wright (myogenin), and S. Konieczny (MRF4). We thank G. Gibney and J. Stauffer for their critical reviews of the manuscript.

The generation of transgenic mice by DNX was supported by the National Institute of Child Health and Human Development contract N01-HD-0-2911. We are grateful to the National Institute of Child Health and Human Development for continued support.

REFERENCES

1. **Baldwin, A. S., E. L. W. Kittler, and C. P. Emerson.** 1985. Structure, evolution, and regulation of a fast skeletal muscle troponin gene. Proc. Natl. Acad. Sci. USA **82**:8080–8084.
2. **Bandman, E.** 1992. Contractile protein isoforms in muscle development. Dev. Biol. **154**:273–283.

2a. **Banerjee-Basu, S., and A. Buonanno.** Unpublished data.

3. **Boxer, L. M., R. Prywes, R. G. Roeder, and L. Kedes.** 1989. The sarcomeric actin CArG-binding factor is indistinguishable from c-*fos* serum response factor. Mol. Cell. Biol. **9**:515–522.
4. **Braun, T., E. Bober, B. Winter, N. Rosenthal, and H. H. Arnold.** 1990. Myf-6, a new member of the human gene family of myogenic determination factors: evidence for a gene cluster on chromosome 12. EMBO J. **9**:821–831.
5. **Braun, T., G. Buschhausen-Denker, E. Bober, E. Tannich, and H. H. Arnold.** 1989. A novel human muscle factor related to but distinct from MyoDI induces myogenic conversion in 10T1/2 fibroblasts. EMBO J. **8**:701–709.
6. **Braun, T., E. Tannich, G. Buschhausen-Denker, and H.-H. Arnold.** 1989. Promoter upstream elements of the chicken cardiac myosin light-chain 2-A gene interact with *trans*-acting regulatory factors for muscle-specific transcription. Mol. Cell. Biol. **9**:2513–2525.
7. **Bucher, E. A., P. C. Maisonpierre, S. F. Konieczny, and C. P. Emerson, Jr.** 1988. Expression of the troponin complex genes: transcriptional coactivation during myoblast differentiation and independent control in heart and skeletal muscles. Mol. Cell. Biol. **8**:4134–4142.
8. **Buckingham, M.** 1992. Making muscle in mammals. Trends Genet. **8**:144–148.
9. **Buonanno, A., L. Casabo, J. Kornhauser, C. M. Crowder, and J. P. Merlie.** 1989. Transcriptional regulation of acetylcholine receptor genes in transfected muscle cells and transgenic mice. NATO ASI Ser. Ser. H **32**:541–552.
10. **Chakraborty, T., and E. N. Olson.** 1991. Domains outside of the DNA-binding domain impart target gene specificity to myogenin and MRF4. Mol. Cell. Biol. **11**:6103–6108.
11. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
12. **Crowder, C. M., and J. P. Merlie.** 1988. Stepwise activation of the mouse acetylcholine receptor δ- and γ-subunit genes in clonal cell lines. Mol. Cell. Biol. **8**:5257–5267.
13. **Cserjesi, P., and E. N. Olson.** 1991. Myogenin induces the myocyte-specific enhancer binding factor MEF-2 independently of other muscle-specific gene products. Mol. Cell. Biol. **11**: 4854–4862.
14. **Davis, R. L., H. Weintraub, and A. B. Lassar.** 1987. Expression of a single cDNA converts fibroblasts to myoblasts. Cell **51**:987–1000.
15. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
16. **Dhoot, D. J., and V. P. Perry.** 1980. The components of the troponin complex and development in skeletal muscle. Exp. Cell Res. **127**:75–87.
17. **Donoghue, M., H. Ernest, B. Wentworth, B. Nadal-Ginard, and N. Rosenthal.** 1988. A muscle specific enhancer is located at the

3′ end of the myosin light-chain 1/3 locus. Genes Dev. **2**:1779–1790.
18. **Donoghue, M. J., J. D. Alvarez, J. P. Merlie, and J. R. Sanes.** 1991. Fiber type- and position-dependent expression of a myosin light chain-CAT transgene detected with a novel histochemical stain for CAT. J. Cell Biol. **115**:423–434.
19. **Edmondson, D. G., T. C. Cheng, P. Cserjesi, T. Chakraborty, and E. N. Olson.** 1992. Analysis of the myogenin promoter reveals an indirect pathway for positive autoregulation mediated by the muscle-specific enhancer factor MEF-2. Mol. Cell. Biol. **12**:3665–3677.
20. **Edmonston, D. G., and E. N. Olson.** 1989. A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes Dev. **3**:628–640.
21. **Ernst, H., K. Walsh, C. A. Harrison, and N. Rosenthal.** 1991. The myosin light chain enhancer and the skeletal actin promoter share a binding site for factors involved in muscle-specific gene expression. Mol. Cell. Biol. **11**:3735–3744.
22. **Frohman, M. A., M. K. Dush, and G. R. Martin.** 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA **85**:8998–9002.
23. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
24. **Gossett, L. A., D. J. Kelvin, E. A. Sternberg, and E. N. Olson.** 1989. A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. Mol. Cell. Biol. **9**:5022–5033.
25. **Graham, F. L., and A. J. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus DNA. Virology **52**:456–467.
26. **Hallauer, P. L., K. E. M. Hastings, and A. C. Peterson.** 1988. Fast skeletal muscle-specific expression of a quail troponin I gene in transgenic mice. Mol. Cell. Biol. **8**:5072–5079.
27. **Konieczny, S. F., and C. P. Emerson, Jr.** 1987. Complex regulation of the muscle-specific contractile protein (troponin I) gene. Mol. Cell. Biol. **7**:3067–3075.
28. **Koppe, R. I., P. L. Hallauer, G. Karpati, and K. E. Hastings.** 1989. cDNA clone and expression analysis of rodent fast and slow skeletal muscle troponin I mRNAs. J. Biol. Chem. **264**:14327–14333.
29. **Kraft, R., J. Tardiff, K. S. Krauter, and L. A. Leinwand.** 1988. Using mini-prep plasmid DNA for sequencing double stranded templates with Sequenase. BioTechniques **6**:544–546, 549.
30. **Lin, H., K. E. Yutzey, and S. F. Konieczny.** 1991. Muscle-specific expression of the troponin I gene requires interactions between helix-loop-helix muscle regulatory factors and ubiquitous transcription factors. Mol. Cell. Biol. **11**:267–280.
31. **Mar, J. H., and C. P. Ordhal.** 1988. A conserved CATTCCT motif is required for skeletal muscle-specific activity of the cardiac troponin T gene promoter. Proc. Natl. Acad. Sci. USA **85**:6404–6408.
32. **Miner, J. H., and B. Wold.** 1990. Herculin, a fourth member of the MyoD family of myogenic factors. Proc. Natl. Acad. Sci. USA **87**:1089–1093.
33. **Miwa, T., and L. Kedes.** 1987. Duplicated CArG box domains have positive and mutually dependent regulatory roles in expression of the human α-cardiac actin gene. Mol. Cell. Biol. **7**:2803–2813.
34. **Murphy, A. M., L. Jones II, H. F. Sims, and A. W. Strauss.** 1991. Molecular cloning of rat cardiac troponin I and analysis of troponin I isoform expression in developing heart. Biochemistry **30**:707–712.
35. **Murre, C. P., S. McCaw, H. Vaessin, M. Candy, C. V. Cabrera, J. N. Baskin, S. D. Hauschka, A. B. Lassar, H. Weintraub, and D. Baltimore.** 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell **58**:537–544.
36. **Nielsen, D. A., J. Chou, A. J. MacKrell, M. J. Casadaban, and D. F. Steiner.** 1983. Expression of a preproinsulin-β-galactosidase gene fusion in mammalian cells. Proc. Natl. Acad. Sci. USA **80**:5198–5202.
37. **Olson, E. N.** 1990. MyoD family: a paradigm for development? Genes Dev. **4**:1454–1461.
38. **Parmacek, M. S., A. R. Bengur, A. J. Vora, and J. M. Leiden.** 1990. The structure and regulation of the murine fast skeletal Tn C gene. J. Biol. Chem. **265**:15970–15976.
39. **Parmacek, M. S., and J. M. Leiden.** 1989. Structure and expression of the murine slow/cardiac troponin C gene. J. Biol. Chem. **264**:13217–13225.
40. **Pette, D., and R. S. Staron.** 1990. Cellular and molecular diversities of mammalian skeletal muscle fibers. Rev. Physiol. Biochem. Pharmacol. **116**:1–76.
41. **Rhodes, S. J., and S. F. Konieczny.** 1989. Identification of MRF4: a new member of the muscle regulator gene family. Genes Dev. **3**:2050–2061.
42. **Rosenthal, N.** 1989. Muscle cell differentiation. Curr. Opin. Cell Biol. **1**:1094–1101.
43. **Rosenthal, N., E. B. Berglund, B. M. Wentworth, M. Donoghue, B. Winter, E. Bober, T. Braun, and H. H. Arnold.** 1990. A highly conserved enhancer downstream of the human MLC1/3 locus is a target for multiple myogenic determination factors. Nucleic Acids Res. **18**:6239–6246.
44. **Shimizu, N., E. Dizon, and R. Zak.** 1992. Both muscle-specific and ubiquitous nuclear factors are required for muscle-specific expression of myosin heavy-chain β gene in cultured cells. Mol. Cell. Biol. **12**:619–630.
45. **Sternberg, E. A., G. Spizz, W. M. Perry, D. Vizard, T. Weil, and E. N. Olson.** 1988. Identification of upstream and intragenic regulatory elements that confer cell-type-restricted and differentiation-specific expression on the muscle creatine kinase gene. Mol. Cell. Biol. **8**:2896–2909.
46. **Stockdale, F. E., and J. B. Miller.** 1987. The cellular basis of myosin heavy chain isoform expression during development of avian skeletal muscles. Dev. Biol. **123**:1–9.
47. **Wade, R., R. Eddy, T. B. Shows, and L. Kedes.** 1990. cDNA sequence, tissue-specific expression, and chromosomal mapping of the human slow-twitch skeletal muscle isoform of troponin I. Genomics **7**:346–357.
48. **Wang, X., H. Tsay, and J. Schimdt.** 1990. Expression of the acetylcholine receptor δ-subunit gene in differentiating chick muscle cells is activated by an element that contains two 16 bp copies of a segment of the α-subunit enhancer. EMBO J. **9**:783–790.
49. **Weintraub, H., R. Davis, R. Tapscott, M. Thayer, M. Krause, R. Benezra, T. K. Blackwell, D. Turner, R. Rupp, S. Hollenberg, Y. Zhuang, and A. B. Lassar.** 1991. The myoD gene family: nodal point during specification of the muscle cell lineage. Science **251**:761–766.
50. **Wentworth, B. M., M. Donoghue, J. C. Engert, E. B. Berglund, and N. Rosenthal.** 1991. Paired MyoD-binding sites regulate myosin light chain gene expression. Proc. Natl. Acad. Sci. USA **88**:1242–1246.
51. **Wilkinson, J. M., and R. J. A. Grand.** 1978. Comparison of amino acid sequence of troponin I from different striated muscles. Nature (London) **270**:31–35.
52. **Wright, W. E., D. A. Sasson, and V. K. Lin.** 1989. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. Cell **56**:607–617.
53. **Yu, Y., R. E. Breitbart, L. B. Smoot, Y. Lee, V. Mahadavi, and B. Nadal-Ginard.** 1992. Human myocyte specific enhancer factor 2 comprises a group of tissue-restricted MADS box transcription factors. Genes Dev. **6**:1783–1798.
54. **Yutzey, K. E., R. L. Kline, and S. F. Konieczny.** 1989. An internal regulatory element controls troponin I gene expression. Mol. Cell. Biol. **9**:1397–1405.
55. **Yutzey, K. E., S. J. Rhodes, and S. F. Konieczny.** 1990. Differential *trans* activation associated with the muscle regulatory factors MyoD1, myogenin, and MRF4. Mol. Cell. Biol. **10**:3934–3944.
56. **Zot, A., and J. D. Potter.** 1987. Structural aspects of troponin-tropomyocin regulation of skeletal muscle contraction. Annu. Rev. Biophys. Chem. **16**:535–539.