Dissimilar Nuclear Protein Binding at Human β-Myosin Heavy Chain Proximal and Distal MCAT Elements in Response to Increased Skeletal Muscle Activity

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Abstract

MCAT regulatory elements mediate muscle-specific transcription and can act as inducible promoter elements in response to hormonal and mechanical stimuli. Herein we investigate whether two distinct MCAT elements [distal MCAT (-290 to -284), proximal MCAT (-210 to –203: βe3 region)] within a region shown by us to confer mechanical overload (MOV) responsiveness onto a 293-base pair human βMyHC transgene (β293) function comparably as MOV-inducible elements. As compared to the distal MCAT element, electrophoretic mobility shift assay (EMSA) analysis revealed low levels of binding at the proximal MCAT element when using skeletal muscle [control plantaris (CP) and MOV-P] nuclear extract and *in vitro* translated transcription enhancer factor 1 (TEF-1) proteins. Direct and competition EMSA analysis showed that only the low mobility binding complex (LMC) formed at the distal MCAT element was highly enriched when using MOV-P nuclear extract, and was only partially competed for by the chicken cardiac troponin T element (cTnT); an element shown to form a high-order LMC involved in muscle-specific expression. Methylation interference (MI) assay identified strong DNA-protein interactions throughout the distal MCAT core element and weaker interactions at the adjacent nucleotides. Our data tentatively indicate that the nuclear protein(s) forming the high-order low mobility complexes at the distal MCAT and cTnT MCAT elements differ, or alternatively, are the same protein(s) binding with different affinity. Also, the proximal and distal MCAT elements display different binding affinities for TEF-1 isoproteins; a property that may determine the magnitude of their involvement in the MOV-induction of transgene β293 in skeletal muscle of adult transgenic mice.

Key words: βMyHC, gene transcription, hypertrophy, MCAT element, mechanical overload, neuromuscular activity, skeletal muscle, TEF-1.

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Native sarcomeric myosin is a major contractile protein that serves a chemomechanical function during muscle contraction, and is comprised of two pairs of dissimilar light chains and two heavy chains (MyHC) [36, 37]. The MyHC subunit is encoded by a multigene family whose members are tightly regulated in a developmental stage-specific manner and in response to numerous physiological and pathologic stimuli [2, 28, 36, 37]. In adult mouse hindlimb skeletal muscle, four primary MyHC isoforms (fast type IIb, IIx/d, IIa, and slow-type I or β) have been detected. It is the differential expression pattern of these MyHC isoforms that contributes to the broad classification scheme that distinguishes four distinct fiber-types termed; fast-type IIb, IIx/d, IIa and slow-type I. Of the MyHC isoforms expressed in adult-stage skeletal muscle, the βMyHC gene displays the most complex expression pattern throughout development. In the day 17 post-coitus mouse fetus, βMyHC expression is detected in the heart and subsequently its expression becomes primarily restricted to adult-stage type I skeletal muscle fibers [18, 21, 22, 38]. Although numerous *in vitro* and *in vivo* studies have defined βMyHC proximal promoter and far upstream regulatory regions involved in its fiber-restricted and

plastic expression [8, 18, 24, 26, 27, 29, 33-35, 38], the precise contribution of each discrete element comprising these regions has not been systematically analyzed in the *in vivo* context.

We have previously undertaken an extensive *in vivo* deletion and mutational analysis of the mouse and human βMyHC promoters using transgenic mice (Figure 1) for the purpose of gaining insight into the mechanistic basis controlling βMyHC slow-fiber restricted expression and responsiveness to mechanical overload (MOV). Our study of the mouse βMyHC promoter revealed that transgenes comprised of either 5600 or 600-base pair (bp) closely mimicked the expression pattern of the endogenous βMyHC gene, i.e., both transgenes expressed in slow-type I fibers and were induced in the fast type II plantaris muscle by MOV [34]. Interestingly, muscle-specific and MOV-induced expression was not lost when three discrete regulatory elements [MCAT, C-

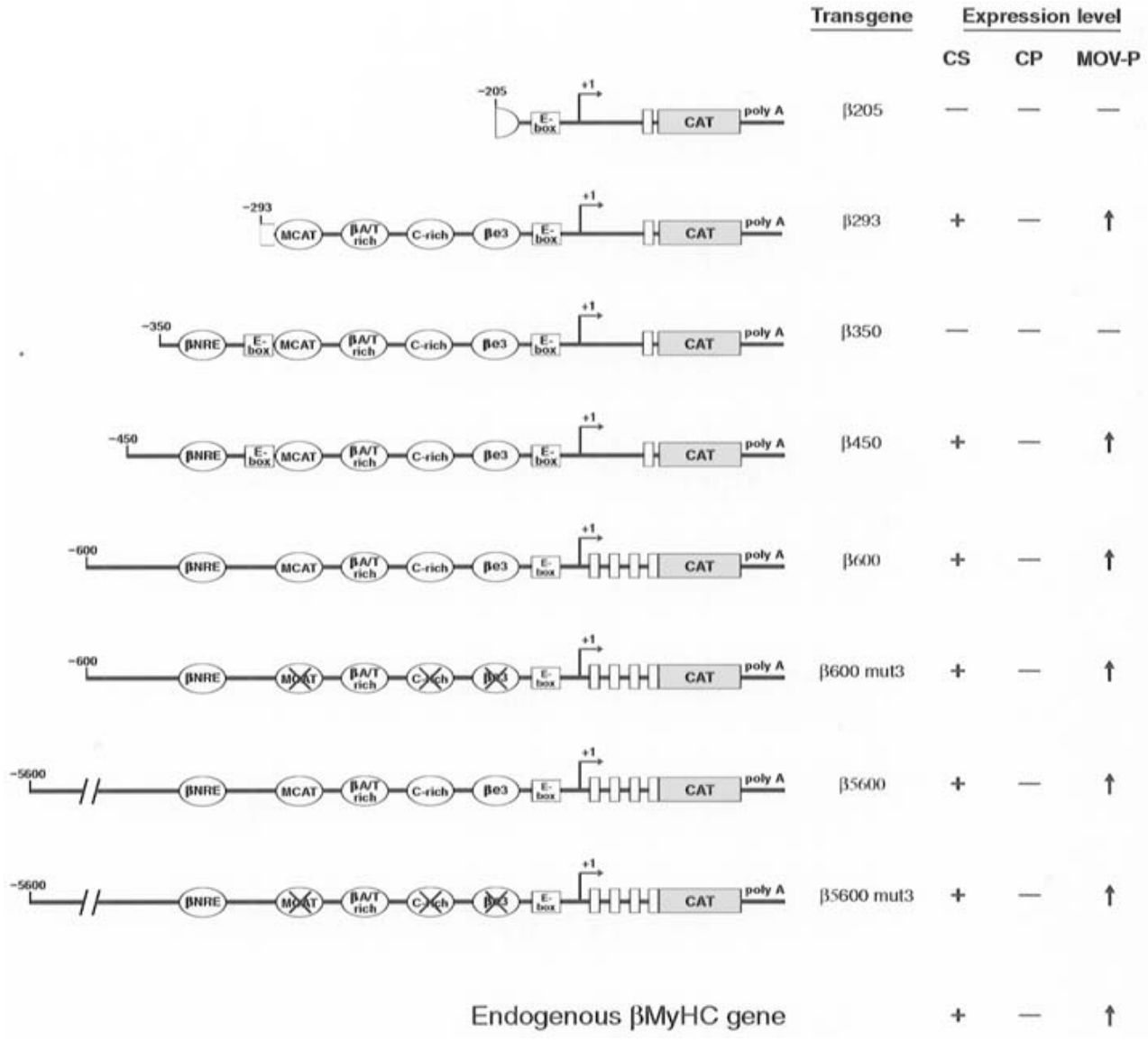

*Figure 1. βMyHC transgene expression in response to MOV activity. Schematic summary of the pattern of chloramphenicol acetyltransferase (*CAT*) reporter gene expression in control soleus (*CS*), control plantaris (*CP*), and mechanically-overloaded plantaris (*MOV-P*) muscles of transgenic mice. Transgenes consist of either 205 bp (β205), 293 bp (β293), 350 bp (β350), or 450 bp (β450) of human βMyHC 5′ promoter sequence and 120 bp of 5′ untranslated region (*UTR*) fused to the bacterial CAT reporter gene. Transgenes β5600 and β600 contain 5600 and 600 bp, respectively, of the mouse βMyHC 5′ promoter sequence and the entire 1600 bp of the 5' UTR linked to the CAT reporter gene. β5600 mut3 and β600 mut3 are structurally identical to β5600 and β600 except they harbor simultaneous mutations within the three major regulatory elements (MCAT, C-rich, and βe3). +, expression; □, barely detectable to no expression; ↑, induced expression. Open boxes within the transgene 5′ UTR represent untranslated exons.* MCAT *denotes distal MCAT.* βe3 *region harbors proximal MCAT element.*

rich, and βe3-contains proximal MCAT element] were simultaneously mutated in transgenes comprising the highly conserved (Figure 2) βMyHC proximal control region (-293 to -180) (Figure 1, [34]).

As concerns the human βMyHC promoter, our *in vivo* deletion analysis has defined a minimal promoter region (a transgene comprised of 293-bp of βMyHC promoter) that closely mimicked the expression pattern of the endogenous βMyHC gene at all developmental stages, and in response to MOV [24, 38]. Importantly, the deletion of an 89-bp region (-293 to –205) resulted in the loss of transgene expression and MOV-responsiveness, thus indicating that this region contains regulatory elements important for both basal and MOV inducible expression of the βMyHC gene [38]. An *in vitro* analysis of this 89-bp region has led to the identification of a putative MOV-element (βA/T-rich element: -269 to –258) that only under MOV conditions showed enriched binding which was antigenically distinct from GATA-4, MEF2A-D, SRF, and Oct-1 [35]. In addition to this A/T-rich element, the 89-bp region also contains a muscle-CAT (MCAT) site; a cis-acting regulatory element previously shown to mediate the muscle-specific gene transcription of numerous genes. Whether this regulatory element plays a functional role in conferring βMyHC fiber type-specific and/or inducible expression has not been investigated as yet.

MCAT regulatory elements (5'-CATTCCT-3') are contained in the promoter region of numerous muscle and nonmuscle genes, and are similar in nucleotide sequence to the simian virus 40 (SV40) enhancer GT-IIC (5'-CATTCCA-3') element [11, 20]. Earlier studies using protein purification and cDNA cloning identified transcription enhancer factor 1 (TEF-1) as the GTIIC protein binding activity in HeLa cell extracts [6, 39], and subsequently, several additional TEF-1 related cDNAs were isolated [1, 30, 40]. It is now known that in vertebrates, a multigene family encodes TEF-1 proteins and that within the TEF-1 family of transcription factors, additional diversity is brought about via alternative splicing [11, 14, 20]. Northern blot and *in situ* hybridization analyses of the various TEF-1 transcripts has revealed an overlapping expression pattern which raises the likely possibility that these factors serve redundant as well as distinct functions [20, 41, 42]. The overlapping and enriched expression pattern of nominal (NTEF-1) and related TEF-1 (RTEF-1) mRNA transcripts in skeletal muscle may represent a mechanism whereby TEF-1 target gene transcription is modulated in a fiber-type manner by specific TEF-1 isoproteins and their associated cofactors. This notion is supported in part by recent studies showing that muscle-specific transcription of the chicken cTnT gene was mediated by the concurrent binding of TEF-1 to the MCAT core sequence and Poly(ADP-Ribose) polymerase to the MCAT 5'-adjacent sequence [4, 19]. An additional example of this type of transcriptional regulation has been reported by Gupta, *et al*., [9], who show that NTEF-1 physically interacts with the transcription factor MAX to positively regulate αMyHC gene expression.

MCAT elements have also been reported to act as inducible promoter elements for several muscle genes. For example, an MCAT element within the skeletal muscle α-actin proximal promoter has been shown to mediate its increased expression during wing-stretch induced hypertrophy of the chicken anterior Latissimus Dorsi muscle [5]. In addition to mechanical signals, hormonal stimuli that activate the α1-adrenergic receptor of neonatal rat cardiocytes led to increased expression of rat βMyHC reporter constructs via an MCAT element contained within the proximal promoter βe3 region [15, 16]. It is noteworthy that two TEF-1 binding sites (distal MCAT and proximal MCAT) are located within a 293 base pair βMyHC promoter region that we have shown is sufficient to confer MOV induction to a βMyHC transgene in adult transgenic mice [24, 38]. Therefore our investigation, herein, of potential functional differences between the human βMyHC distal- and proximal-MCAT elements as well as their involvement in directing MOV inducible expression, is relevant to our goal of delineating the mechanistic basis underlying βMyHC fiber-type and MOV-inducible expression. Our results show the formation of an enriched high-order low mobility nuclear protein binding complex at the distal MCAT element only under MOV-condition, and that this element binds TEF-1 isoproteins with a higher affinity than the proximal MCAT element. Thus, it is possible that the mechanistic basis underlying transgene

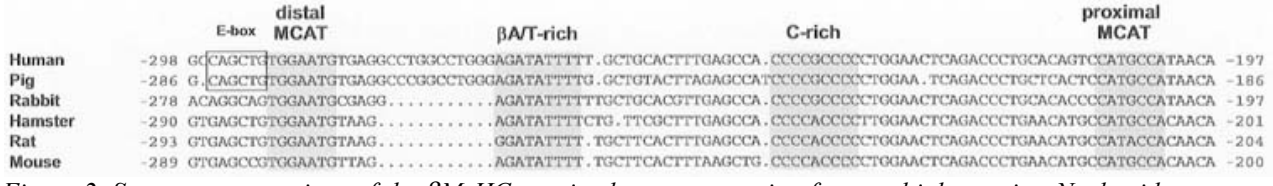

Figure 2. Sequence comparison of the βMyHC proximal promoter region from multiple species. Nucleotide sequences of the human (ref. 1, X52889), pig (ref. 2, L10130), rabbit (ref. 3, unsubmitted), hamster (ref. 4, L12104), rat (ref. 5, X16291), and mouse (ref. 6, L07306) were aligned using the CLUSTALW program provided in the Biology Workbench (http://biology.ncsa.uiuc.edu). Gaps were inserted to optimize the alignment. The position and conserved nucleotides of the distal MCAT, βA/T-rich, C-rich, and βe3 elements are indicated in gray. *The reference and accession number for each sequence is listed in parenthesis.*

β293 MOV induced transcription [24, 38], in part, involves complex interactions involving the distal MCAT element, TEF-1 isoforms, and muscle-specific and/or ubiquitously expressed accessory proteins.

Experimental Procedures

Preparation of nuclear protein extract from adult skeletal muscle

Nuclear extract (NE) was isolated from adult rat control plantaris (CP) and MOV-P muscle as described previously [24, 35]. All procedures were carried out on ice. All buffers contained 2 μg/ml each of aprotinin and leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitors. Ten grams of either CP or MOV-P were harvested from adult female Sprague-Dawley 250-gram rats, and minced in phosphate-buffered saline. Minced muscle tissue was incubated in relaxation buffer 1, for two 15 min intervals, followed by two 10 min washes in relaxation buffer II. The muscle tissue was then homogenized in buffer-A and centrifuged through a 27% Percoll (Amersham Pharmacia, Biotech) density gradient at 27,000 x g for 15 min at 4°C. The pelleted nuclear layer was consolidated and lysed by the addition of 3 M NH_4SO_4 (pH 7.9) to a final concentration of 0.4 M. The lysate was ultra-centrifuged at 126,000 x g for 1 h (4°C) to pellet nuclear debris. Solid NH_4SO_4 (0.3 g/ml) was added slowly to the supernatant, and the precipitated nuclear proteins were concentrated by ultra-centrifugation at 126,000 x g for 30 min. The resulting pellet was resuspended in dialysis buffer and dialyzed for two hours. The nuclear protein extract was stored in aliquots at –80°C. Protein concentration was determined according to Bradford [3].

Electrophoretic mobility shift assay

All oligonucleotide probes used in this study are listed in Table 1 [19, 39]. EMSAs were performed as previously described [24, 35]. The double stranded proximal MCAT and chicken cardiac troponin T (cTnT) MCAT oligonucleotide probes were labeled by fill-in reaction using the Klenow fragment of E. coli DNA polymerase I (Stratagene, La Jolla, Ca) and [α-^{32}P]-dCTP (3000Ci/mmol). All other oligonucleotide probes were end-labeled by T4 polynucleotide kinase (New England Biolabs, Beverly, Ma) and [γ-^{32}P]-ATP (6000 Ci/mmol). Probes were purified by polyacrylamide gel electrophoresis prior to use in EMSA. Binding reactions were performed with 3.5 to 5 μg of either CP or MOV-P nuclear extract and 20,000 cpm of labeled probe for 20 min at room temperature in a 25 μl total volume. Where indicated, 0.5μl of *in vitro*-translated nominal TEF-1 (NTEF-1) (distal MCAT probe), and 2.5 μl of NTEF-1, related TEF-1 (RTEF-1), divergent TEF-1 (DTEF-1) and embryonic TEF-1) ETEF-1 (proximal MCAT probe) protein were used in place of MOV-P or CP nuclear extract. The binding reactions were resolved on a 5% non-denaturing polyacrylamide gel at 220 volts for 2.5 hours at 4°C. Following electrophoresis, the gels were dried and DNA-protein complexes were visualized by autoradiography.

In vitro-transcription/translation (TnT)

In vitro coupled TnT was performed using 1 μg of each TEF-1 expression plasmid in the T7 TnT rabbit reticulocyte kit according to the manufacturer's instructions (Promega). The expression plasmids corresponded to NTEF-1 (pXJ40-TEF-1A, open reading frame (ORF) of human TEF-1 [39]), RTEF-1 (pXJ41-TEF-3, ORF of human TEF-3, [12]), ETEF-1 (pXJ41-TEF-4, ORF of mouse TEF-4 [12]), and DTEF-1 (pXJ41-TEF-5, ORF of human TEF-5, [13]). Parallel TnT reactions were performed in the presence of [^{35}S]-methionine (New England Nuclear). Efficient translation and expected molecular weights of the protein products were verified by resolving the radiolabeled reaction products on a sodium dodecyl sulfate-12% polyacrylamide gel (SDS-

Table 1. Oligonucleotide probes and competitors.

Name	Sequence (sense strand 5'-3')	Position	Reference
bMyHC d-MCAT (distal)	CAGCTG**TGGAATG**TGAGGCCT	human, -296/-276	this study
bMyHC d-MCAT mut	CAGCTG**cGtctTa**TGAGGCCT	human, -296/-276	this study
bMyHC p-MCAT (proximal)	GCACAGTC**CATGCCA**TAACA	human, -216/-197	this study
bMyHC p-MCAT mut	GCACAGTC**CATGttA**TAACA	human, -216/-197	this study
bMyHC C-rich	GCACTTTGAGCCACCCCGCCCC	human, -254/-233	this study
SV40 enhancer GT-IIC	CAGCTG**TGGAATG**TGTGTCAG	275/255	[39]
cTnT MCAT	*TG*CAAGTGTTG**CATTCCT**CTCTG	chicken, -103/-84	[19]

Core MCAT binding elements within the oligonucleotides are delineated in boldface type. E-box motifs are delineated by boxes. *mut*, mutation of the MCAT binding site with base pair alterations shown in lowercase letters. cTnT, cardiac troponin T. Cohesive terminus on cTnT MCAT oligonucleotide is italicized. bMyHC C-rich was used as a non-specific competitor. SV40 enhancer GT-IIC, GT-IIC binding motif from domain B1 of the simian virus 40 enhancer. The position of the oligonucleotide sequence within the enhancer is indicated according to the BBB numbering system coordinantes ([39], and reference within).

PAGE).

Diethyl pyrocarbonate (DEPC) interference assays

DEPC DNA-footprinting assays were performed as described by Sturm, *et al.* [32]. ^{32}P-labeled distal MCAT probe was modified by 2% DEPC for 15 min at 37°C. The modified probe was used for preparative EMSA as described above, except the binding reactions were scaled-up 10-fold. Bands corresponding to the DNA-protein complex and free probe were excised from the EMSA gel, and recovered by electroelution. Base-specific cleavage of the recovered DNA was carried-out in a 100 µl 1M piperidine incubation for 30 min at 90°C which was followed by repeated rounds of lyophilization to remove the piperidine. Equivalent amounts (1000 cpm/lane) of free and bound cleaved probe were resolved on a 20% polyacrylamide-8M urea denaturing sequencing gel. Gels were autoradiographed for 24 hours.

Results

EMSA analyses of nuclear protein binding to the human βMyHC proximal MCAT element

Previous studies have established that the rat βMyHC proximal MCAT element confers hormone inducible gene expression onto rat βMyHC reporter genes in primary cardiac cells [16]. However, it is not known whether this element functions comparably as an inducible element in skeletal muscle in response to MOV. We initiated our investigation into whether the human βMyHC proximal MCAT element acts as an MOV-inducible element by first examining potential differences in binding complex formation at this element between CP and MOV-P nuclear extract using EMSA analyses (Figure 1). Incubation of a double stranded ^{32}P-labeled proximal MCAT probe (Table 1) with 5 µg of CP nuclear extract resulted in the formation of three low intensity binding complexes termed C1, C2, and C3 (Figure 3A lane 1). The same pattern of binding complex formation was detected when MOV-P nuclear extract was used, although the intensity of these complexes was decreased (Fig. 3A, lane 6). In binding reactions containing CP nuclear extract, the addition of 100-fold molar excess unlabeled wild type proximal MCAT element competed strongly for complex C2 while competition with the distal MCAT element completely abolished formation of this complex (Figure 3A, lane 1 vs lanes 2 and 4). EMSA competition with 100-fold molar excess of either cold proximal MCAT mutant probe or the unrelated βMyHC C-rich element, did not compete for complex C2, whereas these competitors did compete for complexes C1 and C3 indicating the non-specific nature of the later two complexes (Figure 3A lane 1 vs 3 and 5).

Since previous work has shown that TEF-1 binds to the rat proximal MCAT element, we conducted binding studies using rabbit reticulocyte lysate-generated *in vitro*-translated TEF-1 isoproteins (Figure 3 inset: ^{35}S-methionine labeled TEF-1 isoproteins) to determine if the human proximal MCAT element could interact with TEF-1 isoproteins. EMSA analysis of these binding reactions revealed the formation of a specific binding complex between the ^{32}P-labeled human proximal MCAT element and *in vitro*-translated NTEF-1, RTEF-1, DTEF-1 and ETEF-1 that was different from the non-specific binding complex formed when unprogrammed lysate was used (Figure 3B, lane 11 vs 12-15). Although not visible in Figure 3B, lane 14, ETEF-1 did bind to the proximal MCAT element and was visible following an extended exposure time (data not shown). Thus, when these binding data are considered with our EMSA competition studies, it is reasonable to hypothesize that TEF-1 protein is likely to be a binding component comprising specific complex C2.

EMSA analyses indicate enriched MOV-P nuclear protein binding at the distal MCAT element

Both *in vitro* and *in vivo* studies have illustrated that the βMyHC distal MCAT element (-296/-284) is required for high levels of muscle specific expression, however, it is not know if this element also functions as an inducible element in striated muscle [8, 18, 29, 33]. As an initial investigation into whether the human βMyHC distal MCAT element represents an MOV-inducible element in adult skeletal muscle, we evaluated binding at the distal MCAT element by EMSA. Incubation of the ^{32}P-labeled distal MCAT probe with CP or MOV-P nuclear extract resulted in the formation of three major binding complexes termed; low mobility complex (LMC), intermediate mobility complex (IMC), and high mobility complex (HMC) (Figure 4, lanes 1-6). As shown in Figure 4, the IMC and HMC contained multiple bands. EMSA analysis of binding reactions containing the ^{32}P-labeled distal MCAT probe revealed the formation of a highly enriched LMC, and slightly reduced binding of the IMC, only when MOV-P nuclear extract was used (Figure 4, lane 1 vs 4). The addition of 100-fold molar excess unlabeled distal MCAT element to binding reactions containing either CP or MOV-P nuclear extract completely abolished formation of all complexes (Fig. 4, lanes 2 and 5). In contrast, the addition of 100-fold molar excess cold distal-MCATmut (d-MCATmut; contained mutation of MCAT core element) did not effectively alter complex formation indicating that the MCAT core sequence was important to the formation of all complexes (Figure 4, lanes 3 and 6). Despite using more nuclear extract in binding reactions containing the proximal MCAT element (5 µg vs 3.5 µg) while maintaining equal film exposure times, these data clearly illustrate enhanced nuclear protein binding

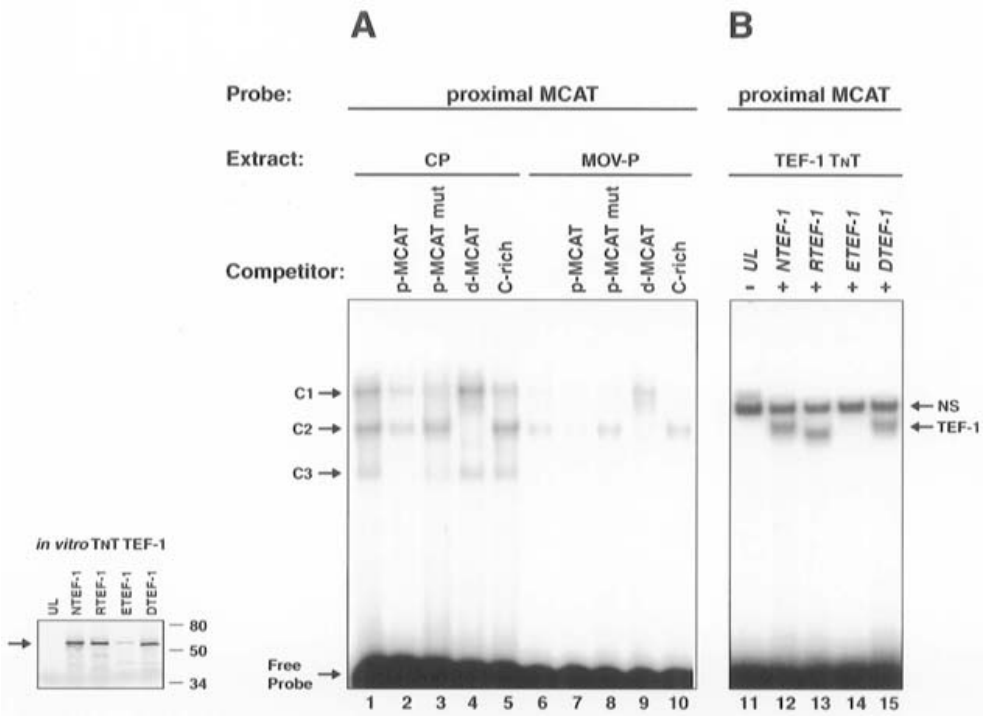

Figure 3. EMSA analysis of DNA-protein interaction at the βMyHC proximal MCAT element. Inset*, [*^{35}*S]methionine-labeled TEF-1. The rabbit reticulocyte lysate system was programmed with 1 μg of circular TEF-1 expression plasmid in the presence of [*^{35}*S]methionine. The TNT product was resolved by 12% SDS-PAGE and exposed to film. Molecular mass markers (in kilodaltons) are shown on the* right*. UL, unprogrammed lysate represents parallel reaction not programmed with TEF-1 expression plasmid.* A*, *^{32}*P-labeled proximal MCAT probe was incubated with 5 μg of either control plantaris (*CP, lanes 1-5*) or mechanical-overload rat plantaris (*MOV-P, lanes 6-10*) nuclear extract, or in* B*, 2.5 μl of either unprogrammed (*UL*,* lane 11*) or TEF-1 cDNA-programmed TNT lysate (*lanes 12-15*). Binding complexes were resolved as described under "Materials and Methods." For competition assays, the following non-radioactive oligonucleotides were added to the reaction mixture at a 100-fold molar excess: proximal MCAT (*lanes 2 and 7*), proximal MCAT mutant (*lanes 3 and 8*), distal MCAT (*lanes 4 and 9*), and C-rich non-specific oligonucleotide (*lanes 5 and 10*). Arrows (*C1, C2, C3*) indicate the positions of the major shifted complexes.* Free probe *represents excess unreacted radiolabeled oligonucleotide.* B*, in lanes 11-15,* NS *represents non-specific binding complex and TEF-1 represents the various transcription enhancer factor-1 isoforms.*

at the distal MCAT element as compared to the proximal MCAT element (Figure 3, lanes 1-10 vs Figure 4, lanes 1-6). Moreover, the formation of a highly enriched LMC only under MOV conditions suggests that this element may play a role in MOV-induction of transgene β293.

Having established enhanced nuclear protein binding at the distal MCAT element we wanted to determine if this element could bind *in vitro*-translated TEF-1 protein, and if so, whether this element bound TEF-1 with a higher affinity than the proximal MCAT element. EMSA analysis of binding reactions containing 0.5 μl of *in vitro*-translated NTEF-1 and the ^{32}P-labeled human distal MCAT probe (Figure 4, lane 7) revealed the formation of a binding complex that was distinct from the complex observed when unprogrammed lysate was used (Figure 3B, lane 11). The addition of 100-fold molar excess unlabeled proximal MCAT element to this reaction hardly altered complex formation (Figure 4, lanes 7 vs 8). In contrast, when 2.5 μl of *in vitro*-translated NTEF-1 protein was reacted with ^{32}P-labeled human proximal MCAT probe, a barely detectable complex formed that was completely abolished by the addition of 100-fold molar excess cold distal MCAT oligonucleotide (Figure 4, lane 9 vs 10). These data clearly show that the distal MCAT element binds *in vitro*-translated NTEF-1 protein with a greater affinity than does the proximal MCAT element, even when the ^{32}P-labeled proximal MCAT element was reacted with a 5-fold greater amount of *in vitro*-translated NTEF-1 protein than the distal MCAT element.

Differences in nuclear protein complex formation at the cTnT and βMyHC distal MCAT element

Previous EMSA analysis using nuclear extract isolated from embryonic chicken leg muscle and the chicken cardiac troponin T (cTnT) distal MCAT element revealed a binding pattern similar to that formed

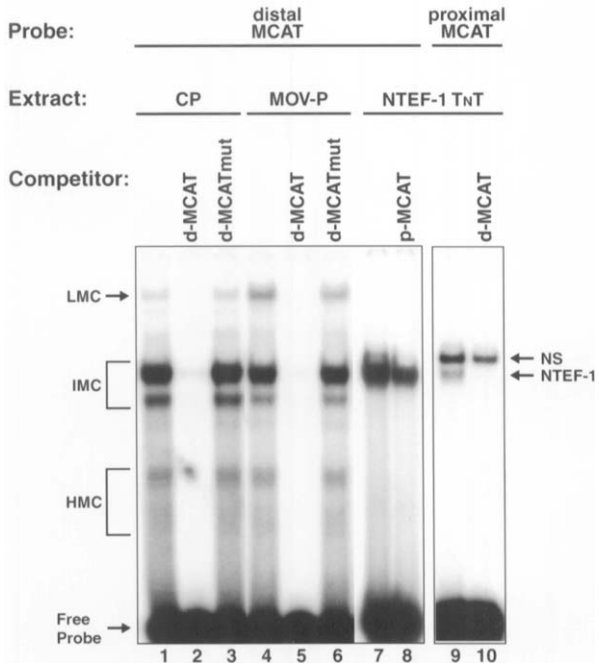

Figure 4. EMSA analysis of sequence-specific DNA-protein interactions at the βMyHC distal and proximal MCAT elements. 3.5 μg of CP (lanes 1-3) and MOV-P (lanes 4-6) nuclear extract and either 1 μl (distal MCAT) or 2.5 μl (proximal MCAT) of NTEF-1 TNT lysate was incubated with radiolabeled MCAT oligonucleotides. Competition EMSA was performed by challenging the binding complexes with a 100-fold molar excess of the following unlabeled oligonucleotides: distal MCAT (lanes 2, 5, and 10), distal MCAT mutant (lanes 3 and 6), and proximal MCAT (lane 8). The three major sets of DNA-protein binding complexes are labeled on the left: LMC, low mobility complex, IMC, intermediate mobility complex, and HMC, high mobility complex. Free probe represents excess unreacted radiolabeled oligonucleotide. NS, non-specific binding complex. NTEF-1 arrow indicates specific DNA-protein complex formed upon incubating NTEF-1 TNT lysate with either distal (lanes 7 and 8) or proximal (lanes 9 and 10) MCAT probes.

at the βMyHC distal MCAT element [19]. In particular, a high-order LMC formed that was shown to bind TEF-1 protein at the MCAT core sequence and the co-factor poly(ADP-Ribose) polymerase at the immediate 5'-flanking sequence [4, 19]. To determine if the LMC formed at the βMyHC and cTnT distal MCAT elements was comprised of the same or different proteins, we performed competition EMSA studies using ^{32}P-labeled cTnT as the binding probe and the distal MCAT element as competitor. EMSA analysis of binding reactions containing CP nuclear extract and the ^{32}P-labeled cTnT probe revealed the formation of three binding complexes (LMC, IMC, and HMC) that were not significantly enriched when MOV-P extract was used (Figure 5, lane 1 vs 4). When 100-fold molar excess cold cTnT probe was added to binding reactions containing either CP or MOV-P nuclear extract the formation of all complexes were abolished (Figure 5, lanes 2 and 5). Interestingly, the addition of 100-fold molar excess unlabeled βMyHC distal MCAT element to these binding reactions resulted in the elimination of IMC and HMC formation, but had no impact on LMC formation (Figure 5, lanes 3 and 6). In contrast, when the ^{32}P-labeled distal MCAT element was incubated with MOV-P nuclear extract in the presence of 100-fold molar excess cold cTnT MCAT element, the enriched LMC was partially competed for (data not shown). The latter findings suggest that the proteins comprising the LMC at these two different MCAT elements may differ, or alternatively, may in part reflect differences in binding affinity.

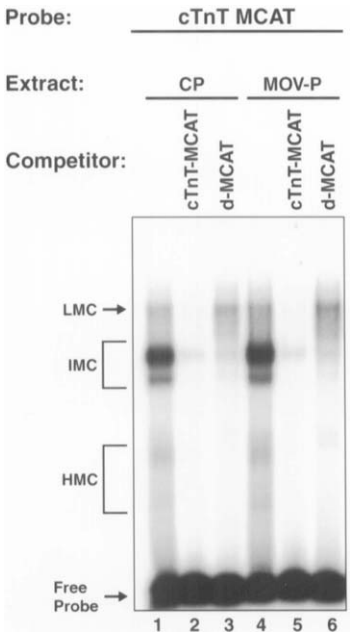

Figure 5. EMSA investigation of DNA-protein binding at the cardiac troponin T MCAT oligonucleotide. ^{32}P-labeled cTnT MCAT probe (20,000 cpm) was incubated with 5 μg of either CP (lanes 1-3) or MOV-P (lanes 4-6) nuclear extract. Three sets of specific DNA-protein complexes are indicated on the left: LMC, low mobility complex, IMC, intermediate mobility complex, and HMC, high mobility complex. Unlabeled competitor oligonucleotides harboring the cTnT MCAT (lanes 2 and 5) and βMyHC distal MCAT (lanes 3 and 6) binding elements were added at a 100-fold molar excess. Free probe represents excess unreacted radiolabeled oligonucleotide.

Diethylpyrocarbonate interference fotoprinting of βMyHC distal MCAT binding complexes

We performed DEPC interference footprinting (interferes with binding at both G and A nucleotides [32]) analyses to identify the exact nucleotides within the βMyHC distal MCAT element that interact with nuclear protein(s) to form the LMC, IMC, and HMC. DEPC footprinting delimited a binding site on the sense strand of the 21-bp distal MCAT probe that encompassed nucleotides –291 to –282 (Figure 6B). Analysis of the IMC and HMC indicated that nucleotide modification by DEPC either partially (positions 6 and 15) or strongly (position 8-11 and 13) interfered with CP nuclear protein binding (Fig. 6). An identical DEPC interference footprint was identified when MOV-P nuclear extract was used except that the partial interference at nucleotide 5 was not observed for the HMC (Figure 6).

DEPC modifications demarcated a binding site on the anti-sense strand of the 21-bp distal MCAT probe that stretched across nucleotides –293 to –283 (Figure 6B). Nucleotide modification by DEPC either strongly (12, 14) or partially (4, 5, 7) interfered with CP nuclear protein binding for the IMC, whereas the partial interference at nucleotide 4 was not observed for the HMC (Fig. 6). DEPC modification strongly (12, 14) and partially (4, 5, 7) disrupted MOV-P nuclear protein binding at the LMC and IMC, while the partial interference at nucleotide 4 was not detected for the HMC (Figure 6). The DEPC interference footprinting experiments revealed that nuclear protein(s) interact strongly within the heptameric distal MCAT core element and that this footprint did not differ significantly between complexes (LMC, IMC, HMC) or type of nuclear extract (CP versus MOV-P).

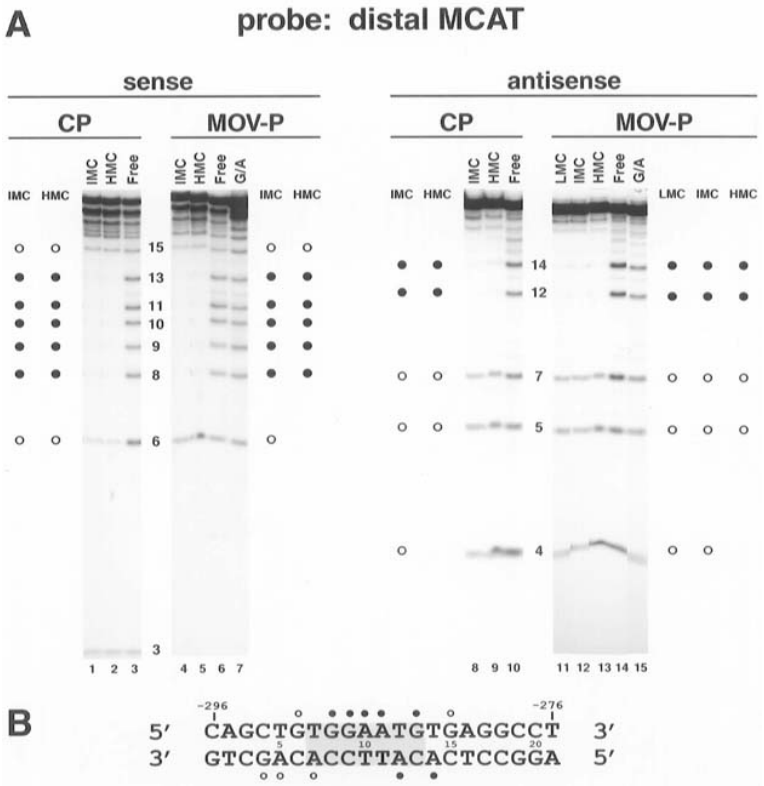

Figure 6. DEPC interference footprinting of the DNA-protein complexes formed at the βMyHC distal MCAT sequence. A, The 21-bp distal MCAT probe was ^{32}P-end-labeled on either the sense or the antisense strand and subjected to partial carbethoxylation with diethyl pyrocarbonate (DEPC). The modified probe was incubated with either CP or MOV-P nuclear extract and resolved by preparative EMSA as described in "Materials and Methods." The base-specific cleavage patterns of the LMC, IMC, and HMC complexes are shown for the sense (lanes 1-2 and 4-5) and antisense (lanes 8-9 and 11-13) strands. The cleavage profile for the unbound free probe is shown for the sense (lanes 3 and 6) and antisense (lanes 10 and 14) strands. Control sequence G/A (lanes 7 and 15) represents Maxam and Gilbert chemical cleavage of the unbound probe. The positions of the modified guanine (G) and adenine (A) residues which either completely (closed circle) or partially (open circle) interfere with LMC, IMC, or HMC formation are shown next to each panel. B, consolidated summary of the DEPC interference footprint within the LMC, IMC, and HMC formed at the distal MCAT oligonucleotide. Sequence numbering begins at the 5′-end of the sense strand. Open circle denotes partial interference; closed circle depicts complete interference.

Discussion

MCAT elements have been implicated as critical regulatory elements that participate in directing inducible as well as muscle- and non-muscle specific gene transcription. Although the molecular properties of MCAT elements have been studied in a variety of cell contexts, its involvement in directing adult-stage skeletal muscle gene transcription in response to altered neuromuscular activity has not been investigated. In our previous transgenic studies, we have identified a transgene comprised of 293-bp of human βMyHC promoter (β293) that is induced to express in the fast type II plantaris muscle by MOV, and that contains two distinct MCAT elements; the distal one located within the MOV-responsive region and the proximal one immediately adjacent to it [24, 38]. In the current study we have used mobility shift and DEPC footprinting analyses to examine whether the βMyHC proximal and distal MCAT elements participate in an equivalent manner during basal and MOV-inducible βMyHC transcription. Our results show that the βMyHC distal MCAT element displays binding properties that are distinct from those of the proximal MCAT element. In EMSA analysis using CP nuclear extracts, the distal MCAT element showed the formation of multiple high-intensity specific binding complexes whereas the proximal MCAT element formed a single low-intensity specific binding complex. In addition, as compared to control binding, when using MOV-P nuclear extract the distal MCAT element formed a highly enriched low mobility complex (Figure 4, lane 1 vs 4), whereas the specific proximal MCAT binding complex C2 was reduced (Figure 3, lane 1 vs 6). Finally, the distal MCAT element bound to *in vitro*-translated TEF-1 protein with a higher affinity than the proximal MCAT element (Figure 4, lane 7 vs 9). These data provide evidence to support the notion that the proximal and distal MCAT elements do not function equivalently during MOV induction of βMyHC transcription in fast type II fibers of adult mouse skeletal muscle.

Low-level nuclear protein binding occurs at human βMyHC proximal MCAT element

Earlier studies using promoter deletion analysis and DNAse I footprinting delimited a highly conserved enhancer-like control region within the βMyHC proximal promoter that contained three important regulatory subregions termed βe2 (distal MCAT), C-rich, and βe3 (proximal MCAT) ([8, 29, 33], Figure 2). Interestingly, further study of the proximal MCAT element by EMSA revealed robust binding at this element when reacted with nuclear extract isolated from a variety of differentiated myogenic cell lines [29, 33]. Mutation of this element within a 600-bp βMyHC promoter representing several species (rat, human, rabbit, mouse) resulted in a significant reduction and/or abolished βMyHC reporter gene expression whether assayed in the *in vitro* or *in vivo* context [18, 29, 33]. Furthermore, this element has been shown to direct α1-adrenergic mediated up-regulation of rat βMyHC reporter constructs in primary rat neonatal cardiocytes, that mechanistically was shown by immunological techniques to involve the formation of a specific binding complex containing TEF-1 protein [15, 16]. By competition EMSA analyses we also observed the formation of a single specific binding complex (termed C2) when the human proximal MCAT element was reacted with adult-stage skeletal muscle nuclear extract (Figure 3), however, the low levels of binding activity in our extracts and the lack of an adeguate TEF-1 antiserapreclude the immunological determination of TEF-1 protein binding at complex C2. Although speculative, several lines of evidence gathered from our study support the notion that TEF-1 is likely to be a binding component of specific complex C2 when either MOV-P or CP nuclear extracts are used. First, when the human distal MCAT element is added to binding reactions as competitor, the formation of complex C2 was completely abolished. This observation is important since the human distal MCAT element is nearly identical in nucleotide (core and 5'-flanking region) sequence to the NTEF-1 binding SV40 GTIIC element (Table 1). Second, we have also shown that *in vitro*-translated TEF-1 isoproteins bind the proximal MCAT element, albeit, with a lower affinity as compared to the distal MCAT element (see discussion below; Figure 3, lanes 11-15 and Figure 4, lanes 7-10). Last, complex C2 has the same relative mobility as the proximal MCAT element when bound by *in vitro*-translated TEF-1 (Figure 3A and B).

Enriched nuclear protein binding occurs at the human βMyHC distal MCAT element

Similar to the proximal MCAT element, previous studies have shown that mutation of the distal MCAT element within the context of a 600-bp βMyHC promoter results in reduced levels of expression *in vitro*, and the loss of muscle restricted expression when analyzed in transgenic mice [8, 18, 27, 29, 33]. However, in contrast to the binding properties of the proximal MCAT element, EMSA analysis of the distal MCAT element displayed higher levels of skeletal muscle nuclear protein binding and the formation of three specific binding complexes termed low (LMC), intermediate (IMC), and high (HMC) mobility complex (Figure 4, lanes 1-6). The aforementioned βMyHC distal MCAT expression and binding data are important because of their striking correlation to what is known about the well studied chicken cardiac troponin T MCAT (cTnT) elements, i.e., they are indispensable for directing embryonic skeletal muscle specific expression [23] and EMSA analysis of binding reactions (Figure 5) revealed the formation of LMC, IMC and HMC similar to the βMyHC distal MCAT element shown herein (Figure 4) [19]. It has also been shown by others, that TEF-1 is a component of all three binding complexes (LMC, IMC, and HMC) formed at the cTnT distal MCAT core element, and that for the LMC, Poly(ADP-Ribose) Poly-

merase (PARP) was determined to be the cofactor bound at the immediate 5'-flanking sequence [4, 7, 19]. Although we have not yet determined the exact identity of the nuclear protein(s) binding at the βMyHC distal MCAT element, TEF-1 is a likely candidate since: 1) the distal MCAT element has high sequence homology to the GTIIC element (Table 1), 2) there is a strong DEPC interference footprint across the MCAT core element (Figure 6), 3) our footprint strongly resembles footprints obtained from other known TEF-1 binding MCAT elements [8, 10, 25, 29], and 4) it has the ability to bind *in vitro*-translated TEF-1 protein (Figure 4). Further support for this idea comes from competition EMSA analysis showing that the βMyHC distal MCAT element abolishes formation of the IMC and HMC, but not the LMC, when the cTnT distal MCAT is reacted with adult-stage skeletal muscle nuclear extract (Figure 5). In the reverse experiment where the human βMyHC distal MCAT element was used as the binding probe and the cTnT distal MCAT acted as competitor, formation of the IMC and HMC complexes were also completely abolished, and the LMC was only partially competed away (data not shown). The differences in competition for the LMC between the βMyHC and cTnT distal MCAT elements likely represents differences in sequence recognition by some or all of the proteins binding at these elements, or alternatively, different binding affinities for the protein(s) interacting at these sites. In fact, recent evidence illustrates that TEF-1 isoproteins can mediate transcriptional activation with different efficiencies; a property which may be specific to each distinct TEF-1 isoprotein and/or related to MCAT element binding affinity [31]. As concerns differences in MCAT element binding affinity, our results show that the βMyHC proximal and distal MCAT elements bind both skeletal muscle nuclear extract (Figure 3, lane 1-10 vs Figure 4 lanes 1-6) and *in vitro*-translated TEF-1 protein (Figures 4, lanes 7-10) with different affinities.

Enriched nuclear protein binding at distal, but not proximal, MCAT element under MOV conditions

There are several examples cited in the current literature that substantiate a role for MCAT motifs as inducible promoter elements in response to both hormonal and mechanical stimuli [5, 16, 25]. For example, in addition to α1-adrenergic mediated α-skeletal actin and βMyHC up-regulation via their proximal MCAT elements [16, 17], mechanical stimuli such as hemodynamic overload [25], and skeletal muscle stretch [5] have also been shown to up-regulate α-MyHC and α-skeletal actin expression, respectively. It is noteworthy that in the later two examples, both mechanical stimuli are associated with increased expression of TEF-1 protein [5, 25].

In this study we have examined the possibility that the human βMyHC proximal and distal MCAT motifs acted as inducible elements by conducting EMSA binding studies using adult-stage MOV-P nuclear extracts. Given the known involvement of the βMyHC proximal MCAT element in βMyHC induction during *in vitro* cardiomyocyte hypertrophy, we were surprised to see a reduction from control levels in specific binding complex C2 formation when MOV-P nuclear extracts were reacted with this element (Figure 3A, lane 1 vs 6). In contrast, we observed an increase in MOV-P nuclear protein binding at the distal MCAT element that was reflected by enriched binding only in the LMC (Figure 4, lane 1 vs 4). Additionally, the cTnT distal element was found to have slightly enriched binding at all complexes (Figure 5, lane 1 vs 4). Although we observed enriched nuclear protein binding at the βMyHC distal element under MOV-conditions, the DEPC interference footprint did not reveal any substantial differences when either CP or MOV-P nuclear extracts were used (Figure 6). The absence of a change in DEPC footprint under CP versus MOV conditions might be accounted for by a possible increase in the protein(s) actively bound at this site, a switch in isoproteins, or an exchange of different proteins with similar size and binding characteristics. Nevertheless, the increased nuclear protein binding at the βMyHC distal MCAT element suggests that it may function as a MOV-inducible element in adult skeletal muscle. Consideration must still be given to the proximal MCAT element since the reduced binding at this site does not eliminate its direct/indirect involvement in MOV-induction of the βMyHC gene. One conceivable explanation that may account for the difference in binding between these two MCAT elements might be the higher affinity displayed by the distal MCAT element making it a more dominant competitor for available transcription factors, such as the TEF-1 protein(s) and their associated cofactors. The reduced binding observed at the proximal MCAT element, whether due to lower binding affinity or modification of bound factor(s) via activated intracellular signaling pathways, may be a requirement for maximum MOV-induction of the βMyHC gene. Alternatively, the proximal MCAT element is perhaps specific to induction of the βMyHC gene in the cardiac context based on the binding of tissue enriched specific TEF-1 isoforms and/or their post-transcriptional modification in response to hypertrophic signals [20].

In summary, we have shown that the βMyHC proximal and distal MCAT elements interact with TEF-1 isoproteins with different affinities which may, in part, confer different functions for these elements in directing adult-stage skeletal muscle-specific and inducible expression. An interesting finding observed during this study, was the formation of a highly enriched LMC formed at the distal MCAT element only under MOV-conditions. Our future investigations will focus on determining whether this enriched LMC will resemble that of the cTnT where TEF-1 binds the heptameric MCAT core sequence and PARP binds at the immediate 5'-flanking sequence resulting in muscle restricted expression. It will also be of interest to determine if the pro-

tein(s) comprising the βMyHC distal MCAT LMC differ depending on the physiological stimulus. For example, in the MOV-plantaris muscle, βMyHC expression is induced and is associated with a fast-to-slow fiber type switch, whereas, its expression is decreased and is associated with a slow-to-fast fiber-type transition in the non-weight bearing soleus muscle [24, 34, 35, 38]. The determination as to whether different TEF-1 isoforms and perturbation specific cofactors bind at the βMyHC distal MCAT element may lend insight into what role MCAT elements play in regulating βMyHC transcription in response to these diverse stimuli, and in modulating the associated fiber phenotype switches.

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