Divergence in species and regulatory role of β-myosin heavy chain proximal promoter muscle-CAT elements

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Tsika, Richard W., John McCarthy, Natalia Karasseva, Yangsi Ou, and Gretchen L. Tsika. Divergence in species and regulatory role of β-myosin heavy chain proximal promoter muscle-CAT elements. Am J Physiol Cell Physiol 283: C1761–C1775, 2002. First published August 22, 2002; 10.1152/ajpcell.00278.2002.—We examined the functional role of distinct muscle-CAT (MCAT) elements during non-weight-bearing (NWB) regulation of a wild-type 293-base pair β-myosin heavy chain (βMyHC) transgene. Electrophoretic mobility shift assays (EMSA) revealed decreased NTEF-1, poly(ADP-ribose) polymerase, and Max binding at the human distal MCAT element when using NWB soleus vs. control soleus nuclear extract. Compared with the wild-type transgene, expression assays revealed that distal MCAT element mutation decreased basal transgene expression, which was decreased further in response to NWB. EMSA analysis of the human proximal MCAT (pMCAT) element revealed low levels of NTEF-1 binding that did not differ between control and NWB extract, whereas the rat pMCAT element displayed robust NTEF-1 binding that decreased when using NWB soleus extracts. Differences in binding between human and rat pMCAT elements were consistent whether using rat or mouse nuclear extract or in vitro synthesized human TEF-1 proteins. Our results provide the first evidence that 1) different binding properties and likely regulatory functions are served by the human and rat pMCAT elements, and 2) previously unrecognized βMyHC proximal promoter elements contribute to NWB regulation.

skeletal muscle hypertrophy; skeletal muscle atrophy; fiber-type transitions; chlormphenicol acetyltransferase

ADULT MOUSE HINDLIMB skeletal muscles express four major myosin heavy chain (MyHC) isoforms (fast IIb, IIx/d, IIa, and slow type I) whose differential expression pattern has contributed to the broad classification scheme that distinguishes four primary fiber types termed fast type IIb, IIx/d, IIa, and slow type I (or β). Each MyHC isoform is thought to serve a specific physiological role; therefore, variation in the proportion and spatial arrangement of each fiber type contributes to the biochemical and functional specialization of each muscle. The notion that each MyHC serves a physiological role is supported by the classic findings that actin-activated myosin ATPase and unloaded shortening velocity (Vmax) are highly correlated to the amount and type of native isomyosin or MyHC comprising a given muscle or muscle fiber (2). For example, type I fibers primarily populate slow-twitch muscles, rely on oxidative metabolism, and express the βMyHC, which is highly efficient in energy utilization while maintaining tension. Thus slow-twitch muscles are primarily used in chronic activities such as posture maintenance and for sustained locomotor activity. On the other hand, fast type IIb and IIx/d fibers are used for high-force burst activities, primarily populate fast-twitch muscles, rely on glycolytic energy production, and are less efficient in energy utilization while maintaining tension (4, 36).

Once established, the adult skeletal muscle phenotype is not static but instead retains the ability to adjust to variations in load bearing and contractile usage patterns, resulting in profound adaptations in morphology, phenotype, and contractile properties (1, 4). The removal of body loading in the microgravity environment of space flight results in decreased bone density, a marked degree of muscle atrophy, and an altered protein phenotype that correlates with a slow-to-fast change in contractile and metabolic properties for both rodents and humans alike (1, 10, 11, 14). Likewise, qualitatively comparable results have been obtained from animal studies by using a rodent ground-based model of simulated microgravity imposed by hindlimb unloading (non-weight bearing [NWB]) (1, 10, 11, 14). In addition to altered muscle mass, strength, and endurance, alterations in the pattern of motor nerve activity have been reported (3, 34). Thus it is not surprising that chronically innervated postural muscles such as the slow-twitch soleus are most susceptible to the effects of a microgravity or simulated microgravity environment. In agreement with the aforementioned findings, our studies on the NWB mouse soleus muscle have documented a loss in mass, a histochemical slow-to-fast fiber type shift,

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and a decrease in endogenous βMyHC mRNA expression (29).

Transcriptional regulation is a fundamental mechanism by which adult-stage skeletal muscle phenotype and its adaptation are controlled. To identify regulatory elements that control βMyHC gene transcription during NWB activity, we have performed a transgenic deletion and mutational analysis of the βMyHC promoter. An expression analysis of transgenes comprised of either 5,600 or 600 base pairs (bp) of wild-type βMyHC promoter revealed that expression had significantly decreased in response to NWB; however, this response was found to be substantially blunted when the βMyHC proximal promoter control region (−300/−170; Fig. 1A) distal muscle-CAT (dMCAT), C-rich, and proximal MCAT (pMCAT) elements were simultaneously mutated (29). Further analysis of transgenes comprising either 350 or 293 bp of wild-type βMyHC promoter revealed that transgene β350wt contained a NWB responsive region (−294) containing the dNRE-S site, which may act as a NWB responsive element(s), a regulatory role possibly served by the negative (−350/−294) element. Our subsequent biochemical analysis of this sequence led to the elucidation of the first putative NWB element, termed dβNRE-S (−332/−311), that bound two distinct proteins highly enriched in NWB soleus (NWB-S) nuclear extract (Fig. 1A; Ref. 30).

Our current questions regarding βMyHC NWB regulation arise from two intriguing observations obtained from our above-cited transgenic analysis. First, the loss of normal NWB regulation of wild-type transgene β293wt occurred when the upstream NWB-responsive region (−600/−294, containing dβNRE-S) was eliminated. Thus it is conceivable that deletion of this NWB-responsive region may have disrupted critical interactions between this element and those elements within the βMyHC control region. In support of this notion, numerous studies using transgenic mice have shown that distinct regulatory modules (enhancers) and/or individual cis-acting elements are required to accurately duplicate diverse aspects of endogenous muscle-specific gene expression (9, 13, 16, 20, 25, 26, 32). Second, the partial loss of NWB regulation of the 5,600-bp βMyHC promoter occurred when the βMyHC control region (−300/−170) dMCAT, C-rich, and pMCAT elements were simultaneously mutated, indi-

Fig. 1. A: schematic representation of β-myosin heavy chain (βMyHC) proximal promoter. Depicted is the non-weight-bearing (NWB)-responsive region (−600/−294) containing the dβNRE-S site, which may act as a NWB element. Also shown is the downstream control region (−300/−170) comprised of the distal muscle-CAT (dMCAT), A/T-rich, C-rich, proximal MCAT (pMCAT), and E-box/nuclear factor of activated T cells (NFAT) elements, which are highly conserved in sequence and location across species. B: schematic illustration of human βMyHC transgenes. Wild-type transgene comprises 293 base pairs (bp) of the human βMyHC proximal promoter plus 120 bp of 5′-untranslated region (+1 to +120) linked to the 5′-end of bacterial chloramphenicol acetyltransferase (CAT) gene-simian virus 40 poly(A) cassette (transgene β293wt). The small box to the left of the CAT gene represents exon 1. Regulatory elements comprising the βMyHC proximal control region are shown from the 5′- to 3′-end and are termed dMCAT, A/T-rich, pMCAT, and E-box/NFAT. Mutant transgene β293Mm is identical to transgene β293wt, except that it harbors a mutated dMCAT element that was generated by bp substitution at nucleotide sites previously shown to be critical for protein-DNA interaction by footprint and scanning mutagenesis analysis (44). Bp substitutions (mutation) are depicted by lowercase letters and are presented 5′ to 3′.
Transgenic mice. Transgenic mice were generated by microinjection of purified transgene DNA into pronuclei of zygotes as described previously (40). Transgenic founder mice were identified by Southern blot analysis, and copy number was then estimated (47). Transgene-positive offspring were identified by PCR amplification by using primers specific for the CAT gene (47). All lines were maintained in a heterozygous state by backcrossing to the nontransgenic FVB/N parental mouse strain.

Animal care and NWB procedure. The Animal Care Committee for the University of Missouri-Columbia approved the NWB procedure used in this study, and the NWB mice were housed in an Association for the Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility. All animals were provided with food and water ad libitum and were housed at room temperature (24°C) with a 12:12-h light-dark cycle in standard rodent cages (control animals) or in cages designed for head-down tilt hindlimb suspension (NWB) as described in detail previously (29).

Adult female wild-type β293 (β293wt) transgenic mice from line 2 are used in this study to show 1) control vs. NWB body and muscle weight and 2) basal and NWB expression levels (Tables 1 and 2). Basal and NWB expression levels for transgenic mice lines 99, 96, and 5 have been reported previously (30) and are used here to facilitate comparisons against basal and NWB expression data collected for transgenic mice carrying mutant transgene β293Mm (Table 2). Adult female β293Mm transgenic mice from lines 4, 6, 7, and 9 (≈22 g) were assigned to one of two groups: 1) a NWB group that used 2 wk of hindlimb suspension to impose NWB conditions (NWB; n = 8), and 2) a group that served as cage ambulatory controls for the NWB group (control; n = 8). After a 2-wk experimental period, both cage control and NWB mice were anesthetized and weighed, and their control soleus (CS) and NWB-soles (NWB-S) muscles were collected for further study (Tables 1 and 2). At the same time, we collected gastrocnemius muscle (≈123 mg) from 40 adult (≈22 g) control mice to be used for the isolation of nuclear extract. The gastrocnemius muscle was used for the isolation of nuclear extract because of the small size of the CS (7–8 mg) muscle. All control and NWB muscles were trimmed clean of fat and connective tissue, weighed, and stored at −80°C until assayed for CAT-specific activity (CS and NWB-S) or used for the isolation of nuclear extract (control gastrocnemius).

All mice designated for terminal sample collection were anesthetized by using 2.5% xylazin at a dosage of 0.017 ml/g of body weight and were euthanized by cervical dislocation while under anesthesia.

Rats were used for the preparation of nuclear extracts utilized in EMSA studies. Each EMSA was repeated using three to four different nuclear extracts in which each extract represented either 20 control rats or 30 NWB rats. Adult
female Sprague Dawley rats (−230 g) were assigned to one of two groups: 1) a NWB group that used 2 wk of hindlimb suspension to impose NWB conditions (NWB; n = 30/extract) and 2) a group that served as cage ambulatory controls for the NWB group (control; n = 20/extract). Rats were prepared for the NWB experiment by a modification of the noninvasive tail traction procedure as described previously (29). After 2 wk of NWB, body weight decreased by 10% (control = 230 ± 4.66 g vs. NWB = 208 ± 3.42 g), whereassoleus weight decreased by 42% (control = 114.4 ± 1.8 mg vs. NWB = 66.35 ± 2.3). After 2 wk of NWB, both control and NWB rats were anesthetized and weighed, and their CS and NWB-S muscles were collected for production of nuclear extracts (30). All rats designated for terminal sample collection were anesthetized by using 0.1 ml/g body wt of anesthetic containing 87 mg/ml ketamine and 13 mg/ml xylazine. Rats were euthanized by CO2 while under anesthesia.

CAT assays. CAT assays were performed as previously described (41). Muscle extracts were prepared from transgenic tissues by using a glass tissue homogenizer to disrupt tissues in 250 mM Tris·HCL (pH 7.8) and 5 mM EDTA. All muscle extracts were prepared from frozen tissue, and each n value represents the number of pooled soleus muscles from one mouse. The protein concentration of the extracts was determined by the method of Bradford (5). Muscle extracts were prepared from frozen tissue, and each n value represents the number of pooled soleus muscles from one mouse. The protein concentration of the extracts was determined by the method of Bradford (5). Muscle extracts were prepared from frozen tissue, and each n value represents the number of pooled soleus muscles from one mouse.

The percent conversion of [14C]chloramphenicol to the acetylated form was quantified by using a PhosphorImager (Storm860) with ImageQuant version 5.1 software. Direct comparisons between and within transgenic lines representing both control and experimental groups (CS and NWB-S) were facilitated by presenting the data as specific CAT activity (picomoles per microgram of protein per minute) (Table 2).

Preparation of nuclear protein extract from adult skeletal muscle. Nuclear extracts (NE) were isolated from adult rat CS and NWB-S muscle and from mouse control gastrocnemius muscle as previously described (30). Three independent batches of CS, NWB-S, and control gastrocnemius nuclear extract were isolated and used in EMSA analysis and yielded the same results. Protein concentration was determined according to Bradford (5).

EMSA. All oligonucleotide probes used in this study are listed in Table 3. EMSAs were carried out as previously described (30, 43, 44). The double-stranded human and rat proximal MCAT oligonucleotide probes were labeled by fill-in reaction using Klenow fragment of Escherichia coli DNA polymerase I (Stratagene, La Jolla, Ca) and [γ-32P]dCTP (3,000Ci/mmol). The human distal MCAT oligonucleotide probe was end-labeled by T4 polynucleotide kinase (New England Biolabs, Beverly, Ma) and [γ-32P]dATP (6,000Ci/ mmol). All probes were purified by polyacrylamide gel electrophoresis before use in EMSAs. Binding reactions were performed by using either rat CS or NWB-S nuclear extract (dMCAT probe = 4.0 μg, pMCAT probe = 5.0 μg) or mouse control gastrocnemius nuclear extract (pMCAT probe = 5.5 μg) and 20,000 cpm of labeled probe for 20 min at room temperature in a 25-μl total volume. Where indicated, binding reactions contained 1 μl (rat pMCAT element) or 5 μl (human pMCAT probe) of in vitro-translated human NTFE-1 protein in place of muscle nuclear extract. The binding reac-

Table 1. Body and muscle weights from control and NWB transgenic mice

<table>
<thead>
<tr>
<th>Weight</th>
<th>Wild-Type β293WT Line 2</th>
<th>Mutant β293Mm Lines 4, 6, 7, 9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Control</td>
</tr>
<tr>
<td>Body (initial), g</td>
<td>8</td>
<td>24.5 ± 0.4</td>
</tr>
<tr>
<td>Body (final), g</td>
<td>8</td>
<td>24.8 ± 0.5</td>
</tr>
<tr>
<td>Soleus, mg</td>
<td>16</td>
<td>6.32 ± 0.2</td>
</tr>
<tr>
<td>Soleus, mg/g</td>
<td>16</td>
<td>0.25 ± 0.00</td>
</tr>
</tbody>
</table>

Weight data for mutant β293Mm lines 4, 6, 7, and 9 are compiled. Weight data for wild-type β293wt line 2 are reported and although morphometric data for β293wt lines 5, 96, and 99 are not shown here, they have been reported previously (44) and are in good agreement with our results reported herein. All values are means ± SE. Statistical significance was accepted at P < 0.05.*P < 0.05; †P < 0.01; ‡P < 0.001; control vs. NWB-S. NWB-S, non-weight-bearing soleus.

Table 2. Response of mutant transgene β293Mm to NWB.

<table>
<thead>
<tr>
<th>Line No.</th>
<th>Copy No.</th>
<th>n</th>
<th>CS</th>
<th>NWB-S</th>
<th>Fold increase</th>
<th>Line No.</th>
<th>Copy No.</th>
<th>n</th>
<th>CS</th>
<th>NWB-S</th>
<th>%Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>12</td>
<td>8</td>
<td>163 ± 9</td>
<td>414 ± 12</td>
<td>+2.5†</td>
<td>4</td>
<td>6</td>
<td>8</td>
<td>91.6 ± 3.4</td>
<td>60.8 ± 2.3</td>
<td>-33.7*</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
<td>8</td>
<td>167 ± 38</td>
<td>392 ± 75</td>
<td>+2.4*</td>
<td>6</td>
<td>35</td>
<td>8</td>
<td>135.5 ± 3.1</td>
<td>6.2 ± 1.1</td>
<td>-54.1†</td>
</tr>
<tr>
<td>96</td>
<td>64</td>
<td>8</td>
<td>1254 ± 54</td>
<td>2172 ± 147</td>
<td>+1.7*</td>
<td>7</td>
<td>43</td>
<td>6</td>
<td>47.3 ± 8.6</td>
<td></td>
<td></td>
</tr>
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<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>25.9 ± 5.3</td>
</tr>
<tr>
<td>99</td>
<td>84</td>
<td>8</td>
<td>490 ± 57</td>
<td>1111 ± 135</td>
<td>+2.3*</td>
<td>9</td>
<td>1</td>
<td>8</td>
<td>6.6 ± 0.4</td>
<td>4.0 ± 0.8</td>
<td>-40.3*</td>
</tr>
</tbody>
</table>

Values for chloroamphenicol acetyltransferase (CAT) specific activity (in pmol·μg protein −1·min −1) are expressed as means ± SE. CAT-specific activity and protein extracts were incubated with 20 mM acetyl-CoA and [14C]chloramphenicol at 37°C. Incubation conditions for control soleus (CS) or NWB-S muscle protein extracts for β293wt line 2 were 10 μg/60 min. β293wt lines 5, 96, and 99 have been reported previously (30) as well as CS data for β293Mm (44) and are presented here for ease of comparison. Incubation conditions for CS and NWB-S muscles from mutant β293Mm (distal MCAT mutation) transgenic lines were as follows: lines 4 and 7, 30 μg/60 min; line 6, 15 μg/60 min; line 9, 10 μg for 17 h. *P < 0.05; †P < 0.01; ‡P < 0.001; CS vs. NWB-S. Comparison of transgene expression for wild-type (β293wt) lines vs. mutant (β293Mm) lines within the CS were significantly (P < 0.001; not denoted in table) decreased in all cases.

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RESULTS

The formation of a highly specific and enriched low-mobility complex at the βMyHC dMCAT decreases after 2 wk of NWB. To determine whether the binding properties of the βMyHC dMCAT element differed between control and NWB conditions, we performed a direct and competition EMSA analysis using either CS or NWB nuclear extract. Binding reactions containing the 32P-labeled wild-type βMyHC dMCAT probe (Table 3) and either CS or NWB-S nuclear extract showed the formation of multiple binding complexes termed low-mobility complex (LMC), intermediate-mobility complex (IMC), and high-mobility complex (HMC) (Fig. 2). The formation of a LMC when using NWB-S nuclear extract was substantially reduced compared with the LMC formed when using CS nuclear extract, whereas those comprising the HMC increased (Fig. 2, lane 1 vs. 6). The addition of 100-fold molar excess cold wild-type βMyHC dMCAT probe to binding reactions containing either CS or NWB-S nuclear extract completely abolished complex formation, indicating that these complexes are specific (Fig. 2, lanes 2 and 7). Interestingly, the addition of 100-fold molar excess cold cTnT MCAT1 probe [an element previously shown to form LMC, IMC, and HMC binding complexes (28)] to binding reactions containing either CS or NWB-S nuclear extract prevented IMC and HMC formation and partially competed away the LMC formation (Fig. 2, lane 3 vs. 8). The addition of 100-fold molar excess cold muscle creatine kinase (MCK) transcriptional regulatory factor x (Trex) element [an element that has relative sequence similarity to consensus MCAT site but that is shown not to bind TEF-1 (12)] to binding reactions containing either CS or NWB-S nuclear extract did not effectively compete for complex formation (Fig. 2, lane 4 vs. 9). Because the immediate 5′-flanking region of the βMyHC dMCAT element contains a consensus E-box element, we examined whether E-box binding proteins were components of the LMC. The addition of 100-fold molar excess high-affinity MCK E-box as competitor to binding reactions containing either CS or NWB-S nuclear extract did not alter complex formation (Fig. 2, lane 5 vs. 10). These data show that specific binding complex formation at the dMCAT element differed when using CS vs. NWB-S nuclear extract, and

Table 3. Oligonucleotides

<table>
<thead>
<tr>
<th>Oligo Name</th>
<th>Sequence (Sense Strand 5′→3′)</th>
<th>Species</th>
<th>Position</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>βMyHC dMCAT (distal)</td>
<td>AAGCTTGGAGATCTGGAAGCTTCTT</td>
<td>human</td>
<td>−296−276</td>
<td>44</td>
</tr>
<tr>
<td>βMyHC dMCATmut</td>
<td>AAGCTTGGCTCTTGGAAGCTTCTT</td>
<td>human</td>
<td>−296−276</td>
<td>44</td>
</tr>
<tr>
<td>βMyHC pMCAT (proximal)</td>
<td>GCCAATGTCCAGTCAGGAACATGC</td>
<td>human</td>
<td>−216−197</td>
<td>this study</td>
</tr>
<tr>
<td>βMyHC pMCATmut</td>
<td>GCCAATGGACGTGTAACAAAC</td>
<td>human</td>
<td>−216−197</td>
<td>this study</td>
</tr>
<tr>
<td>βMyHC pMCAT (proximal)</td>
<td>GCCAATGCTGACCCACCAAA</td>
<td>rat</td>
<td>−223−204</td>
<td>this study</td>
</tr>
<tr>
<td>βMyHC pMCATmut</td>
<td>GCCAATGCTGTGAAACCAAA</td>
<td>rat</td>
<td>−223−204</td>
<td>this study</td>
</tr>
<tr>
<td>cTnT MCAT</td>
<td>TCAGCTGTTCTGGCTCTCTCTCTTG</td>
<td>chicken</td>
<td>−103−84</td>
<td>28</td>
</tr>
<tr>
<td>MCK Trex</td>
<td>GCAGGATGCCATGAGCTCTGTATA</td>
<td>mouse</td>
<td>−1213−1192</td>
<td>12</td>
</tr>
<tr>
<td>MCK E-box</td>
<td>ATGGCCCATACGCTTCTGCGA</td>
<td>mouse</td>
<td>−1163−1139</td>
<td>28</td>
</tr>
</tbody>
</table>

Core consensus binding elements within the oligonucleotides are delineated in boldface type. Lowercase letters represent mutant sequence.
this difference was characterized by a striking reduction in LMC formation only when using NWB extract. Also, known muscle regulatory factors (MyoD, myogenin) previously shown to bind the MCK E-box are likely not components of the specific binding complex formed at the βMyHC dMCAT element.

dMCAT element core nucleotides are required for DNA-protein binding complex formation. To determine whether different nucleotides within the 21-bp βMyHC dMCAT element interact with CS vs. NWB-S nuclear protein, we performed competition EMSA analysis using scanning mutagenesis. In these experiments, we introduced nucleotide substitutions 2 bp at a time, starting within the immediate 5′-flanking region (E-box) and extending throughout the core MCAT element and its immediate 3′-flanking region (Fig. 3, A and B). The resulting unlabelled oligonucleotides were then added in 100-fold molar excess to binding reactions containing either CS or NWB-S nuclear extract or the 32P-labeled wild-type dMCAT probe (Fig. 3A). As shown previously, the addition of excess wild-type βMyHC dMCAT probe to binding reactions containing either CS or NWB-S nuclear extract completely abolished the formation of all binding complexes (Fig. 3A, lanes 2 and 12). Similarly, all binding complexes were effectively competed away by the addition of either dMCAT mut-1, mut-2 (mutations within E-box), or mut-7 (3′-flanking region) to binding reactions containing either CS (Fig. 3A, lane 1 vs. 3, 4, and 9) or NWB-S (Fig. 3A, lane 11 vs. 13, 14, and 19) nuclear extract. Importantly, mutant MCAT probes carrying nucleotide substitutions within the core MCAT element (mut-3, 4, 5, and 8) did not act as effective competitors of complex formation when added to binding reactions containing CS (Fig. 3A, lane 1 vs. 5–7 and 10) or NWB-S (Fig. 3A, lane 11 vs. 15, 16, 18, and 20) nuclear extract. Interestingly, the addition of dMCAT mut-6 probe to binding reactions containing CS (Fig. 3A, lane 1 vs. 8) or NWB-S (Fig. 3A, lane 11 vs. 18) nuclear extract partially competed for LMC formation, but not IMC or HMC. These data show that nucleotides comprising the core βMyHC dMCAT element are critical for the formation of all binding complexes when using either CS or NWB-S nuclear extract (Fig. 3C).

**NTEF-1, PARP, and Max comprise the LMC formed at the βMyHC distal MCAT element.** Although we have shown previously that NTEF-1, PARP, and Max comprise the LMC formed at the dMCAT element when using CS nuclear extract, it is possible that different nuclear proteins form the LMC under NWB conditions (44). Thus we performed antibody EMSAs using polyclonal antibodies that recognize NTEF-1, PARP, or Max to determine whether these proteins comprised the LMC formed at the βMyHC dMCAT element when using NWB-S nuclear extract or whether other proteins were components of this complex. The specific binding complexes formed at the 32P-labeled human βMyHC dMCAT element when reacted with NWB-S nuclear extract were not altered by preincubation with PI (Fig. 4, lane 2 vs. 3). The addition of polyclonal NTEF-1 antibody to binding reactions containing NWB-S nuclear extract supershifted only the top band of the IMC doublet and the prominent HMC band, whereas the LMC was partially abolished (Fig. 4, lane 2 vs. 4). Interestingly, the addition of either polyclonal PARP or Max antibody to binding reactions using NWB-S extract essentially immunodepleted the LMC, which resulted in an embellishment of the top band of the IMC, which entirely comprises NTEF-1 protein (Fig. 4, lane 2 vs. 5 and 6). When all combinations of the three polyclonal antibodies were added to binding reactions using NWB-S nuclear extract, the LMC was completely immunodepleted (Fig. 4, lane 2 vs. 7). The preincubation of binding reactions containing either CS or NWB-S nuclear extract with antibodies recognizing other E-box-binding bHLH (basic helix-loop-helix) and bHLH-Zip proteins (MyoD, myogenin, E2A, HEB, and USF) did not alter βMyHC dMCAT element binding complex formation or mobility (unpublished obser-

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**Table 1.**

<table>
<thead>
<tr>
<th>Probe:</th>
<th>βMyHC distal MCAT</th>
<th>Extract:</th>
<th>CS</th>
<th>NWB-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Competitor:</td>
<td>dMCAT</td>
<td>C(\text{hTf} ) MCAT</td>
<td>MCK - E-box</td>
<td>dMCAT</td>
</tr>
<tr>
<td>LMC</td>
<td>Lane 1</td>
<td>Lane 2</td>
<td>Lane 3</td>
<td>Lane 4</td>
</tr>
<tr>
<td>IMC</td>
<td>Lane 7</td>
<td>Lane 8</td>
<td>Lane 9</td>
<td>Lane 10</td>
</tr>
<tr>
<td>HMC</td>
<td>Lane 13</td>
<td>Lane 14</td>
<td>Lane 15</td>
<td>Lane 16</td>
</tr>
<tr>
<td>Free Probe</td>
<td>Lane 19</td>
<td>Lane 20</td>
<td>Lane 21</td>
<td>Lane 22</td>
</tr>
</tbody>
</table>

**Fig. 2.** Competition electrophoretic mobility shift assay (EMSA) assessment of sequence-specific DNA-protein binding at the βMyHC distal MCAT element. 32P-labeled βMyHC distal MCAT (dMCAT) oligonucleotide probe was incubated with 4.0 μg of either control soleus (CS, lanes 1–5) or NWB-soleus (NWB-S, lanes 6–10) nuclear extract. To characterize the sequence specificity of binding at the dMCAT, the following cold (nonradioactive) competitors were added to the reaction mixture at a 100-fold molar excess before the addition of the probe: dMCAT (lanes 2 and 7), cardiac troponin T (c\(\text{hTf} \)) MCAT (lanes 3 and 8), muscle creatine kinase (MCK) Trex (lanes 4 and 9), and the MCK high-affinity right E-box (lanes 5 and 10). Lane 11 contains no extract (free). Low-, intermediate-, and high-mobility complexes are designated LMC, IMC, and HMC, respectively. Free probe represents excess unreacted radiolabeled probe. A decrease in HMC formation and a concomitant increase in LMC formation was consistently observed with NWB-S nuclear extract (lane 6) relative to CS (lane 1). These experiments were repeated using 3 independent batches of nuclear extracts, which yielded the same results.
These data support several noteworthy conclusions:
1) Proteins antigenically related to NTEF-1, PARP, and Max comprise the LMC formed at the human H9252MyHC dMCAT element;
2) Protein antigenically related to NTEF-1 likely constitutes the prominent HMC band and the top band of the IMC doublet; and
3) Decreased binding of NTEF-1, PARP, and Max at the dMCAT element under NWB conditions may, in part, contribute to NWB decreases in H9252MyHC gene expression.

Morphological changes after NWB. Body and soleus muscle weight data reported for transgenic mice harboring mutant transgene H9252Mm represent pooled weights from transgenic lines 4, 6, 7, and 9 (Table 1). Compared with control values, 2 wk of NWB activity imposed by hindlimb suspension resulted in a significant decrease in the body wt of H9252Mm (−15.8%) and H9252wt (−19.3%) transgenic mice (Table 1). Similarly, significant decreases were measured in both absolute (H9252Mm = −27.1%; H9252wt = −31.9%) and normalized (H9252Mm = −17.1%; H9252wt = −16%) NWB-S muscle weight compared with control values (Table 1). These data are consistent with our previous findings showing that a 2-wk NWB treatment produces statistically significant decreases in both mouse body and muscle mass (29, 30).

Mutation of distal MCAT element restores NWB regulation and alters basal slow muscle expression of wild-type transgene H9253wt. We examined the hypothesis that the dMCAT element is responsible for the unexpected upregulation of transgene H9253wt in the absence of the upstream NWB-region by studying multi-
To examine the impact of dMCAT element mutation on basal transgene expression, we performed expression assays. Measured CAT specific activity (picomoles per microgram of protein per minute) in CS muscle extract of transgenic mice harboring mutant transgene β293Mm was significantly decreased compared with CS muscle extract of mice carrying wild-type transgene β293wt (Table 2). These results confirm our previous findings by demonstrating that the dMCAT element acted as a strong positive cis-acting element that is required for high basal levels of wild-type transgene β293wt expression (44). We next examined what effect mutation of the dMCAT element would have on NWB regulation of mutant transgene β293Mm. After 2 wk of NWB, the CAT specific activity measured in NWB-S muscle extract obtained from transgenic mice representing each of four independent transgenic lines carrying mutant transgene β293Mm showed decreases ranging from −33.7 to −54.1% compared with that measured in CS muscle extract of β293Mm mice (Table 2). These results were in sharp contrast to the CAT-specific activity measured in NWB-S extract of mice carrying wild-type transgene β293wt, which showed increases ranging from 1.7- to 2.5-fold above those measured in CS muscle extract of β293wt mice (Table 2). Because NWB downregulation was consistently obtained for each of four independent β293Mm transgenic lines that carried different transgene copy numbers (Table 2), it is unlikely that our results are due to chromosomal integration site or seldom encountered copy number effects. Our findings are consistent with the well-documented fact that not all transgenes express in a copy number-dependent fashion, in contrast to the claim by Giger et al. (15). In fact, the incidence of transgenes that do express in a copy number dependent manner is rare, and these transgenes have been shown to be associated with a locus control region (27, 35). Thus our findings indicate the following: 1) the dMCAT element is required for high-level basal slow muscle expression of wild-type transgene β293wt, 2) the unexpected upregulation of wild-type transgene β293wt in response to NWB activity is driven by the dMCAT element, and 3) previously unidentified element(s) located downstream from the dMCAT (−290/−284) element contribute to decreases in βMyHC expression under NWB conditions.

**Dissimilar binding of rat nuclear extract between human and rat βMyHC pMCAT elements suggests a species difference in regulatory roles.** The pMCAT element (−210/−203) resides downstream from the dMCAT element (−290/−284) and has been shown to play a regulatory role in the downregulation of injected rat βMyHC reporter plasmids in the soleus muscle of spinal cord isolated rats (19). Because spinal cord isolation and NWB are associated with decreased soleus muscle βMyHC gene expression, we have used an EMSA analysis to evaluate whether the human pMCAT element (−210/−203) serves a regulatory role in the downregulation of injected rat βMyHC reporter plasmids. Dissimilar binding of rat nuclear extract between human and rat βMyHC pMCAT elements suggests a species difference in regulatory roles. The pMCAT element (−210/−203) resides downstream from the dMCAT element (−290/−284) and has been shown to play a regulatory role in the downregulation of injected rat βMyHC reporter plasmids in the soleus muscle of spinal cord isolated rats (19). Because spinal cord isolation and NWB are associated with decreased soleus muscle βMyHC gene expression, we have used an EMSA analysis to evaluate whether the human pMCAT element (−210/−203) serves a regulatory role in response to NWB. Incubation of 32P-labeled human pMCAT element with rat CS nuclear extract resulted in the formation of three binding complexes (termed C1, C2, C3) that did not change in intensity when rat NWB-S nuclear extract was used (Fig. 5A, lanes 1 and 6). The addition of 100-fold molar excess cold wild-type human pMCAT probe to binding reactions containing rat CS nuclear extract competed away complex C2 only, whereas competition with 100-fold molar excess of mutant human pMCAT (pMCATm) element did not compete away complex C2 but did compete away com-
plexes C1 and C3, indicating that only complex C2 represents specific binding (Fig. 5A, lanes 2 and 3). When binding reactions containing rat CS or NWB-S nuclear extract were preincubated with PI, complex formation at the human pMCAT element was not altered (Fig. 5A, lanes 1 vs. 4 and 6 vs. 7), whereas addition of polyclonal NTEF-1 antibody led to a supershift (SS) of only complex C2 (Fig. 5A, lanes 1 vs. 5 and 6 vs. 8). These data indicate that a nuclear protein antigenically related to NTEF-1 forms the low-intensity C2 specific binding complex at the human pMCAT element. However, because the intensity of this complex did not differ between control and NWB conditions, it is unlikely that the human pMCAT element plays a regulatory role under NWB conditions.

Our findings for the human pMCAT element prompted us to evaluate whether the rat pMCAT element contributes to NWB regulation, a role not yet assessed for this element. Given that the human and rat pMCAT elements differ in nucleotide composition

### Table: pMCAT Element Species Comparison

<table>
<thead>
<tr>
<th>Species</th>
<th>pMCAT Element</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>human</td>
<td>pMCAT</td>
<td>CATGCATGCACATA</td>
</tr>
<tr>
<td>rat</td>
<td>pMCAT</td>
<td>CATGCAACACACCACA</td>
</tr>
</tbody>
</table>

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**Fig. 5.** Competition and antibody EMSA analysis of human and rat pMCAT elements using CS or NWB-S extract. A: ³²P-labeled human pMCAT probe was incubated with 5.0 µg of either CS (lanes 1–5) or NWB-S (lanes 6–8) nuclear extract. EMSA analysis of sequence specific binding at the human pMCAT element was determined by the addition of 100-fold molar excess nonradioactive (cold) wild-type or mutant human pMCAT element before the addition of the ³²P-labeled human pMCAT probe. Note that only complex C2 is competed away by the addition of wild-type pMCAT element (lane 1 vs. 2), whereas the addition of mutant pMCATm probe does not compete for complex C2 (lane 1 vs. 3). For antibody supershift, EMSA rat CS or NWB-S nuclear extract was preincubated with 2 µl of PI (lanes 4 and 7) or polyclonal NTEF-1 antibody (lanes 5 and 8) for 30 min at room temperature before the addition of the probe. B: ³²P-labeled rat pMCAT probe was incubated with 5.0 µg of either CS (lanes 9–13) or NWB-S (lanes 14–16) nuclear extract. Specificity of binding at the rat pMCAT element was determined by the addition of 100-fold molar excess nonradioactive (cold) wild-type or mutant rat pMCAT element before the addition of the ³²P-labeled rat pMCAT probe. Note that only complex C2 is competed away by the addition of wild-type rat pMCAT element (lane 1 vs. 2), whereas the addition of mutant pMCATm probe does not compete for complex C2 (lane 1 vs. 3). For antibody supershift, EMSA rat CS or NWB-S nuclear extract was preincubated with 2 µl of PI (lanes 12 and 15) or polyclonal NTEF-1 antibody (lanes 13 and 16) for 30 min at room temperature before the addition of the probe. The anti-NTEF-1 antibody produced a supershift of binding complex C2 (human pMCAT, lanes 5 and 8; rat pMCAT, lanes 13 and 16). This assay was performed by using 3 different batches of nuclear extract which yielded the same results. C: Alignment of MCAT core elements is delineated in bold type. Nucleotide differences between the human vs. rat pMCAT element are underlined on the human sequence. The overlapping rat pMCAT element is boxed.
(Fig. 5C and Table 3), it is possible that the binding properties of these elements may differ. Thus, to evaluate whether differences in nucleotide composition between the human and rat pMCAT elements would influence nuclear protein binding and/or NWB regulation, we performed an EMSA analysis. Incubation of \(^{32}\)P-labeled rat pMCAT element with either rat CS or NWB-S nuclear extract resulted in the formation of three binding complexes (C1, C2, C3); however, the intensity of complex C2 was robust and decreased considerably when NWB-S nuclear extract was used (Fig. 5B, lane 9 vs. 14). Competition studies revealed that complex C2 represents specific binding because the addition of 100-fold molar excess cold wild-type rat pMCAT probe to binding reactions containing rat CS nuclear extract competed away only complex C2 (Fig. 5B, lane 9 vs. 10), whereas 100-fold molar excess mutant rat pMCAT (pMCATm) element did not compete away complex C2 (Fig. 5B, lane 9 vs. 11). The preincubation of binding reactions containing rat CS or NWB-S nuclear extract with PI did not interfere with complex formation at the rat pMCAT element (Fig. 5B, lanes 9 vs. 12 and 14 vs. 15), whereas addition of polyclonal NTEF-1 antibody supershifted complex C2 (Fig. 5B, lanes 9 vs. 13 and 14 vs. 16). In contrast to our findings of weak binding at the human pMCAT element, these data clearly demonstrate that a nuclear protein antigenically related to NTEF-1 interacts more avidly with the rat pMCAT element under basal and NWB conditions. Moreover, our EMSA findings of a decrease in the intensity of specific complex C2 when using NWB-S nuclear extracts suggest that the rat pMCAT element likely plays a regulatory role in directing decreased rat \(\beta\)MyHC expression under NWB conditions.

Use of mouse nuclear extracts or in vitro-synthesized human TEF-1 proteins confirms a species difference in binding properties of TEF-1 at the human vs. rat \(\beta\)MyHC pMCAT elements. To clarify whether the dissimilar binding of nuclear TEF-1 at the human vs. rat pMCAT element was due to differences in nucleotide composition and not species difference in nuclear TEF-1 protein, we performed an EMSA analysis using either mouse nuclear extract or in vitro synthesized human TEF-1 protein. EMSA analysis of binding reactions containing mouse control gastrocnemius nuclear extract and a \(^{32}\)P-labeled human pMCAT element revealed three binding complexes (C1, C2, C3; Fig. 6) that were of very low intensity like those obtained when using rat nuclear extract. When 100-fold molar excess cold wild-type pMCAT element was added to the binding reaction, only complex C2 was abolished (Fig. 6, lanes 1 vs. 2). The addition of PI to the binding reaction did not alter the complex formation (Fig. 6, lane 3), whereas addition of polyclonal NTEF-1 antibody resulted in a SS of complex C2 only (Fig. 6, lane 4). Similarly, three binding complexes formed at the \(^{32}\)P-labeled rat pMCAT element when mouse control gastrocnemius nuclear extract was used, and only complex C2 was competed away with the addition of 100-fold molar excess unlabeled wild-type pMCAT probe (Fig. 6, lane 5 vs. 6). In contrast, formation of complex C2 at the rat pMCAT element was highly enriched compared with the formation of complex C2 when using the human pMCAT element (Fig. 6, lane 1 vs. 5). Preincubation of binding reactions with PI did not alter complex formation at the rat pMCAT element (Fig. 6, lane 7), whereas addition of polyclonal NTEF-1 antibody resulted in a SS of complex C2 (Fig. 6, lane 8).

We next performed an EMSA analysis using in vitro-synthesized human NTEF-1 protein. EMSA analysis of binding reactions containing in vitro-translated human NTEF-1 (Fig. 7, inset, \[^{35}\]S)methionine-labeled NTEF-1) and the \(^{32}\)P-labeled human pMCAT element revealed the formation of a binding complex whose intensity was barely detectable and distinct from the complex observed when UL was used (Fig. 7, lane 1 vs. 2). The addition of 100-fold molar excess unlabeled wild-type human pMCAT probe to binding reactions inhibited complex formation (Fig. 7, lane 3). Preincubation of binding reactions with PI did not interfere with complex formation at the human pMCAT element (Fig. 7, lane 4), whereas addition of polyclonal NTEF-1

![Fig. 6. Analyses of mouse nuclear extract binding at the human and rat pMCAT elements. All binding reactions contained 5.5 \(\mu\)g of control mouse gastrocnemius (CG) nuclear extract and either \(^{32}\)P-labeled human pMCAT (lanes 1–4) or rat pMCAT (lanes 4–8) element. Specific binding was determined by the addition of 100-fold molar excess cold wild-type human (lane 2) or rat (lane 6) pMCAT before the addition of the \(^{32}\)P-labeled human or rat pMCAT probe.](http://ajpcell.physiology.org/)
antibody resulted in a supershifted complex (Fig. 7, lane 5). In contrast, EMSA analysis using the $^{32}$P-labeled rat pMCAT element in binding reactions containing in vitro-translated human NTEF-1 showed the formation of a very robust binding complex distinct from the complex observed when UL was used (Fig. 7, lane 6 vs. 7). When 100-fold molar excess cold rat pMCAT probe was added to binding reactions, complex formation was abolished (Fig. 7, lane 8). The preincubation of binding reactions with PI did not alter complex formation (Fig. 7, lane 9), whereas addition of polyclonal NTEF-1 antibody led to a supershifted complex (Fig. 7, lane 10). When NTEF-1 antibody was reacted with either the human or rat pMCAT element (Fig. 7, lanes 12 and 13), complex formation was not observed, revealing that the supershifted bands shown in Fig. 7, lanes 5 and 10, are not due to antibody-DNA complex formation.

Collectively, our EMSA results conclusively show that when using rat or mouse nuclear extract or in vitro-synthesized human NTEF-1 protein, the rat pMCAT element binds more avidly than does the human pMCAT element, supporting the notion that the difference in nucleotide composition between these elements underlies this dissimilar binding.

**DISCUSSION**

The genetic regulatory programs that control the adaptation of adult-stage skeletal muscle phenotype in

![Fig. 7. EMSA analyses of human and rat pMCAT element binding of recombinant NTEF-1. Inset reveals the correct size of $[^{35}$S]methionine-labeled human NTEF-1. Rabbit reticulocyte lysate system was programmed with 1 µg of circular NTEF-1 in the presence of $[^{35}$S]methionine. The transcription/translation product was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and exposed to film. Molecular weight markers (in kDa) are shown at left. The lane marked UL (unprogrammed lysate) represents parallel reaction not programmed with TEF-1 expression plasmid. EMSA of $^{32}$P-labeled human pMCAT and rat pMCAT element reacted with in vitro-translated human NTEF-1 TnT product. Binding assays contained 1 µl of UL (lanes 1 and 6) or 5 µl of NTEF-1 TnT protein for the human pMCAT probe (lanes 2–5) or 1 µl of NTEF-1 TnT protein for the rat pMCAT probe (lanes 7–10). Sequence specific binding was determined by the addition of 100-fold molar excess cold wild-type human (lane 3) or rat (lane 8) pMCAT before the addition of the $^{32}$P-labeled human or rat pMCAT probe. Note the enriched binding of NTEF-1 at the rat pMCAT element (lane 7) vs. the human pMCAT element (lane 2). Antibody supershift EMSA was performed by preincubation of in vitro synthesized human NTEF-1 antibody (lanes 5 and 10) for 30 min at room temperature before the addition of the probe. Lane 11 represents free labeled human pMCAT probe, whereas lanes 12 and 13 show that NTEF-1 antibody reacted with either the human or rat pMCAT element, respectively, does not form a complex.
response to decreased weight bearing are complex and remain incompletely understood. To gain insight into this process, our current study utilizes a transgenic mutagenesis and EMSA analysis to investigate whether two distinct βMyHC proximal promoter MCAT elements (dMCAT and pMCAT) serve regulatory roles during NWB. Our transgenic study provides the first in vivo evidence that: 1) the dMCAT element is responsible for the unexpected upregulation of human transgene β293wt in response to NWB, and 2) regulatory sequence(s) located downstream from the dMCAT element are sufficient to direct NWB responsiveness. Our EMSA experiments are the first to reveal the existence of a species (human vs. rat) difference in βMyHC pMCAT element binding of NTEF-1 under both control and NWB conditions, suggesting a possible species divergence in functional roles served by this element.

Transgenic mutagenesis analysis reveals that the dMCAT element is required to achieve high-level basal expression of transgene β293wt. MCAT regulatory elements are frequently found in the control region of numerous muscle genes and have been shown to mediate muscle-specific and inducible gene transcription. In our previous transgenic mutagenesis study, we found that mutation of the dMCAT element did not abolish induction of transgene β293Mm expression in the functionally overloaded adult plantaris muscle; however, basal expression levels of this transgene were significantly reduced (44). Likewise, our current transgenic analysis shows that mutation of the dMCAT element significantly decreases transgene β293Mm expression in the adult CS muscle, indicating that in the context of a 293-bp βMyHC promoter, this element acts as a strong positive cis-acting sequence (Table 2).

Transgenic mutagenesis analysis reveals that the dMCAT element is responsible for the increase in transgene β293wt expression after NWB. Our observation that the dMCAT element acts as a strong positive cis-acting sequence offers insight into how the dMCAT element mediates the uncharacteristic upregulation of transgene β293wt in response to NWB. In this regard, it is plausible that in the absence of the upstream NWB-responsive region (−600/−294; Fig. 1A), interactions required for NWB downregulation would be disrupted, thereby allowing the dMCAT element (−290/−284) to exert a dominant positive effect on transgene β293wt expression in response to NWB. The transgenic results presented here support this notion by showing NWB decreased expression of a transgene containing a mutated dMCAT element (β293Mm). In fact, decreases in transgene β293Mm expression were similar to those previously reported by us for larger (5,600 or 600 bp) βMyHC promoter/transgenes (Table 2; Ref. 29), decisively demonstrating in vivo that the dMCAT element is responsible for the unexpected upregulation of wild-type transgene β293wt in response to NWB.

Of direct relevance to our current findings are the numerous transgenic studies showing that gene expression is specified by unique combinations of regulatory element(s) that, when disrupted by deletion or mutation, alter the level and pattern of gene expression (Ref. 13 and references within). Specifically, an uncharacteristic fiber type-specific expression pattern has recently been reported for several fast myosin light chain-1/3 and troponin I fast transgenes (9, 20, 25, 26, 32). Importantly, these findings were consistently documented by using multiple independent transgenic lines, thereby showing that integration site or rarely encountered transgene copy number effects were not responsible for these observations. Moreover, the requirement for individual cis-acting regulatory elements and/or enhancer (module) regions for directing correct patterns of gene expression have recently been demonstrated by using gene targeting. In these studies, the homozgyous targeted deletion/mutation of distinctly located enhancer regions associated with either the myosin light chain-1/3 or MyoD gene locus led to defective myosin light chain or MyoD expression patterns, respectively (8, 22).

EMSA analysis suggests a role for the dMCAT element during NWB regulation of the βMyHC gene. In this study, we have used EMSA analysis to investigate how a strong positive cis-acting regulatory element might function during a physiological process that normally results in negative regulation. One conceivable mechanism by which a strong positive cis-acting element can contribute to decreased expression levels of a given gene is by incurring changes in the type and/or amount of transcription factor binding. As concerns change in the type of transcription factor binding, our current antibody EMSA experiments show that the proteins interacting at the dMCAT element do not differ between control and NWB conditions. Specifically, antibody EMSA analysis determined that proteins antigenically related to PARP, NTEF-1, and Max comprised the LMC that formed at the βMyHC dMCAT element when using either control or NWB nuclear extract (Fig. 4). This analysis also showed that other factors, such as the E-box binding proteins MyoD, myogenin, E2A, HEB, and USF, were not components of the binding complexes formed at the dMCAT element (data not shown). Furthermore, we showed by EMSA scanning mutagenesis that no difference existed in protein-DNA interactions at the dMCAT element because the same nucleotides comprising only the core MCAT element were involved in both control and NWB nuclear protein binding (Fig. 3).

The one notable difference observed was the decrease in LMC formation when using NWB-S nuclear extracts compared with CS nuclear extracts. Although minor differences in complex formation were observed for the IMC and HMC, the decrease observed for the LMC was the most striking. This finding extends our previous observation showing that the formation of a LMC at the βMyHC dMCAT element varied in accordance with the proportion of slow type I fibers populating the muscle from which the nuclear extract was isolated (44). Our EMSA analysis supports the idea that the dMCAT element contributes, at least in part, to NWB decreases in βMyHC gene expression as a result of reduced binding of the positive-acting tran-
scription factors NTEF-1, PARP, and Max. When considering this potential NWB mechanism in the context of the endogenous βMyHC gene, it is logical to speculate that under NWB conditions a decrease in occupancy of transcriptional activator proteins at a positive cis-acting element would minimize transcriptional activation sufficiently to allow negative regulation to proceed in the presence of other NWB element(s). In this manner, global changes in the transcription of genes representative of the slow type I phenotype could be coordinately downregulated, thereby contributing to the slow-to-fast fiber type transition induced by NWB. Whether the decrease of positive cis-acting element occupancy under NWB conditions occurs by regulation of transcription factor(s) at the level of transcription and/or posttranscriptional modification remains to be determined.

Previously unidentified NWB element(s) reside downstream of the dMCAT element. In addition to determining that the dMCAT element directs the uncharacteristic upregulation of transgene β293wt, our transgenic analysis showing downregulation of transgene β293Mm expression after NWB provides the first in vivo evidence that previously unidentified NWB responsive element(s) reside downstream from the dMCAT element (Fig. 1A). Candidate elements that may fulfill this role that are located downstream from the dMCAT element (−290/−284) are the A/T-rich (−269/−258), C-rich (−242/−231), pMCAT (−210/−203, examined herein), and E-box/NFAT (−179/−171) elements. Our previous transgenic analysis revealed that the independent mutation of the A/T-rich element in the context of a 293-bp βMyHC promoter/transgene resulted in the complete loss of expression in all 21 independent lines examined (CAT = 15 lines, luciferase = 6 lines) (44, 46). Similarly, E-box/NFAT mutation in 11 independent lines resulted in 4 lines that did not express and 7 lines whose basal expression levels were very low-rendering results, with NWB being unreliable (unpublished observation). Interestingly, a molecular model that accounts for slow fiber-specific gene expression and involves both A/T-rich and NFAT elements has been recently proposed. This model suggests that sustained elevations of intracellular calcium coactivate the calcineurin and calmodulin-dependent protein kinase signaling pathways and the subsequent transcriptional activation of slow fiber genes by various members of the myocyte enhancer factor-2 (MEF-2) and/or nuclear factor of activated T-cell (NFAT) transcription factor families (31, 33, 49, 50). Because NWB has been shown to result in a decrease in total muscle electrical activity and a slow-to-fast fiber-type transition (3, 34), it is conceivable that a decrease in nuclear protein binding at the A/T-rich and/or NFAT elements may contribute to reduced βMyHC expression under NWB conditions. In fact, we have previously reported reduced levels of NFAT protein binding at the βMyHC NFAT element (46). Although not all agree with the proposed model of slow fiber-specific expression (7), it will be instructive to reevaluate the involvement of the A/T-rich and E-box/NFAT elements in regulating βMyHC expression during the NWB induced slow-to-fast fiber-type by using larger βMyHC promoter/transgenes.

Divergence in pMCAT element sequences underlies species difference in NTEF-1 binding. The pMCAT element (−210/−203) is located downstream from the dMCAT element and represents a viable element that could confer NWB regulation. Previous EMSA analysis has revealed robust binding at this element when reacted with nuclear extracts isolated from a variety of differentiated myogenic cell lines (37, 38). In addition, 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 when assayed in the in vitro context (37, 38). The pMCAT element has also been shown to confer inducible expression of the rat βMyHC gene in response to α1-adrenergic treatment of primary rat neonatal cardiomyocytes (23, 24). Mechanistically, the latter was shown by immunological techniques to involve the formation of a single binding complex (termed C2) that was comprised of TEF-1 protein. More recently, it was reported that the pMCAT element plays a role in the downregulation of rat βMyHC plasmids injected into the soleus muscle of spinal cord isolated rats (19).

Although the βMyHC pMCAT element has been shown to contribute to both basal and inducible expression, a role for this element under NWB conditions has not been investigated. Our results indicate that the human βMyHC pMCAT element is unlikely to play a role in NWB regulation because EMSA analysis detected weak binding of NTEF-1 that did not differ between rat CS or NWB-S nuclear extract (Fig. 5A). The weak binding of rat NTEF-1 at the human pMCAT element cannot be attributed to species differences in transcription factors because we also observed weak binding when using either in vitro synthesized human TEF-1 protein or mouse control gastrocnemius nuclear extract (Figs. 6 and 7). Furthermore, all members of the TEF-1 family are well conserved at the amino acid level and, in particular, throughout the DNA binding domain (Ref. 6 and references within). Although our EMSA results suggest that the human pMCAT element does not serve a regulatory role during NWB, at present we cannot eliminate the possibility that post-transcriptional modifications of NTEF-1 could allow NWB regulation to occur through this element without affecting the degree of NTEF-1 binding. In this regard, various TEF-1 isoforms have been shown to modulate gene transcription as a result of posttranscriptional modification (18, 42). Also of significance, we provide evidence that the two distinct human βMyHC proximal promoter MCAT elements are not equivalent in that the dMCAT element binds TEF-1 proteins more avidly than the pMCAT element, suggesting that the dMCAT element is a stronger activator of muscle gene transcription.

In striking contrast to our findings with the human pMCAT element, our EMSA experiments provide evidence in support of a NWB regulatory role for the rat pMCAT element. In competition and antibody EMSAs,
we detected robust binding of CS nuclear NTEF-1 at the rat pMCAT element that decreased when using NWB-S nuclear extract (Fig. 5B). We also found robust binding at this element when using either nuclear extract isolated from the mouse gastrocnemius muscle or in vitro-synthesized human NTEF-1 protein (Figs. 6 and 7). Therefore, it is likely that the difference in NTEF-1 binding at the human vs. rat pMCAT elements can be attributed to a divergence in nucleotides comprising the core and flanking regions of these two elements (Fig. 5C). Previous work has illustrated the importance of flanking sequences in transcription factor binding at regulatory elements (6, 17, 28). In fact, the region located between nucleotides –215 and –203 of the rat proximal promoter contains two overlapping consensus MCAT elements (Fig. 5C), a configuration that may contribute to enhanced NTEF-1 binding and thus regulated expression. Although speculative, this example of a species difference in cis-acting regulatory element binding, and presumably function, may partially underlie the difference in βMyHC expression in the human (predominately αMyHC) vs. small rodent heart (predominately αMyHC), as well as responses to pathophysiological and physiological signals.

In summary, our transgenic analysis of the βMyHC dMCAT element demonstrates that this element function in a context-dependent manner because, in the absence of the upstream NWB-responsive region, it confers a dominant positive effect on transgene β293wt transcription, resulting in upregulation in response to NWB. Importantly, this analysis also revealed that previously unidentified NWB responsive element(s) resides further downstream within the βMyHC proximal promoter. Our EMSA analysis suggests that the dMCAT element contributes, at least in part, to the NWB response by incurring decreased binding occupancy of NTEF-1, PARP, and Max, thereby allowing negative regulation to proceed in the presence of other intact NWB responsive elements. In addition, we provide evidence for a species difference in βMyHC pMCAT element function during NWB.

The current challenge will be to decipher crucial regulators (cis-acting elements, transacting factors, and signaling pathways) that underlie NWB induced slow-to-fast phenotypic transitions. It is clear that tight control of βMyHC gene transcription involves multiple DNA regulatory elements that integrate information from numerous signaling pathways into fiber type- and perturbation-specific expression patterns. On the basis of our findings herein, it will be important to determine whether one or more of the βMyHC proximal promoter elements downstream from the dMCAT element (A/T-rich, NFAT, C-rich) participates in NWB regulation or whether a previously unrecognized element serves this role. Furthermore, once the downstream NWB responsive element(s) are identified, it will be important to examine possible interaction between these elements and the upstream NWB responsive region with an emphasis on the dNRE-S element (Fig. 1A).

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