M-CAT Binding Factor, a Novel *trans*-Acting Factor Governing Muscle-Specific Transcription

JANET H. MAR AND CHARLES P. ORDAHL*

Department of Anatomy, University of California, San Francisco, San Francisco, California 94145-0452

Received 22 January 1990/Accepted 2 May 1990

The cardiac troponin T (cTNT) promoter contains a highly muscle specific distal promoter element capable of conferring muscle-specific transcription from a heterologous TATA box-transcription initiation site. Three sequence motifs within this distal promoter element are conserved in the promoter and regulatory regions of many sarcomeric protein genes. Mutational analysis demonstrated that homologies to two of these conserved motifs (CArG/CBAR and MEF 1) were not required for activity of cTNT promoter-marker gene constructs in transfected embryonic skeletal muscle cells. In contrast, disruption of either or both copies of the conserved M-CAT motif (5'-CATTCT-3') inactivated the cTNT promoter in these cells. Both M-CAT motifs were protected from DNase I cleavage in solution footprint assays by an M-CAT binding factor (MCBF) present in nuclear extracts from embryonic muscle tissue. M-CAT mutations that inactivated the cTNT promoter also disrupted MCBF binding, indicating that MCBF may be a key *trans*-acting factor required for muscle-specific expression of the cTNT promoter. MCBF also bound to the M-CAT motif in the distal promoter region of the skeletal α -actin gene, suggesting that it may play a role in the regulation of this and perhaps other muscle genes that contain M-CAT motifs.

Myogenesis is a complex, multistep process involving (i) progenitor cell determination to the myogenic lineage, (ii) migration of myogenic stem cells to appropriate locations, (iii) multiplication and alignment of myogenic precursor cells with one another and with nonmyogenic cells such as those in tendons, (iv) terminal differentiation and sarcomerogenesis, and (v) modulation of the terminally differentiated state in response to developmental and physiological cues. Myogenic terminal differentiation is readily reproduced in culture, and as a result, a great deal of progress has recently been made regarding the regulation of sarcomeric protein genes that are coexpressed during terminal differentiation (6, 7, 13, 24, 58). A central question has been whether coexpression of the various unlinked contractile protein genes is governed by common or diverse *trans*-acting factors. Pursuant to this question, a number of potential *cis* regulatory sequences conserved among many contractile protein genes have been identified (8, 40, 45, 47).

Recently, the role(s) of such conserved sequence motifs has begun to yield to experimental analysis. A simple, common regulatory mechanism has not emerged. For example, transcription of sarcomeric actin genes is governed by the interaction between a distal promoter element, dubbed the CArG or CBAR motif (1, 40), and nuclear factors in skeletal muscle nuclei (23, 44, 55) which resemble the ubiquitous serum response factor (2, 48, 53). On the other hand, enhancer-type elements located either upstream or downstream of the promoter region appear to be important for the muscle-specific expression of the genes encoding myosin light chain 1/3 (14) and creatine kinase (25, 26, 51). The skeletal (fast) troponin I gene contains a muscle-specific enhancer in its first intron (30, 60), and recent experiments indicate that the promoter of this gene also contains multiple interactive elements which govern muscle-specific expression (46). At least one essential component of the enhancer of the mouse creatine kinase gene, and probably the enhancers of the myosin light-chain 1/3 and skeletal troponin I genes

as well, is a conserved MEF 1 motif (8) that is similar to the E12 enhancer motif of immunoglobulin κ -chain genes (43). Recent evidence indicates that the MEF 1 motif interacts in a sequence-specific fashion with MyoD (33), a myogenic determination factor that can convert many cell types to the muscle lineage (12, 57). Since the region of MyoD which binds to the MEF 1 motif is highly conserved among the growing family of myogenic determination factors (5, 16, 35, 52, 59) and other more ubiquitous transcriptional regulatory factors (43), it is becoming increasingly evident that at least one of the roles of such factors is to govern transcription of individual muscle-specific genes. Thus, the presence of potentially MyoD-responsive, MEF 1 binding motifs on many muscle-specific genes suggests a mechanism by which expression of diverse genes might be coordinated (33). Moreover, the increased DNA-binding affinity of heterodimers between MyoD and other transcription factors (42) strongly suggests that coordination among diverse muscle regulatory elements may be mediated via combinatorial protein-protein interactions. However, as indicated above, not all muscle genes have MEF 1 binding homologies in regions known to be critical for their expression. In addition, many of the sarcomeric protein genes that are expressed in the skeletal muscle lineage are also expressed in the cardiac muscle lineage. Since MyoD and related proteins are not expressed in cardiac muscle, it is likely that cardiac muscle may have its own complement of complex regulatory factors acting upon these same genes. It is unknown whether the activation and regulation of such genes is independent of or coordinated with those which are MyoD responsive.

We have chosen to study the regulation of the cardiac troponin T (cTNT) gene because its developmental expression indicates that it is subject to common as well as divergent regulatory programs during striated muscle development. The cTNT gene is constitutively transcribed throughout cardiac development, although its rate of transcription increases dramatically during the fetal period (9, 36). In developing skeletal muscle, on the other hand, the cTNT gene is transcribed only transiently. Its transcription

* Corresponding author.

is activated at the onset of terminal differentiation along with the other sarcomeric protein genes. During mid-fetal development, its transcription is abruptly repressed (36), and it remains inactive in adult muscle unless it is activated in satellite cells during skeletal muscle regeneration (T. A. Cooper and E. Bandman, unpublished observation).

Our previous experiments indicated that separate and distinct upstream regions are required for expression of the cTNT gene in embryonic cardiac and skeletal muscle cells (38). Expression in skeletal muscle cells requires only the minimal promoter (about 130 nucleotides upstream of the transcription initiation site at +1), whereas expression in cardiac myocytes requires, in addition to the minimal promoter region, an upstream segment residing between -268 and -201 (38; R. Iannello and C. Ordahl, submitted for publication). Chimeric promoter experiments further indicate that the distal segment of the cTNT minimal promoter (nucleotides -129 to -50) can confer skeletal muscle-specific expression when recombined with appropriate proximal segments of a heterologous, nonmuscle promoter (39). Thus, discrete regulatory domains in the upstream region of the cTNT gene interact to bring about its cell-specific regulation during development.

Here we identify the sequence elements within the skeletal muscle-specific distal promoter region of the cTNT minimal promoter that are required for its activity in skeletal muscle cells. Our results indicate that although nominal CARG/CBAR and MEF 1 motifs are present in this region, both are dispensable for muscle-specific transcriptional activity. On the other hand, disruption of either or both copies of a conserved M-CAT motif, 5'-CATTCCT-3' (39; this report), completely inactivates the cTNT promoter in skeletal muscle cells. Using DNase I footprint and gel shift analyses, we show further that the activity of the M-CAT motifs is correlated with binding of a novel transcriptional regulatory factor, MCBF (M-CAT binding factor). The possible relationship(s) between MCBF and the other muscle gene regulatory factors mentioned above is discussed.

MATERIALS AND METHODS

Plasmid constructions, probes, and competitors. The construction of recombinant clones containing cTNT promoter sequences ligated directly in front of the bacterial chloramphenicol acetyltransferase (CAT) (21) gene to direct its expression has been described (38, 39). The cTNT-CAT clone containing only 129 base pairs (bp) of cTNT promoter sequence (cTNT-129-CAT; Fig. 1a; see references 38 and 39 for construction) was modified by deletion of the polylinker sites between the cTNT promoter and the CAT gene by partial *Sma*I and *Hind*III digestion, followed by religation. 5' deletion mutants were generated from the resulting plasmid by BAL 31 digestion from the unique *Cla*I site at the 5' end of cTNT-129-CAT. Except for clone d (Fig. 1), which was generated by direct recircularization, a *Cla*I linker was added to the deleted ends, and the plasmid was secondarily digested with *Hind*III. Truncated cTNT promoter fragments were recloned into pBRCAT between the *Cla*I and *Hind*III sites. To facilitate subsequent mutations, all cTNT-CAT constructs were recloned into pBluescript K/S (+) between the *Cla*I and *Bam*HI sites. Clustered point mutants i to l (Fig. 1) were generated by substitution of the unique *Nar*I fragment in cTNT-129-CAT (Fig. 1a):

5'-CGCCGGGACATTCTGCTGCTGCTGCCCGCCCCGGGGTGGG -3'
3'-GGCCCGTGAAGGACGACGAGACGGGCGGGCCCCACCCGC-5'

with a double-stranded synthetic oligonucleotide (Operon

Technologies, San Pablo, Calif.), the sequence of which corresponds to the above fragment but contains specific nucleotide changes as indicated in Fig. 1. The construction of mutant h has been previously described (39). Mutant m was generated by ligation of the mutant *Nar*I oligonucleotide into *Nar*I-digested mutant h. All mutations and deletions were confirmed by restriction endonuclease mapping and sequenced by using Sequenase (U.S. Biochemical Corp., Cleveland, Ohio). Spacing mutants between the two M-CAT motifs were generated by using mutant i, which contains a *Bam*HI site between the M-CAT motifs as a result of the oligonucleotide-directed mutation. The nucleotide changes in mutant i do not affect the activity of the cTNT promoter (see Results and Fig. 1). The spacing between the two M-CAT motifs was decreased by four nucleotides by *S*1 nuclease treatment after partial *Bam*HI digestion, followed by recircularization. Four additional nucleotides were introduced between the M-CAT motifs by partial digestion of mutant i with *Bam*HI, followed by recircularization after filling in the overhanging ends with reverse transcriptase. To insert 10 nucleotides between the M-CAT motifs, a synthetic *Bam*-*Sma* adaptor was directly ligated into the *Bam*HI site of mutant i. Insertion mutants with longer inserts (100 and 400 bp) between the M-CAT motifs were generated by ligation of size-selected *Escherichia coli* *Mbo*I-digested DNA fragments into the *Bam*HI site of mutant i. The structure of each of these insertion and deletion mutants was confirmed by restriction mapping and sequencing.

Probes for DNase I footprinting were derived from cTNT-129-CAT (Fig. 1a) by partial *Nar*I digestion, followed by complete digestion with either *Kpn*I or *Xho*I. The fragments corresponding to the cTNT sequence between -129 to -41 were cloned into pBluescript K/S (+) between the *Cla*I and *Kpn*I or *Xho*I sites of the polylinker. Probes for DNase I footprinting were generated by cleavage at the *Xho*I and *Xba*I sites in the polylinker. This produced a DNA fragment that contains the cTNT promoter sequence and approximately 20 bp of polylinker sequence at both the 5' and 3' ends of the cTNT sequence. The coding strand was labeled with [α -³²P]dideoxy-ATP (5,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) and terminal transferase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The noncoding strand was ³²P labeled by using γ ATP (6,000 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) and T4 polynucleotide kinase (Pharmacia, Inc., Piscataway, N.J.). Chicken skeletal α -actin promoter probe was isolated from α -actin-CAT (C. P. Ordahl, unpublished construction) by *Hind*III and *Sma*I digestion. The resultant fragment spans the actin promoter region from -196 to +28. Coding and noncoding strands were labeled as described above.

Competitors in either DNase I footprint or mobility shift assay consisted of gel-isolated cTNT fragments or double-stranded synthetic oligonucleotides as indicated in the figure legends. The sequence of the M-CAT 1 oligonucleotide is 5'-CGTGTGCATTCCTCTCTGGATC-3'; and sequence of the mutant M-CAT 2 oligonucleotide sequence is 5'-CGCCGGGACAGTACCTGCTGCTCTGCCCGCCCCGGGGTGGG-3'. Complementary strands of each oligonucleotide were annealed before they were used either as probes or as competitors. Oligonucleotide probe was ³²P labeled by using γ ATP and T4 polynucleotide kinase. All recombinant DNA methods were performed by standard protocols (37), and enzymes were used as directed by the manufacturers.

Cell culture, transfection, and CAT assay. Primary breast muscle cells and embryonic fibroblasts were obtained from day 11 chicken embryos as previously described (38). For

transfection experiments, cells were plated at 10^6 cells per 60-mm culture dish. Cells were transfected by the DNA-calcium phosphate precipitation method as described previously (38), using 5 μ g of test plasmid per dish. After 16 to 18 h of incubation at 37°C, the medium containing the precipitates was removed, and the cells were washed with serum-free medium and fed with fresh medium. Forty-eight hours after transfection, cells were harvested and assayed for CAT enzymatic activity, using [14 C]chloramphenicol (Amersham) (20). Activity of each cTNT-CAT construction was determined from multiple transfection experiments, using precautions as previously described (38) to minimize differences due to transfection variability.

Preparation of nuclear extracts from chicken tissues. Muscle nuclear extracts were prepared from skeletal or heart muscle tissues from day 12 to 13 and day 18 to 19 chicken embryos. Muscle nuclei were isolated by using published protocols (22, 34), with the following modifications. Breast or heart muscle tissues were dissected from embryos, rinsed in phosphate-buffered saline, and stored in ice-cold buffer A (61) containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM sodium pyrophosphate (pH 6.8), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 μ g each of leupeptin and aprotinin per ml. After 15 min, the tissues were rinsed twice with ice-cold buffer B (61) consisting of 50 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 1 mM sodium pyrophosphate (pH 6.8), 1 mM PMSF, and 2 μ g each of leupeptin and aprotinin per ml. The tissues (10 to 15 g) were then washed with 100 ml of homogenization buffer (10 mM HEPES [pH 7.6], 25 mM KCl, 1 mM EDTA, 1 mM dithiothreitol [DTT], 0.15 mM spermine, 0.5 mM spermidine, 1.8 M sucrose, 5% glycerol, 0.5 mM PMSF, 2 μ g of leupeptin per ml, and 2 μ g of aprotinin per ml) and then transferred to 200 ml of fresh homogenization buffer. The tissues were disrupted by mechanical homogenization with an Ultra-Turrax homogenizer (Tekmar) at 60% power with two to three 30-s pulses. Care was taken to avoid foaming during homogenization. Cell lysis and liberation of nuclei were monitored microscopically. Typically, greater than 90% of the muscle nuclei were liberated after three 30-s pulses. Samples (27 ml) of the homogenate were layered over a 10-ml cushion of the same homogenization buffer in centrifuge tubes and centrifuged at 25,000 rpm for 60 min at 4°C in a Beckman SW27 rotor. Pelleted nuclei were suspended in lysis buffer (10 mM HEPES [pH 7.6], 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.76 mM spermidine, 0.167 mM spermine, 0.1 mM PMSF, 2 μ g each of leupeptin and aprotinin per ml, 10% glycerol). The procedures for lysis of nuclei and extraction of nuclear proteins were exactly as described by Lichtsteiner et al. (34) except that protease inhibitors were added to the dialysis buffer. Protein concentration was determined according to Bradford (3). Nuclear extract was quick-frozen in an ethanol-dry ice bath and stored at -80°C . Extracts stored at -80°C remain active for several months.

The aforementioned modifications were required because the myofibrils in muscle tissues interfere with isolation of nuclei. Muscle tissues, washed in buffers A and B, remained in a relaxed state, resulting in more efficient liberation of nuclei. The changes in the concentrations of sucrose and glycerol in the homogenization buffer were empirically determined to better effect separation of myofibers from nuclei during centrifugation and results in nuclear preparations from heart tissues and early embryonic skeletal muscle that are virtually free of contaminating myofibrils, as judged by microscopic examination.

The same procedure was used to prepare nonmuscle

nuclear extracts (brain and liver) except that the tissues were dissected, rinsed in buffered saline, and put directly into the homogenization buffer without pretreatment in buffers A and B.

DNase I footprinting. DNase I footprinting (18) reaction mixtures consisted of 5 to 10 μ l of dialyzed nuclear extract (30 to 40 μ g of protein) or dialysis buffer, 10 mM HEPES (pH 7.9), 20 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, and 1 to 2 μ g of double-stranded poly(dI-dC) as a nonspecific competitor DNA (Pharmacia). Reaction volume was made up to 50 μ l with distilled water. This mixture was preincubated at room temperature for 10 min; an end-labeled DNA fragment (0.2 to 0.5 ng) was added, and the binding reaction was allowed to proceed at room temperature for another 10 min. Competing DNA was added at the same time as addition of probe. No significant difference was observed whether competitor DNA was added during the preincubation period or at the same time as the probe. DNase I digestion was initiated by addition of 50 μ l of 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 1 μ l of various concentrations of DNase I (1-mg/ml stock) diluted just before use in 10 mM HEPES (pH 7.6)–10 mM $MgCl_2$ –5 mM $CaCl_2$. Digestion was for 60 s at room temperature; the reaction was stopped by addition of equal volume of stop buffer containing 100 μ g of yeast tRNA per ml, 0.1% sodium dodecyl sulfate, and 10 mM EDTA. The samples were vortexed vigorously, extracted with phenol-chloroform and chloroform, and then ethanol precipitated. The DNA pellet was washed with 70% ethanol, dried, suspended in formamide-dye solution, and run on a 6 or 8% sequencing gel. After electrophoresis, the gel was exposed to film at -80°C with an intensifier screen.

Electrophoretic mobility shift assay. The gel shift assay (17, 19) reaction mixtures contained 50 mM Tris hydrochloride (pH 7.9), 2 mM $MgCl_2$, 50 mM KCl, 0.5 mM DTT, 0.5 mM EDTA, and 5% glycerol. Each reaction contained 0.1 to 0.2 ng of ^{32}P -labeled double-stranded synthetic M-CAT 1 oligonucleotide, 100 ng of poly(dI-dC) as a nonspecific competitor, and 0.5 to 1.0 μ g of protein in 25 μ l (final volume). The binding reaction (without labeled DNA) was preincubated for 10 min at room temperature. After addition of probe, the reaction mixture was incubated for another 10 min before it was electrophoresed through a 5% native polyacrylamide gel (40:1, acrylamide/bisacrylamide) in $0.5\times$ TBE (37) buffer for 1.5 to 2 h at 11 V/cm at room temperature. Where appropriate, unlabeled double-stranded oligonucleotides or DNA fragments were added as test competitors. Competitors were added to the reaction just before addition of probe. After electrophoresis, the gels were dried and autoradiographed with an intensifier screen.

RESULTS

Deletion and mutation analyses of the cTNT distal promoter region. Figure 1a shows the nucleotide sequence of the cTNT minimal promoter region from nucleotides -129 to -1 . The distal promoter region, between nucleotides -129 to -50 (shaded area in Fig. 1), was previously shown to contain a sequence element(s) sufficient to direct skeletal muscle-specific expression when recombined with a heterologous proximal promoter region (39). To localize the sequences within the cTNT distal promoter region that are responsible for its cell-specific expression, a series of promoters with deletions or clustered point mutations were analyzed for their ability to direct expression of the heterologous marker CAT gene (21) after transient transfection into embryonic skeletal muscle and fibroblast cells in culture

FIG. 1. Nucleotide sequence of deletion and clustered point mutant cTNT promoters. (a) Nucleotide sequence of the wild-type minimal skeletal muscle-specific cTNT promoter (cTNT-129). Shaded region was previously shown to contain determinants of muscle specific transcription (39). The nominal transcription elements TATA and Sp1 are indicated. The sequences designated CArG and MEF 1 show sequence similarity to regulatory motifs present in other muscle-specific genes (see text). M-CAT 1 and M-CAT 2 sequences are highlighted. (b to g) Deletion mutants generated by BAL 31 digestion of cTNT-129. (h to m) Clustered point mutants of the cTNT-129 promoter. Mutations were generated by oligonucleotide-directed mutagenesis (see Materials and Methods). Specific bases altered within each mutant are identified by black dots over nucleotides. The CAT activity of each mutant promoter relative to that directed by cTNT-129 (a), which is arbitrarily set to 100%, is derived from the absolute CAT activity values presented in Table 1.

TABLE 1. CAT activity of cTNT promoters in skeletal muscle and fibroblast cells^a

Promoter	CAT activity (10 ⁻² U) (± SEM) ^b	
	Muscle	Fibroblast
a	149 ± 10 (10)	0.59 (3)
b	140 ± 13 (4)	0.61 (3)
c	128 ± 18 (4)	0.54 (2)
d	64 ± 7 (5)	0.52 (2)
e	1.8 ± 0.8 (3)	0.56 (1)
f	1.0 ± 0.4 (3)	0.49 (1)
g	0.7 ± 0.2 (3)	0.52 (1)
h	2.4 ± 1 (7)	0.48 (3)
i	128 ± 13 (12)	0.56 (2)
j	1.0 ± 0.2 (6)	0.55 (2)
k	127 ± 20 (6)	0.55 (2)
l	41 ± 9 (7)	0.55 (2)
m	0.7 ± 0.2 (3)	0.51 (3)
pBS-CAT	1.2 ± 0.2 (10)	0.24 (3)

^a Skeletal muscle and fibroblast cells were transfected by the calcium phosphate method and harvested for assay 48 h after transfection.

^b Calculated as described elsewhere (38); the activity of the promoterless pBS-CAT has been subtracted in each case. Numbers in parentheses is the number of independent determinations.

(38). In fibroblast cells, each of the cTNT-CAT constructs showed CAT activity values no more than one to two times above background (Table 1). This result indicates that no potential negative element has been uncovered by either deletion or mutation of sequences within the cTNT minimal promoter.

In skeletal muscle cells, 5' cTNT promoter deletions with endpoints at either nucleotide -110 or nucleotide -105 are unchanged with respect to the level of CAT expression (Fig. 1b and c; see Table 1 for CAT activity data). Deletion to -99 reduced CAT activity approximately twofold (Fig. 1d), whereas deletion to more downstream positions (-92, -76, and -66) essentially eliminated CAT activity (Fig. 1e to g). These results indicate that sequences between -92 and -99, and possibly sequences downstream of -92, are essential for the activity for the minimal cTNT promoter in skeletal muscle cells.

To determine which sequences between -99 and -50 are essential for activity, a series of clustered point mutations was introduced by using oligonucleotide-directed mutagenesis (see Materials and Methods). We chose five domains for mutation: two M-CAT motifs (Fig. 1h and j), the distal-most of which was previously implicated in the activity of this promoter region (39); two G+C-rich domains (Fig. 1i and l); and a C+T-rich domain (Fig. 1k). Mutations within either M-CAT motif reduced activity of the cTNT promoter more than 50-fold. Mutations outside these M-CAT motifs had relatively little effect except for mutation within the most proximal G+C-rich domain (Fig. 1l), which reduced promoter activity approximately fourfold. A double mutation, introduced into both M-CAT motifs, also resulted in an inactive promoter (Fig. 1m). Thus, both M-CAT motifs are required for activity of the cTNT minimal promoter in skeletal muscle cells.

DNase I footprinting of the cTNT distal promoter region. To determine whether nuclear factors interact with the M-CAT motifs, we performed DNase I footprinting experiments, using nuclear protein extracts from embryonic skeletal muscle tissues. Crude nuclear extracts were prepared from the breast muscle of chicken embryos at days 12 to 13 of development, a stage of chicken development when the endogenous cTNT gene is most active in developing skeletal

FIG. 2. DNase I footprint of the distal region of the cTNT promoter. (A) Schematic drawing of the cTNT promoter and the nucleotide sequence of the region footprinted (shaded). Conserved sequence motifs indicated on the schematic drawing are shown as boldface type in the sequence. Open boxes superimposed over the sequence indicate the nucleotides protected from DNase I cleavage; asterisks indicate DNase I-hypersensitive sites. (B) Autoradiographs of footprint gels, using nuclear extracts from chicken embryonic day 12 to 13 skeletal muscle tissue. Lanes 1 and 2 and lanes 9 and 10 represent DNase I digestion patterns of noncoding and coding strands, respectively, without nuclear extract. In lanes 1 and 9, DNA was digested with 1 µl of a 1:300 dilution of DNase I stock (1 mg/ml). In lanes 2 and 10, DNA was digested with 1 µl of a 1:500 dilution of DNase I stock. Lanes 3 to 5 and 6 to 8 represent DNase I patterns of the noncoding and coding strands incubated with 35 µg of nuclear extract, respectively. Reactions were treated with 1 µl of a 1:50 dilution of DNase I (lanes 1 and 6), 1 µl of a 1:100 dilution of DNase I (lanes 4 and 7), and 1 µl of a 1:200 dilution of DNase I (lanes 5 and 8). Lane G/A, G + A sequence ladder of the probe. Bars indicate protected regions A, B, and C; asterisks indicate DNase I-hypersensitive sites. Apparent footprinting upstream of region A on the lower strand is in a polylinker.

muscle (9, 10). The cTNT promoter segment spanning nucleotides -129 to -41 (Fig. 1a) was isolated, and the coding or noncoding strand was end labeled with ³²P by terminal transferase or polynucleotide kinase, respectively. These end-labeled probes were then incubated with and without crude nuclear extract from skeletal muscle before partial degradation with DNase I as described in Materials and Methods.

In the presence of embryonic muscle nuclear extract, three regions (labeled A, B, and C in Fig. 2B) of the coding (lanes 6 to 8) and noncoding (lanes 3 to 5) strands of the cTNT promoter were protected from DNase I cleavage. The nucleotide sequence of the protected regions is shown in Fig.

2A. Protected region A spans nucleotides -125 to -110 on the coding strand and nucleotides -129 to -112 on the noncoding strand. Protected region B spans nucleotides -101 to -85 on the coding strand and nucleotides -103 to -88 on the noncoding strand. Protected region C spans nucleotides -76 to -61 on the coding strand and nucleotides -81 to -64 on the noncoding strand. Protected regions B and C are both centered around the M-CAT motifs, whereas protected region A centers around the nominal CArG/CBAR motif (see Discussion). No other protected regions were observed. In addition to these protected regions, several DNase I-hypersensitive sites were consistently observed (indicated by the asterisks in Fig. 2).

To determine whether protection at regions B and C is due to binding of the same factor in the muscle nuclear extract, we performed competition binding assays, using probes containing either the M-CAT 1 motif region or the M-CAT 2 motif region (Fig. 3). The M-CAT 1 oligonucleotide (cTNT nucleotides -101 to -82; competitor a in Fig. 3A) consisting of the M-CAT 1 protected region (region B in Fig. 2) competed for DNase I cleavage at both regions B and C (Fig. 3B; compare lanes 3 and 4 with lanes 5 to 8). Similarly, either a fragment or a synthetic oligonucleotide containing the M-CAT 2 protected region (region C in Fig. 2) was also effective in competing for protection at both sites (Fig. 3B, lane 9, competitor b). These competition experiments indicate that the same nuclear factor binds to regions B and C.

Competition assays, using oligonucleotides with mutated M-CAT motifs, were then used to determine whether the nucleotide sequence of the M-CAT motif is required for binding of the factor(s) protecting regions B and C from DNase I digestion. Neither a double-stranded oligonucleotide containing the cTNT promoter region from -81 to -41 but containing a three-base mutation in the M-CAT 2 motif (mutant M-CAT 2 oligonucleotide; competitor c in Fig. 3A) nor a cTNT fragment from -129 to -89 with the same base changes in the M-CAT 1 motif (Fig. 3A, competitor d) was an effective competitor for binding activity at regions B and C (Fig. 3B, lanes 10 and 11 and lane 12, respectively). Therefore, the factor(s) affording DNase I protection at regions B and C requires an intact M-CAT motif to bind. Since the M-CAT sequence appears to be required for binding, we have tentatively named this factor MCBF, for M-CAT binding factor.

M-CAT mutants disrupt MCBF binding. The data summarized in Fig. 1 show that specific mutations in the M-CAT motifs abolish promoter activity. The footprinting experiments described above show that a nuclear factor, MCBF, binds specifically to the M-CAT motif. If the MCBF identified by footprint analysis is the factor responsible for cTNT promoter activity, we would expect M-CAT mutations to disrupt MCBF binding. To determine the effect of M-CAT mutations on MCBF binding at regions B and C, probes covering the region between -129 to -41 and containing a three-base mutation (CATTICCT to GGTACCT) in either the M-CAT 1 or M-CAT 2 motif were isolated and analyzed for DNase I protection by muscle nuclear extracts. In contrast to the wild-type cTNT segment, which showed protection at regions A, B, and C, the M-CAT mutant fragments showed protection only at site A and the unmutated M-CAT site (Fig. 4). In the fragment containing a mutation at the M-CAT 1 motif but an intact M-CAT 2 motif, only regions A and C were protected (compare wild type and M-CAT 1 mutant in Fig. 4). Conversely, the fragment that had a mutation at the M-CAT 2 motif but an intact M-CAT 1 motif showed the same protection pattern in regions A and B as observed for

FIG. 3. Competition footprint of the cTNT distal promoter region. (A) Schematic representation of cTNT promoter and fragments used for competition footprinting. Competitors: a, double-stranded oligonucleotide containing the M-CAT 1 (M1) motif; b, gel-isolated cTNT promoter fragment -81/-41 containing M-CAT 2 (M2) motif and nominal Sp1 (S) motif; c, double-stranded oligonucleotide identical to b but containing a mutant M-CAT 2 motif; d, gel-isolated fragment -129/-82 containing a nominal CArG/CBAR (C) motif and a mutant M-CAT 1 motif; T, TATA motif. (B) DNase I digestion pattern for the lower strand of the cTNT distal promoter region as described in the legend to Fig. 2 and in Materials and Methods. Lanes: G + A, GA sequence ladder; 1 and 2, probe without nuclear extract and digested with 1 μ l of 1:300 and 1:500 dilutions of DNase I stock, respectively; 3 and 4, with 35 μ g of embryonic skeletal muscle nuclear extract digested with 1 μ l of 1:100 and 1:200 dilutions of DNase I but without competitor; 5 to 12, DNase I footprint pattern in the presence of indicated competitor at 50-, 100-, 250-, or 500-fold molar excess digested with 1 μ l of a 1:50 dilution of DNase I. Protection of region A is unaffected at the concentration of competitors used in these experiments. Competition of region A requires significantly higher concentration of competitor d. Bars denote protected regions A, B, and C.

the wild-type fragment but lost most of its protection in the M-CAT 2 region (Fig. 4, M-CAT 2 mutant). We do not yet know the significance of the residual protection of some bands within the mutant M-CAT 2 motif. These results demonstrate that binding of MCBF at either M-CAT motif does not require binding at the other. In addition, they show that M-CAT motif mutations which disrupt cTNT promoter activity in embryonic skeletal muscle cells also disrupt

FIG. 4. Footprint patterns of cTNT wild-type or mutant M-CAT motifs. (A) Footprint pattern of wild-type cTNT promoter fragment; (B) same cTNT promoter fragment as in panel A but containing a mutated M-CAT 1 motif (CATTCCT to GGTACCT); (C) same cTNT promoter fragment but containing a mutated M-CAT 2 motif (CATTCCT to GGTACCT). Lanes 1, 2, 6, 7, 11, and 12, DNase pattern of respective fragments with no nuclear extract. In lanes 1, 6, and 11, fragments were digested with 1 μ l of a 1:300 dilution of DNase I stock; in lanes 2, 7, and 12, digestion was with 1 μ l of a 1:500 dilution of DNase. Lanes 3 to 5, 8 to 10, and 13 to 15, Footprint patterns of fragments reacted with 35 μ g of embryonic skeletal muscle nuclear extract. Within each set of lanes, fragments were digested with 1:50, 1:100, and 1:200 dilutions of DNase I. Bars indicate the protected regions (A, B, and C) observed for the wild-type fragment. Lane G/A, G + A sequence ladder.

MCBF binding to the M-CAT motif regions of the cTNT promoter.

Spatial relationship between the M-CAT motifs. The center-to-center spacing between the two M-CAT motifs of the cTNT promoter is 23 nucleotides (Fig. 1a). To determine whether this spacing is important for promoter activity, we changed the spacing between the M-CAT motifs by either deleting or introducing additional nucleotides into the *Bam*HI site of mutant i (Fig. 1). Deletion or addition of as few as four nucleotides between the two M-CAT motifs resulted in a 10- or 4-fold reduction in CAT activity (Table

2). When 10 nucleotides (representing an integral helix turn) instead of 4 were inserted, cTNT promoter activity was not restored but was further reduced. Insertion of 100 or 400 nucleotides between the M-CAT motifs to allow for looping interactions also did not restore activity (Table 2). These results indicate that the activity of the cTNT promoter is extremely sensitive to spacing changes between the M-CAT motifs. Despite this effect on activity, these spacing mutants had no effect on the binding of MCBF at either M-CAT motif in the mutant containing either 4 or 10 additional nucleotides (Table 2 and data not shown).

MCBF recognizes the M-CAT motif in the skeletal α -actin promoter. The M-CAT motif has been found in the promoter regions of many contractile protein genes (45). To determine whether MCBF binds to M-CAT motifs in genes other than the cTNT gene, we analyzed the footprint pattern of the α -actin promoter region, which also contains an M-CAT motif in the same approximate relative position as those found in the cTNT promoter. In the presence of embryonic skeletal muscle nuclear extract, two regions of the skeletal α -actin promoter were protected from DNase I digestion (Fig. 5). Region I covers 14 and 13 nucleotides on the coding and noncoding strands, respectively, and includes the CARG motif previously shown to be required for skeletal α -actin promoter activity (1, 40; see Discussion). Region II spans 22 nucleotides on the coding strand and 25 nucleotides on the noncoding strand and is centered around the M-CAT motif.

To determine whether the MCBF that binds to the cTNT promoter is the same factor which protects the M-CAT motif

TABLE 2. CAT activity of cTNT M-CAT spacing mutants in embryonic skeletal muscle cells in culture^a

Promoter	CAT activity (10 ⁻² U, mean $\pm$ SEM) ^b	% of parent activity	Footprint of M-CAT motifs ^c
Parent	149 $\pm$ 19 (6)	100	+
-4 bp	17 $\pm$ 2 (2)	11	ND
+4 bp	37 $\pm$ 5 (6)	25	+
+10 bp	17 $\pm$ 3 (4)	11	+
+100 bp	6 $\pm$ 3 (4)	4	ND
+400 bp	7 $\pm$ 3 (4)	5	ND

^a Insertion and deletion mutants were generated as described in Materials and Methods. Transfection into skeletal muscle cultures and calculation of CAT activity were performed as described in the footnotes to Table 1.

^b Number in parentheses is the number of independent determinations.

^c +, Protection of M-CAT 1 and M-CAT 2 regions in DNase I footprint assays (data not shown); ND, not determined.

FIG. 5. M-CAT footprint in the skeletal α -actin promoter. (A) Schematic representation of the skeletal α -actin promoter showing relative positions of CARg, M-CAT, Sp1, and TATA motifs and the nucleotide sequence of the region footprinted (shaded), in which these motifs are shown in boldface type. Open boxes over sequence show nucleotides protected from DNase I cleavage in regions I and II. (B) Autoradiograph of DNase I footprints of the upper and lower strands of the skeletal α -actin promoter in the presence of 35 μ g of embryonic skeletal muscle nuclear extract and with or without M-CAT 1 oligonucleotide (see Materials and Methods) as competitor. Only the portion of the promoter containing footprinted regions is shown. Lanes: G/A, G + A sequence ladders; 1 and 9, No extract and digested with 1 μ l of a 1:300 dilution of DNase I stock; 2 and 10, No extract, 1 μ l of a 1:500 dilution of DNase I stock; 3 and 11, with extract and digested with 1 μ l of a 1:100 DNase I dilution; 4 and 12, with extract and 1 μ l of 1:200 DNase I dilution. Lanes 5 to 8 of the noncoding strand and lanes 13 to 16 of the coding strand contain as competitor the M-CAT 1 oligonucleotide (see Materials and Methods) at 500- or 1,000-fold molar excess and were digested with 1 μ l of a 1:25 (lanes 7 and 15), 1:50 (lanes 5, 8, 13, and 16), or 1:100 (lanes 6 and 14) dilution of DNase I. Bars indicate protected regions I and II.

in the chicken α -actin promoter, we asked whether synthetic cTNT M-CAT 1 oligonucleotide could compete for the DNase I protection in region II of the α -actin promoter. In the presence of the cTNT M-CAT 1 oligonucleotide, protection at region II was abolished, but the protection at region I was unaffected (Fig. 5, lanes 5 to 8 and 13 to 16). The M-CAT 2 oligonucleotide that contains a mutated M-CAT 2 motif did not affect protection at region II (data not shown). Except for the M-CAT motif, there is no obvious sequence homology between the competing cTNT oligonucleotide and the actin promoter. Therefore, the ability of the cTNT oligonucleotide to compete for factor binding at region II

FIG. 6. Specificity of MCBF binding in a mobility shift competition assay. A 23-nucleotide double-stranded oligonucleotide representing protected region B of the cTNT promoter (M-CAT 1 oligonucleotide; also see Fig. 1 and Materials and Methods) was labeled and used as probe in a binding reaction containing embryonic skeletal muscle nuclear extract with and without indicated double-stranded competitor. The reaction was carried out as described in Materials and Methods. Competitors: M-CAT, unlabeled M-CAT 1 oligonucleotide; mutant M-CAT, M-CAT 2 oligonucleotide containing a mutant M-CAT 2 motif; myoglobin enhancer, sequence 5'-GGGACCCAACCTCCTCCCTTCTTTTC-3'; MEF 1, sequence from the mouse creatine kinase enhancer, 5'-GATCCCCC CAACACCTGCTGCTGA-3'. S1, S2, and S3, Visible gel-shifted bands; P, labeled cTNT M-CAT 1 oligonucleotide probe.

indicates that both the α -actin M-CAT motif and the cTNT M-CAT motif bind the same factor, namely, MCBF.

MCBF is unrelated to other known muscle *trans* factors. Results of competition experiments using oligonucleotides or cTNT promoter fragments containing either an intact or mutated M-CAT motif suggest that the binding of MCBF to the cTNT promoter is specific to the sequence of the M-CAT motif. For example, an intact nominal CARg/CBAR motif was not capable of competing for MCBF binding to either M-CAT motif of the cTNT promoter (Fig. 3B, lane 12), nor could the M-CAT 1 oligonucleotide compete for binding of the CARg/CBAR footprint on the skeletal α -actin promoter (Fig. 5). Thus, MCBF is not the CARg box binding factor (2, 55). To determine whether MCBF is similar to other factors that bind to muscle-specific regulatory elements, we used the gel mobility shift assay to test the ability of other muscle gene regulatory sequences to compete for MCBF binding. The probe used in these assays is the cTNT M-CAT 1 oligonucleotide (see Materials and Methods) that includes the footprinted region B (Fig. 2 and 3). In the presence of embryonic skeletal muscle nuclear extract and nonspecific DNA competitor poly(dI-dC), migration of the M-CAT 1 oligonucleotide was shifted to one major (S3) and two minor (S1 and S2) positions (Fig. 6). The two minor S1 and S2 bands were not consistently observed. To determine whether the band shifted at position S3 represents specific interaction of MCBF to the probe, the binding reaction was challenged with the following competitors: (i) unlabeled

FIG. 7. Tissue distribution and specificity of M-CAT binding activity. The double-stranded M-CAT 1 oligonucleotide (see Fig. 6) was used as a probe (P) in a gel shift assay using nuclear extracts from muscle and nonmuscle tissues. Reactions were carried out as described in Materials and Methods, and the same amount of nuclear protein (1 μ g) was used in all reactions. S3 represents the M-CAT-specific band. Competitor for lanes 3, 6, 9, 12, 15, and 18 was unlabeled M-CAT 1 oligonucleotide. For lanes 4, 7, 10, 13, 16, and 19, the competitor was the M-CAT 2 oligonucleotide containing a mutated M-CAT-2 motif. A 100-fold molar excess of each competitor was used.

M-CAT 1 oligonucleotide and (ii) a 41-bp oligonucleotide (mutant M-CAT 2 oligonucleotide; see Materials and Methods) containing cTNT sequence around and including foot-printed region C (Fig. 2) except that the M-CAT 2 motif was mutated as in Fig. 1. When competed with itself (Fig. 6, lanes 3 to 5) or an oligonucleotide containing an intact M-CAT 2 motif (data not shown), the intensity of the S3 band was greatly reduced. However, the intensity of S3 was not significantly reduced when the competitor contained a mutated M-CAT motif (Fig. 6, lanes 6 to 8). This result and the observation that the intensity of both S1 and S2 bands was unaffected by the competing oligonucleotides indicate that the S3 band represents a specific band shift due to binding of MCBF to the M-CAT motif.

Competition for the S3 band shift was then tested by using oligonucleotides containing the mouse creatine kinase gene enhancer element, which binds a factor termed MEF 1 (from J. Buskin and S. D. Hauschka, University of Washington), and the muscle-specific, enhancerlike element of the human myoglobin gene (from R. S. Williams, Duke University). Even in the presence of 100-fold molar excess of either the mouse creatine kinase or myoglobin enhancer element, the intensity of the M-CAT-specific gel-shifted band S3 was not diminished (Fig. 6, lanes 9 to 11 and 12 to 14, respectively). These competition experiments show that the M-CAT binding factor specifically recognizes and binds to the M-CAT motif and is different from factors that bind to other known muscle-specific regulatory elements.

Tissue and developmental stage occurrence of M-CAT binding activity. Since binding of MCBF to the cTNT M-CAT motifs correlates with the muscle-specific activity of the cTNT promoter, we wanted to determine whether M-CAT binding activity is itself muscle specific. The signature S3 band shift was observed with nuclear extracts from both early and late stages of skeletal muscle development (Fig. 7,

lanes 2 to 4 and 17 to 19, respectively) and early and late stages of cardiac muscle development (lanes 11 to 13 and 14 to 16, respectively). In each case, the S3 band was competed for by an oligonucleotide containing a wild-type M-CAT motif but not by an oligonucleotide containing a mutated M-CAT motif (Fig. 7; compare lanes 3, 12, 15, and 18 with lanes 4, 13, 16, and 19, respectively), indicating that the S3 band represents authentic MCBF activity in all extracts.

Two nonmuscle tissues were also tested for the presence of M-CAT binding activity. Nuclear extract from brain tissue produced a gel shift pattern similar to that observed for muscle extracts (Fig. 7, lane 8), although the overall intensity of the gel shift was reduced. Liver extract, on the other hand, gave a strong gel shift, whose pattern was different from that obtained with muscle or brain nuclear extract (Fig. 7, lane 5). The significance of this altered gel shift pattern is not yet clear. Nevertheless, the binding activity found in these nonmuscle tissues appears to be dependent on a sequence-specific interaction with the M-CAT motif, since oligonucleotides containing a wild-type, but not a mutant, M-CAT motif serve as efficient competitors for binding (compare lanes 6 and 7 and lanes 9 and 10 of Fig. 7, respectively). We conclude, therefore, that MCBF activity is not tissue specific (see Discussion).

Although the preliminary results presented in Fig. 7 suggest a correlation between the relative levels of MCBF and transcriptional activity of the cTNT gene at different stages of heart and skeletal muscle development (see introduction), we emphasize that for the case of skeletal muscle, this quantitative change is not reproducible, and some day 19 skeletal muscle extracts have levels of MCBF activity comparable to that present at day 12 (data not shown). The variability in MCBF activity levels in day 19 skeletal muscle extracts may be related to the greater difficulty of isolating nuclei free of myofibrillar contamination from such highly

differentiated muscle (see Materials and Methods). The resulting higher but variable levels of myofibrillar contamination in these preparations could either dilute or inhibit MCBF activity in these extracts. Further experiments will be necessary to determine the quantitative and qualitative changes in MCBF during muscle development.

DISCUSSION

The goal of the experiments presented here was to define the regulatory element(s) responsible for the activity of the cTNT promoter in embryonic skeletal muscle cells. Previous studies indicated that the cTNT distal promoter region, between nucleotides -129 and -50, contains the sequence element(s) essential for skeletal muscle-specific transcription (39). Here, using more detailed deletion analysis, we show that the segment between -129 and -100 is nonessential for cTNT promoter activity in embryonic skeletal muscle cells. The -129/-100 segment contains sequence with similarity to two conserved sequence motifs, each of which has been demonstrated to be important in the regulation of other muscle-specific genes (Fig. 1). One is the nominal CARG/CBAR motif, which has been shown to be an essential component of sarcomeric actin gene promoters from a wide variety of vertebrates (1, 40, 41). The second is a nominal MEF 1 motif that has been shown to be the core component of the enhancer governing the muscle-specific expression of the creatine kinase gene and may be an important component of the enhancers of several other contractile protein genes (8). The results presented here indicate that neither nominal homology to these conserved motifs is required for activity of the cTNT promoter and suggest that the mechanism(s) by which this promoter is regulated in embryonic skeletal muscle may be distinct from those regulating either the sarcomeric actin genes or the muscle creatine kinase gene.

M-CAT motifs are essential *cis* elements within the muscle-specific domain of the cTNT promoter. The deletion experiments outlined above demonstrate that the cTNT promoter region that is essential for muscle-specific expression resides between nucleotides -99 and -50. To determine which sequences within this region are absolutely required, a scanning series of clustered point mutations were introduced at five locations in this segment (Fig. 1h to m). The results of these experiments indicate that two sites are highly sensitive to mutation, disruption of either of which reduces activity of the cTNT promoter by 2 orders of magnitude (Fig. 1h, j). Mutation at the other sites within this region had relatively little effect (Fig. 1i, k, and l; see also below). Both of the highly sensitive sites contain the sequence 5'-CATTCT-3', which is found in the promoter regions of several muscle-specific genes (29, 45, 49, 56) and which we have named the M-CAT motif (39). Because mutation of either or both M-CAT motifs inactivates the cTNT promoter, we conclude that two tandem copies of the M-CAT motif are required for cTNT promoter activity in embryonic skeletal muscle cells and that neither motif alone is sufficient for activity.

Two other regions outside the M-CAT motifs, although not required for expression of the cTNT promoter, had a modest effect on the overall level of activity when mutated or deleted (Fig. 1d and l). First, mutation of an Sp1 binding site homology within a G+C-rich domain reduces cTNT promoter activity by approximately fourfold (Fig. 1l). Although footprint analysis using crude muscle nuclear extracts did not show protection in this region, Sp1 binding has been shown to affect activity of other promoters (15, 27,

28). It seems possible that it may also play a similar role in the cTNT promoter. Second, deletion of nucleotides -105 through -99 results in a twofold decrease in cTNT promoter activity, suggesting that these nucleotides may be important for optimal promoter activity. This region contains part of the nominal MEF 1 motif (Fig. 1). Although it is possible that this deletion blocks factor binding to the nominal MEF 1 motif, two additional observations argue against such a conclusion. First, if this nominal MEF 1 site were important for activity of the cTNT promoter, we would expect its disruption to reduce promoter activity more than twofold, because its deletion lowers activity of the creatine kinase promoter 10- to 50-fold (33). Second, in footprint experiments only a portion of the nominal MEF 1 motif is protected, and that protection is competed for by oligonucleotides containing M-CAT motifs (see Fig. 3) but not by oligonucleotides containing a MEF 1 motif (data not shown). An alternative possibility is that deletion of the -105 to -100 segment may affect the activity of MCBF bound to M-CAT 1, but further work will be required to clarify this matter.

The results from the deletion and mutagenesis experiments reported here indicate that the two M-CAT motifs play central roles in governing the expression of the cTNT promoter in embryonic skeletal muscle cells. Sequences outside the M-CAT motifs are not required for the muscle-specific expression of the cTNT promoter but may play an auxiliary role that is subordinate to the role played by the M-CAT motifs.

cTNT promoter activity is dependent on interaction between the M-CAT motifs and a nuclear factor in muscle. Using DNase I protection and gel shift assays, we show that the M-CAT regions of the cTNT promoter interact with a factor present in embryonic skeletal muscle nuclei. Both M-CAT regions appear to bind the same factor because either region can efficiently compete for binding and protection of both M-CAT regions. Several lines of evidence indicate that the M-CAT motif is the central sequence element responsible for binding this factor and that this binding interaction is essential for cTNT promoter activity. First, DNase I protection experiments using segments of the cTNT promoter show footprints that are centered around the M-CAT motifs. Second, DNase I protection at the M-CAT regions can be competed for with excess oligonucleotides containing wild-type M-CAT motifs but not with oligonucleotides containing mutant M-CAT motifs. Third, mutant cTNT promoters, in which either M-CAT motif has been mutated, rendering the promoter inactive in transient transfection experiments, lose DNase I protection specifically in the mutant M-CAT regions. Thus, there is a direct correlation between the requirement for an intact M-CAT motif for both cTNT promoter activity and binding to a specific factor in embryonic skeletal muscle nuclei. Therefore, the M-CAT binding factor (MCBF) is a transcription factor that is essential for activity of the cTNT promoter in embryonic skeletal muscle cells.

Both M-CAT motifs must bind MCBF in an interactive fashion to activate transcription. As indicated above, cTNT promoter activity depends on two intact M-CAT motifs, since mutation of either of the two M-CAT motifs inactivates the cTNT promoter. Footprint experiments show that MCBF binding at one M-CAT motif does not require binding at the other because promoters with one mutant M-CAT motif show a normal footprint over the wild-type M-CAT motif. The requirement for MCBF occupancy at both M-CAT motifs suggests that cTNT promoter activity depends on interaction between these two sites. In the cTNT promoter, the center-to-center spacing between the two M-CAT

motifs is 23 nucleotides, indicating that both M-CAT motifs are likely to face the same side of the DNA helix, approximately two helix turns apart. Deletion and insertion mutants show that the activity of the cTNT promoter is sensitive to alterations in the distance between the M-CAT motifs (Table 2). For example, deletion or insertion of four nucleotides (slightly less than one-half helix turn) between the M-CAT motifs reduced cTNT promoter activity to 11 or 25%, respectively, of wild-type activity. While this result is consistent with the notion that alignment of M-CAT sites on the same side of the DNA helix is important for promoter activity, insertion of an integral helix turn of 10 instead of 4 nucleotides further reduced activity to 10% of wild type. Thus, the activity of the M-CAT motifs may be more sensitive to distance per se than to stereospecific alignment. Although such alterations in spacing had a profound effect on transcriptional activity, they had no effect on binding of MCBF to either M-CAT motif (Table 2), further supporting the conclusion that MCBF can bind independently at the two M-CAT sites. These results indicate that mere binding of MCBF to the M-CAT motifs is not sufficient for optimal transcriptional activity but that efficient promoter activity also requires a relatively precise spatial register between bound M-CATs. Promoter activity is not restored by insertion of longer segments of spacer DNA (100 or 400 nucleotides; Table 2) between the two M-CAT sites, indicating that the spatial registration requirement cannot be alleviated by looping out of intervening DNA. We therefore conclude that each M-CAT motif is capable of binding to an MCBF and that transcriptional activation of the cTNT promoter requires a short-range (presumably protein-protein or intraprotein) interaction between these two sites.

MCBF is a novel transcription factor. MCBF does not appear to be related to either the CArG box binding factor, which binds to an essential regulatory sequence within the promoter region of α -actin genes (23, 44, 54), or MEF 1/MyoD, which binds to the enhancer of the mouse creatine kinase gene (8, 33). In addition to the dissimilarity of their binding site sequences, there are two additional reasons which lead us to believe that MCBF is distinct from either the CArG box binding factor or MEF 1/MyoD. First, the cTNT promoter contains nominal CArG/CBAR and MEF 1 motifs in a nonessential region (see above). Although footprint analysis shows protection of the nominal CArG/CBAR motif, deletion analysis indicates that neither it nor the nominal MEF 1 motif (which does not show DNase I protection in our experiments) is required for activity of the cTNT promoter in embryonic skeletal muscle cells in culture, unlike the two M-CAT motifs, both of which footprint and are both absolutely required for activity. Second, in both footprint and gel shift assays, DNA fragments containing either CArG/CBAR or MEF 1 motifs are not capable of competing for MCBF binding (nor can oligonucleotides containing the consensus sequences for CTF/NF-1, AP1, AP2, AP3 or Sp1; data not shown). Thus, MCBF is a novel gene regulatory factor that is required for the muscle-specific activity of the cTNT promoter.

M-CAT binding activity is present in nonmuscle tissues. Using the gel mobility shift assay, we show that the factors which bind in a sequence-specific fashion with the M-CAT motif are present also in cardiac muscle and in nonmuscle tissues (Fig. 7). It is not yet clear whether the M-CAT binding factors present in nonmuscle tissues are the same or different from those present in muscle tissues. Sequence-specific DNA binding activity can result from both cell-specific and ubiquitous binding factors. For example, al-

though MyoD is a muscle-specific nuclear factor that recognizes a specific nucleotide motif in the muscle creatine kinase gene enhancer, this sequence motif is also bound by factors present in nonmuscle cells which are distinct gene products (33, 42). Definitive determination of the identity or nonidentity of MCBF from different sources will require nucleic acid or immunological probes that can discriminate between different gene products with similar activities.

Multiple muscle regulatory elements and factors. Although MCBF was initially identified by its specific interaction with the M-CAT motifs of the cTNT promoter, we show that MCBF also binds specifically to the single M-CAT motif in the promoter region of the chicken skeletal α -actin gene. The specificity of this binding suggests that MCBF may be involved in some manner with the regulation of the skeletal α -actin gene and possibly other muscle-specific genes in which the M-CAT motif is found. The actin M-CAT motif is conserved in skeletal α -actin promoters of both birds and mammals and resides downstream of the most proximal CArG/CBAR motif. The CArG/CBAR motif has been shown to be essential for the activity of this promoter in skeletal muscle cells. We do not know what role MCBF might play in regulation of the skeletal α -actin gene; however, it is interesting that the skeletal α -actin promoter contains a single M-CAT motif, a configuration which renders the cTNT promoter inactive. Thus, it is possible that the consequences of M-CAT-MCBF interaction may be different in the skeletal α -actin promoter than in the cTNT promoter.

The conservation of the M-CAT, CArG/CBAR, and MEF 1 motifs among diverse muscle gene promoters suggests some functional roles of these sequences in the regulation of the genes on which they are found. How then can one account for the simultaneous binding of two or more regulatory factors to their cognate sequences in one muscle gene promoter where some of these motifs do not have any apparent functional roles in transfection experiments? One likely possibility is that transfection of cells in culture assays only a subset of the regulatory activities governing a specific promoter. During muscle development in vivo, contractile protein genes are subject to modulation of their transcriptional activity, which may depend on the combinatorial action of many muscle transcription factors. That transcription factors and elements may have different roles depending on the context of their environment or stage in development has been shown, for example, for the RAP1 activator-silencer sequence in yeast cells (4, 50) and the *Drosophila* homeotic genes *fushi tarazu* (31, 32) and *daughterless* (11). The recent demonstration that regulatory molecules with similar structural motifs (such as Fos and Jun or E12 and MyoD) may form heterodimers with binding properties that are different from those of homodimers (42) also serves to expand the repertoire of functions of any one regulatory factor. Thus, the pattern of expression characteristic of a given muscle gene during development may be the result of a combination of common and specific regulatory elements that can respond to the selective expression, differential modification, or interaction of a limited set of transcription factors. If such a regulatory scheme operates in muscle cells during development, then it may account for the presence of M-CAT in the chicken α -actin promoter and the presence in the cTNT promoter of nominal CArG/CBAR and MEF 1 motifs (as well as the regulatory region upstream of -129 that is required for cTNT expression in cardiac cells). The fact that such regulatory sequences are apparently not required for expression during transient transfection of skeletal muscle cells may simply reflect the necessity of address-

ing the role of such elements in muscle gene expression under physiological conditions.

ACKNOWLEDGMENTS

We thank our colleagues at the University of California, San Francisco, for advice and encouragement and our co-workers in the laboratory for critically reading the manuscript and for fruitful discussions. We also thank William Odegard and Nina Kostanian for dedicated technical assistance and S. D. Hauschka, J. Buskin, and R. S. Williams for their generous gifts of oligonucleotides.

This work was supported by Public Health Service grants GM32018 and HL35561 from the National Institutes of Health to C.P.O. J.M. was supported by a Public Health Service postdoctoral fellowship from the National Institutes of Health.

ADDENDUM IN PROOF

Recent experiments indicate that the nominal CARG/CBAR motif from the cTNT promoter cannot compete with the skeletal α -actin CARG/CBAR motif in gel shift binding assays. Thus, the cTNT nominal CARG/CBAR motif may bind a novel factor distinct from that which binds to the CARG/CBAR motifs of actin promoters.

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