Nuclear Protein Binding at the β-Myosin Heavy Chain A/T-rich Element Is Enriched following Increased Skeletal Muscle Activity*

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Dharmesh R. Vyasa, John J. McCarthyb, and Richard W. Tsikac,b

From the aDepartment of Biomedical Sciences, School of Veterinary Medicine, the bDepartment of Biochemistry, School of Medicine, and the cDalton Cardiovascular Research Center, University of Missouri-Columbia, Columbia, Missouri 65211

In adult mouse skeletal muscle, β-myosin heavy chain (βMyHC) gene expression is primarily restricted to slow-type I fibers but can be induced in fast-type II fibers by mechanical overload (MOV). Our previous transgenic analyses have delimited an 89-base pair (bp) MOV-responsive region (−293 to −205), and shown that mutation of the MCAT and C-rich elements within this region did not abolish βMyHC transgene induction by MOV. In this study we describe an A/T-rich element (βA/T-rich; −269 to −258) located within this 89-bp region that, only under MOV conditions, revealed enriched binding as characterized by electrophoretic mobility shift assays and dimethyl sulfoxide and diethyl pyrocarbonate interference footprinting. Direct, competition, and supershift electrophoretic mobility shift assays revealed highly enriched specific binding activity at the βA/T-rich element that was antigenically distinct from GATA-4, MEF2A–D, SRF, and Oct-1, nuclear proteins that were previously shown to bind A/T-rich elements. In vitro translated GATA-4, MEF2C, SRF, and Oct-1 bound to consensus GATA, MEF2, SRE, and Oct-1 elements, respectively, but not to the βA/T-rich element. Two-dimensional UV cross-linking of the bromodeoxyuridine-substituted βA/T-rich element with mechanically overloaded plantaris (MOV-P) nuclear extract detected two proteins (44 and 48 kDa). Our results indicate that the βA/T-rich element may function in vivo as a βMyHC MOV-inducible element during hypertrophy of adult skeletal muscle by binding two distinct proteins identified only in MOV-P nuclear extract.

Myosin is an abundantly expressed contractile protein comprised of two heavy chain subunits and two pairs of dissimilar light chains. The myosin heavy chain (MyHC) subunit is encoded by a multigene family comprised of eight members that are regulated in a tissue-specific manner throughout development and in response to various physiological stimuli (1, 2). The heterogeneous spectrum of vertebrate sarcomeric MyHC isoforms and their differential expression pattern underlies the broad classification scheme that histochemically (myofibrillar ATPase) distinguishes four primary adult-stage skeletal muscle fiber-types. Because each MyHC isoform is thought to serve a specific physiological role, variation in the proportion and spatial arrangement of each fiber-type underlies the biochemical and functional specialization of each muscle. This notion is underscored by the classic finding that actin-activated myosin ATPase activity and unloaded shortening velocity (V0) are highly correlated to the amount and type of isomyosin or MyHC comprising a given muscle or muscle fiber (3–5). More recently, insight into the function of individual MyHC isoforms was gained from studies employing the genetic strategy of homologous recombination to target the inactivation of either the fast type IIb or IId/x MyHC genes. Functional analyses of muscle from either the type IIb or IId/x MyHC knock-out mice revealed altered contractile properties that were unique to each null mutation despite the compensatory activation of the endogenous fast type IId/x and IIa genes, respectively (6, 7). In contrast to our current knowledge concerning the diversity of adult-stage MyHC isoforms and their distinct functional properties, there exists a paucity of information regarding the mechanisms that govern MyHC fiber-type-specific gene expression and their differential regulation in response to various modes of neuromuscular activity.

It has been well documented that the phenotype of adult-stage skeletal muscle can be profoundly altered in response to specific mechanical perturbations, such as mechanical overload (MOV) or non-weight bearing (NWB), which presumably reflect altered neuromuscular activity. To better understand this phenotypic plasticity in molecular terms, we have used the βMyHC gene as a model system since βMyHC expression is primarily restricted to slow-type I fibers in the adult mouse but can be induced in fast-type II fibers following MOV (8). In addition, βMyHC expression is decreased in slow-type I fibers in response to NWB activity (9, 10). Our investigation into the regulatory mechanism(s) underlying the antibiotic expression pattern of the βMyHC gene in response to these two diverse stimuli have established that NWB- and MOV-responsive element(s) are distinct and segregated within the proximal promoter of the βMyHC gene (see Fig. 1, Refs. 8–10). More specifically, our transgenic studies have delimited a 156-bp βMyHC NWB-responsive promoter region (nucleotides −450 to −294), and within this region we have identified a negative regulatory element (dJNRE-S: −332 to −311) that binds two distinct proteins found only in NWB soleus nuclear extract (10). As concerns MOV, we have identified an 89-bp βMyHC MOV-responsive promoter region (−293 to −205), and shown

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† To whom correspondence should be addressed: University of Missouri-Columbia, Dept. of Veterinary Biomedical Sciences and Department of Biochemistry, 1600 E. Rollins Ave., W112 Vet Medicine Bldg., Columbia, MO 65211. Tel.: 573-884-4547; Fax: 573-884-6890; E-mail: tsikar@missouri.edu.

‡ The abbreviations used are: MyHC, myosin heavy chain; MOV, mechanical overload; bp, base pair; NWB, non-weight bearing; CAT, chloramphenicol acetyltransferase; MCAT, muscle-CAT; MEF2, myocyte enhancer factor 2; CP, control plantaris; DMS, dimethyl sulfate; DEPC, diethyl pyrocarbonate; MCAT, mechanically overloaded plantaris; PAGE, polyacrylamide gel electrophoresis; cTnC, cardiac troponin C; BNP, B-type natriuretic peptide; UL, unprogrammed lysate; MCK, muscle creatine kinase; SRF, serum response factor; SRE, serum response element.

2 J. J. McCarthy, and R. W. Tsika, unpublished observation.
that mutation of the muscle-CAT (MCAT) and C-rich elements within this region did not abolish transgene induction, suggesting that an MOV element(s) resides within this region (see Fig. 1, Refs. 8 and 10).

Examination of the nucleotide sequence comprising this 89-bp βMyHC MOV-responsive region revealed that, in addition to containing MCAT and C-rich elements, there is an A/T-rich motif (−269 5′GGAGATATTTTG−258) that is highly conserved in nucleotide sequence and location across species (Fig. 2, Refs. 11, 12, 14, and 16) and has a high degree of homology to both the consensus myocyte enhancer factor 2 (MEF2) element [CTA(A/T)4TAG/A] and the consensus GATA element [(A/T)GATA(A/G)]. In support of the hypothesis that a MOV element may reside within the βMyHC 89-bp MOV-responsive region, Hasegawa et al. (17) have reported that this A/T-rich element (referred to as a GATA element by them) within the proximal promoter region of rat βMyHC reporter genes acts as an inducible element following direct injection into pressure-overloaded adult rat hearts. This response was presumably conferred by GATA-4 binding at this element. In light of this finding, and because the A/T-rich element (referred to as βA/T-rich hereafter) contains a GATA/MEF2-like homology, it is noteworthy that McGrew et al. (18) have recently reported the detection of GATA-2 and -3 transcripts in skeletal muscle since GATA factor expression is thought to be absent in this tissue. Furthermore, these investigators also demonstrated that an intact GATA motif is required for full transcription in response to mechanical perturbations of skeletal muscle since GATA factor expression is thought to be absent in this tissue. Furthermore, these investigators also demonstrated that an intact GATA motif is required for full transcriptional activation of the fast alcali myosin light chain-3 (FMLC3) promoter in transient expression assays using primary cultures of neonatal skeletal muscle cells. In contrast to GATA, the involvement of MEF2 proteins in activating muscle gene transcription in response to mechanical perturbations of adult skeletal muscle has not been reported as yet.

In addition to GATA and MEF2 proteins, A/T-rich elements have been reported to interact with a diverse group of transcription factors including the ubiquitously expressed POU-domain octamer-binding factor; Oct-1, the homeodomain protein; MHOx, the MADS-box (MCM, Agamous, Deficiens, Serum response factor) factor; serum response factor (SRF), and the high-mobility group I and II (HMG-I, HMG-II) architectural response factor factor; serum response factor (SRF), and the homeodomain protein; MHOx, the MADS-box (MCM, Agamous, Deficiens, Serum response factor) factor; serum response factor (SRF), and the high-mobility group I and II (HMG-I, HMG-II) architectural proteins were present only in MOV-P nuclear extract, bind the βA/T-rich element under MOV conditions. The latter result implicates these proteins in the MOV-induction of βMyHC transgene expression in fast type II skeletal muscle fibers that normally do not express the βMyHC to any significant degree.

**EXPERIMENTAL PROCEDURES**

**Preparation of Nuclear Protein Extract from Adult Skeletal Muscle—**

Nuclear extract was isolated from adult rat control plantaris (CP) and MOV-P muscle as described previously (10). All procedures were carried out on ice. All buffers contained 2 μg/ml each of aprotinin and leupeptin, 0.5 μM phenylmethylsulfonyl fluoride, and 100 μM sodium orthovanadate. Ten grams of either CP or MOV-P were harvested from adult female Harlan Sprague-Dawley 200-gram rats and minced in phosphate-buffered saline. Minced muscle tissue was incubated in relaxation buffer I, 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 × g for 15 min at 4 °C. The pelletted nuclear layer was consolidated and lysed by the addition of 3 M NH₄SO₄ (pH 7.9) to a final concentration of 0.4 M. The lysate was ultracentrifuged at 126,000 × g for 1 h (4 °C) to pellet nuclear membrane debris. Solid NH₄SO₄ (0.3 g/ml) was added slowly to the supernatant, and the precipitated nuclear protein more were centrifuged by ultra-centrifugation at 126,000 × g for 30 min.

**Electrophoretic Mobility Shift Assay—**

All oligonucleotide probes used in this study are listed in Table I (17, 26–31). Electrophoretic mobility shift assays (EMSA) were performed as described previously (10). The double-stranded A/T-rich oligonucleotide probe (nucleotides −275 to −252) was labeled by fill-in reaction using the Klenow fragment of *Escherichia coli* DNA polymerase I (Stratagene, La Jolla, CA) and [α-32P]dCTP (3000 Ci/mmol). All other oligonucleotide probes were end-labeled by T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and [γ-32P]ATP (6000 Ci/mmol). Probes were purified by polyacrylamide gel electrophoresis prior to use in EMSA. Binding reactions were performed with 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, 1 μl of *in vitro* translated GATA-4, 2 μl of MEF2C, 1 μl of SRF, or 0.5 μl of Oct-1 protein was used in place of MOV-P nuclear extract. The binding reactions were resolved on a 5% non-denaturing polyacrylamide gel at 220 volts for 2.5 h at 4 °C. Supernatants as were performed with 5 μg of either CP or MOV-P nuclear extract and 20,000 cpm of probe for 20 min at room temperature prior to the addition of the 32P-labeled DNA probe. 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 expression plasmid in the T7 TnT wheat germ (GATA-4, MEF2C) and rabbit reticulocyte TnT (Oct-1, GATA-4) lysate systems according to the instructions of the manufacturer (Promega). Prior to use in the wheat germ TnT reaction, the expression plasmids pCDNA GATA-4 (1.7-kb insert of mouse GATA-4 cDNA, Ref. 32), pCDNA MEF2C (1.4-kb insert of mouse MEF2C cDNA, Ref. 29), and pT7SRFΔATG (1.6-kb insert of human SRF cDNA, Ref. 34) were linearized with *XhoI*, *XbaI*, and EcoRI, respectively. Circular p6HisOct-1 expression plasmid (33) containing the full-length human Oct-1 cDNA was used in the rabbit reticulocyte kit. Parallel TnT reactions were performed in the presence of [35S]methionine (NEN Life Science Products). 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-PAGE).

**Antibodies—**

The antibodies used in this study are as follows: GATA-4, affinity-purified goat polyclonal antibody raised against mouse GATA-4 carboxyl terminus amino acids 420–439 (Santa Cruz Biotechnology, Inc.); MEF2A, rabbit polyclonal antibody raised against fusion protein GST-MEF2A (human, amino acids 129–253) (35); MEF2C, rabbit polyclonal antibody raised against GST-MEF2C (human, amino acids 88–206) (36); MEF2A, affinity-purified rabbit polyclonal antibody raised against human MEF2C carboxyl terminus amino acids 487–507 (Santa Cruz Biotechnology, Inc.); SRF, affinity-purified rabbit polyclonal antibody raised against a peptide within the carboxyl terminus of human SRF (Santa Cruz Biotechnology, Inc.); and Oct-1 rabbit polyclonal raised against human Oct-1.

**Two-dimensional UV Cross-Linking Analysis—**

*P. K. Umeda, personal communication.*

*J. G. Edwards, D. F. Bonilla, and E. Morkin, unpublished data.*
cross-linking was performed essentially as described previously by us (10). The first dimension of this assay involved EMSA using the bromodeoxyuridine-substituted A/T-rich probe and MOV-P nuclear extract since only this reaction revealed the formation of a highly enriched DNA-protein complex. EMSA was performed as described above, except that the reaction mixture was scaled up 5-fold. Immediately following electrophoresis, the gel was exposed to UV irradiation (312 nm) for 30 min at 4 °C. The specific band corresponding to the cross-linked DNA-protein complex was excised, transferred to a sample well of a SDS-12% polyacrylamide gel, and electrophoresed at 150 V for 75 min (second dimension PAGE). Following electrophoresis, the gel was placed on Whatman filter paper and dried, and the DNA-protein complexes were visualized by autoradiography.

**RESULTS**

**The Identification of Enriched MOV-P Nuclear Protein Binding Activity That Interacts at the Human βA/T-rich Element—**
Our previous work has identified an 89-bp MyHC MOV-responsive region (2293 to 205) which contains a highly conserved A/T-rich motif that harbors an overlapping GATA/MEF2-like homology (Figs. 1 and 2). Since the βA/T-rich element appears to function, in part, as an inducible element of injected rat βMyHC reporter constructs in pressure overloaded adult rat hearts, it was important that we determine whether the human βA/T-rich element functions in vivo as an MOV-inducible element (MOV-E) in adult skeletal muscle. We initiated this investigation by first examining the binding properties of the βA/T-rich element by performing gel EMSAs using CP and MOV-P nuclear extract. The incubation of the double stranded 24-bp 32P-labeled βA/T-rich probe was modified by either 0.7% DMS or 2% DEPC for 15 min at 25 °C and 37 °C, respectively. The probe was used for preparative EMSA as described above, except that the reaction mixture was 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 1 M piperidine incubation for 30 min at 90 °C which was followed by repeated rounds of lyophilization to remove the piperidine. Equivalent amounts (1000 cpm/lanes) of free and bound cleaved probe were resolved on a 20% polyacrylamide, 8 M urea denaturing sequencing gel. Gels were autoradiographed for 24 h.

**Transgene** | **Expression level** |
--- | --- |
CS | CP | MOV-P |

β205 | – | – | – |
β293 | + | – | – |
β350 | – | – | – |
β450 | + | – | – |
β600 | + | – | – |
β600 mut3 | + | – | – |

**Endogenous βMyHC gene** | + | – | ↑

**FIG. 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 (β205), 293 (β293), 350 (β350), or 450 bp (β450) of human βMyHC 5' promoter sequence and 120 bp of 5' untranslated region fused to the bacterial CAT reporter gene. Transgene β600 contains 600 bp of the mouse βMyHC 5' promoter sequence and the entire 1600 bp of the 5' untranslated region linked to the CAT reporter gene. Transgene β600 mut3 is structurally identical to β600 except it harbors 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'-untranslated region represent untranslated exons.
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### Table I

| Name          | Sequence (sense strand 5’→3’) | Position | Reference |
|---------------|-------------------------------|----------|-----------|
| βMyHC A/T-rich| GCCCTGGGAGATAATTTTCTGCTGA    | Human, −275→252 | This study |
| βMyHC A/T-rich mut| GCCCTGGGAGagTCTTTTGTGCTGA | Human, −190→252 | This study |
| βMyHC A/T-rich BrdU| GCCCTGGGAGaUUUUUUGCTGA | Human, −275→252 | This study |
| βMyHC GATA    | AATGTGAGGATATTITGCTCATTGTGA  | Rat, −280→252  | 17        |
| cTnC GATA     | GGGCCCGGGCTGAGATCTGGCTGCTGTA | Rat, −193→255 | 15        |
| cTnC GATA mut | CACCGCTGGAGGATATTITGCTCATTGTGA | Mouse, −120→100 | 27        |
| BNP GATA      | TGTGTGGCTGAAATCAAGAATGATTTACA | Rat, −100→97 | 28        |
| MCK MEF2      | GTTCCTGCTCAGAAAATGATCATTGCTGCTG | Mouse, −100→1058 | 29        |
| Desmin MEF2   | GCTTCCTGCTAATATAAATGATCATTGCTG | Mouse, −848→848 | 30        |
| c-fos SRE     | GGAATGCGGAAATTACATCCTATT       | Mouse, −193→255 | 31        |
| Consensus Oct-1| TGTGGATGCGGAAATTACATCCTATT | Santa Cruz Biotechnology, Inc. |         |

mut) probe (Fig. 4, lanes 3 versus 9) did not. The nucleotides mutated within the βA/T-mut probe corresponded to DNA-protein contact points elucidated in our DMS and DEPC footprinting experiments (Fig. 3, B and C). These data establish that highly enriched binding activity exists in MOV-P nuclear extract as compared with CP nuclear extract and support the notion that the βA/T-rich element may function as an MOV element that confers induction to βMyHC transgenes in adult skeletal muscle.

To identify the exact nucleotides involved in the highly enriched DNA-protein binding complex formed at the βA/T-rich element, we performed DMS and DEPC interference footprinting analyses. DMS footprinting delimited a binding site on the sense strand of the 24-bp βA/T-rich probe that encompassed nucleotides −269 to −266 wherein methylation of guanine residues either partially (positions 7 and 8) or strongly (position 10) interfered with nuclear protein binding (Fig. 3, B and C). In contrast, DMS modification of the antisense strand did not distinguish a DNA-protein interaction. The modification of adenine residues by DEPC treatment resolved a protein binding site demarcated by strong interference at positions 11 and 13 on the sense strand, and 12, 14, 15, and 16 on the antisense strand (Fig. 3, B and C). Overall, our DMS and DEPC interference footprinting experiments revealed that MOV-P nuclear protein(s) interact at a site spanning nucleotides −269 to −266 wherein methylation of guanine residues may be important determinants specifying binding affinity and classification of transcription factor(s) binding at this site (Fig. 3C).

EMSA Analyses Indicate That GATA-4 Does Not Bind to the Human βA/T-Rich Element during MOV Induction of the βMyHC Gene—The βA/T-rich element contains a GATA-like consensus element (5/6 nucleotides match, 83%) that has recently been reported to mediate pressure overload induction of injected rat βMyHC reporter genes and to bind GATA-4 protein (17). In contrast to GATA-2 and -3 mRNAs, the detection of GATA protein in skeletal muscle has not been reported as yet; however, it is possible that intracellular signals generated by MOV may activate the transcription of GATA isoforms during the hypertrophic growth of adult skeletal muscle. To determine whether GATA protein(s) represent a component of the enriched binding activity identified in MOV-P nuclear extract, we performed competition EMSAs. As discussed previously, incubation of the 32P-labeled βA/T-rich probe with MOV-P nuclear extract formed a highly enriched specific binding complex in comparison to that formed when CP nuclear extract was used (Fig. 3A and Fig. 4, lanes 1–3 versus 7–9). Competition EMSA made use of probes harboring GATA elements previously shown to bind GATA-4. When using CP or MOV-P nuclear extract, complex formation was not inhibited by the addition of 100-fold molar excess of cold mouse cardiac troponin C GATA (cTnC GATA; Fig. 4, lanes 10), rat α-MyHC GATA (a-MyHC; Fig. 4, lanes 1 and 2), or rat GATA-4 (Fig. 5, lanes 1 and 2). A binding complex was not formed when either unprogrammed lysate (UL) or in vitro translated GATA-4 was re-

![Image](Image 541x656 to 62x667)

**Fig. 2.** Sequence comparison of the βMyHC proximal promoter region from multiple species. Nucleotide sequences of the human (Ref. 11, X52889), pig (Ref. 12, L10130), rabbit (Ref. 14, I21204), rat (X16291), and mouse (Ref. 16, L07306) were aligned using the CLUSTALW program provided in the Biology Workbench (available on the WWW). Gaps were inserted to optimize the alignment. Sequences are shown in gray. Conserved nucleotides of the βA/T-rich sequence are stippled and in boldface. The reference and accession numbers for each sequence are listed in parenthesis.

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binding reactions containing either the aMyHC, the cTnC, or the BNP GATA element (Fig. 3A, lanes 5, 7, and 9). However, the addition of in vitro translated GATA-4 to binding reactions containing either the aMyHC, the cTnC, or the BNP GATA probe resulted in the formation of binding complexes (Fig. 5A, lanes 6, 8, and 10). These results provide evidence that in vitro translated GATA-4 is capable of binding a genuine GATA site and support the conclusion that GATA-4 is not a component of the enriched MOV-P-binding activity.

The failure of in vitro translated GATA-4 to bind the human βA/T-rich element was not expected since GATA-4 has been reported to bind to the rat βMHC A/T-rich element (17). To investigate if this discrepancy was because of species-specific divergences in nucleotide sequences flanking the GATA site, we conducted binding experiments using a 31-bp 32P-labeled rat βA/T-rich probe and rabbit reticulocyte lysate-generated in vitro translated GATA-4 (Fig. 5B). A specific binding complex between in vitro translated GATA-4 and the rat βA/T-rich element was not formed, but rather, a series of identical nonspecific binding complexes formed when using either UL or programmed lysate (Fig. 5B, lanes 1–4). These nonspecific complexes were not competed for by addition of 100-fold molar excess of either the rat βA/T-rich element or the high affinity BNP GATA binding site, indicating that the endogenous binding activity in rabbit reticulocyte lysate was not GATA-4 (Fig. 5B, lanes 1–4). Similarly, multiple nonspecific binding activities have been observed by another investigator in studies examining the binding of rabbit reticulocyte lysate-produced GATA-4 protein to the cTnC GATA site (also referred to as CEF-1) (24). Interestingly, these nonspecific complexes were notably absent when using wheat germ lysate (Fig. 5, A and C versus B).

To complete our investigation into whether GATA-4 might be a component of the enriched MOV-P-βA/T-rich binding complex, we performed supershift EMSAs using a polyclonal antibody that specifically recognizes GATA-4. The enriched specific binding complex formed when MOV-P nuclear extract was reacted with the 32P-labeled human βA/T-rich element was clearly not altered by preincubation with either preimmune serum or polyclonal GATA-4 antibody (Fig. 5C, lane 1 versus lanes 2 and 3). The binding complex formed between either the aMyHC (lanes 4 versus 5), the cTnC (lane 8 versus 9), or the BNP (lane 12 versus 13) GATA elements and in vitro translated GATA-4 was self-competed away by addition of 100-fold molar excess of each respective cold probe to the binding reaction thereby revealing specific binding (Fig. 5C).

Preincubation of preimmune serum in binding reactions containing in vitro translated GATA-4 and either the aMyHC (lanes 6 versus 7), the cTnC (lanes 10 versus 11) or the BNP (lanes 14 versus 15) GATA probes did not alter complex formation, whereas the addition of polyclonal GATA-4 antibody either depleted or supershifted these binding complexes (Fig. 5C). When taken together, the results gathered from our direct, competition, and supershift EMSA experiments support the notion that GATA proteins, in particular GATA-4, are not a component of the enriched MOV-P-βA/T-rich binding complex. In addition, our EMSA results indirectly indicate that GATA-5, GATA-6, and HMG-II are not components of the MOV-P-βA/T-rich binding complex since the cTnC GATA element, previously shown to bind these factors, did not compete for complex formation (Fig. 4, lanes 4 and 10) (24, 38, 39). However, these experiments do not eliminate the possibility that unidentified GATA-related proteins exist.
Fig. 5. A, EMSA analysis of in vitro transcribed-translated GATA-4 protein. Inset, [35S]methionine-labeled GATA-4. The wheat germ lysate system was programmed with 1 μg of linearized mouse GATA-4 expression plasmid in the presence of [35S]methionine. The transcribed-translated (TnT) product was resolved on 12% SDS-PAGE and exposed to film. Molecular mass markers (in kilodaltons) are shown on the right. , represents parallel reaction not programmed with GATA-4 expression plasmid. Right panel, EMSA of [32P]-labeled βAT-rich, α-MyHC GATA, cTnC GATA, and BNP GATA oligonucleotides with in vitro TNT GATA-4. Binding assays contained 1 μl of either unprogrammed (lanes 3, 5, 7, and 9) or GATA-4 cDNA programmed TNT product (lanes 2, 6, 10, and 14). Note GATA-4 interacts with the α-MyHC, cTnC, and BNP GATA elements but not with the βAT-rich sequence. CP and MOV-P binding to the βAT-rich probe are provided for reference (SC, lanes 1 and 2). B, EMSA analysis of GATA-4 TNT protein binding at the rat βAT-rich element. EMSA of [32P]-labeled rat βMyHC GATA oligonucleotide with in vitro TNT rabbit reticulocyte GATA-4 product. The binding assays contained 1 μl of either unprogrammed (lane 1) or GATA-4 cDNA programmed (lanes 2–4) TNT protein. Competition reactions were performed with 100-fold molar excess of either self (lane 3) or BNP GATA (lane 4) oligonucleotides. No difference was observed in sequence-specific DNA-protein binding between the unprogrammed and programmed reactions. C, antibody supershift EMSA analysis of MOV-P and GATA-4 TNT binding complexes. Supershift EMSAs were performed by preincubation of MOV-P nuclear extract and GATA-4 TNT product with 2 μl of polyclonal anti-GATA-4 antibody for 30 min at room temperature prior to the addition of the labeled probe. Either immunodepletion or supershift (SS) of the DNA-protein complex was observed with the α-MyHC, cTnC, and BNP GATA probes (lanes 7, 11, and 15). In contrast, the GATA-4 antibody did not disrupt the βAT-rich MOV-P complex (lane 3). Control reactions were performed with preimmune serum (PI, lanes 2, 6, 10, and 14). Sequence-specific binding of the GATA-4 TNT to the α-MyHC, cTnC, and BNP GATA elements is demonstrated by the eradication of binding complex formation upon addition of 100-fold molar excess of the corresponding unlabeled oligonucleotide (lanes 5, 9, and 13).

MEF2 Proteins Are Not a Component of the Enriched MOV-P Binding Activity—Members of the MEF2 family play a pivotal role during mouse embryogenesis by collaboratively regulating the expression of muscle genes that are critical for striated muscle differentiation (40). However, it is not known what role, if any, MEF2 proteins serve in regulating gene expression during adult skeletal muscle hypertrophy. Since the βAT-rich element contains an MEF2-like homology, it was important to determine whether MEF2 proteins bind the βAT-rich element in response to skeletal muscle MOV. To address this possibility, we performed competition and supershift EMSAs, as well as binding reactions using in vitro translated MEF2C (Fig. 6, A and B). The addition of 100-fold molar excess cold muscle creatine kinase MEF2 (MCK MEF2) or desmin MEF2 probe to the binding reaction as a competitor resulted in the partial inhibition of [32P]-labeled βAT-rich MOV-P complex formation (Fig. 6A, lane 1 versus lanes 2 and 3). This result was not surprising given the high degree of sequence homology between these elements (Table I). Although binding complexes formed when in vitro translated MEF2C (Fig. 6A, inset, [35S]methionine-labeled MEF2C) was added to binding reactions containing the [32P]-labeled human βAT-rich element, these complexes did not differ from those obtained when UL was used and therefore must be considered nonspecific (Fig. 6A, lanes 4 and 5). In contrast, when in vitro translated MEF2C was added to binding reactions containing a [32P]-labeled MCK MEF2 probe, a binding complex formed that had a lower mobility than the nonspecific binding complex that formed when using UL (Fig. 6A, lanes 6 versus 7). In competition experiments, 100-fold molar excess cold MCK MEF2 probe completely abolished com-
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**A**

| Probe: | βA/T-rich | MCK MEF2 |
|-------|-----------|----------|
| Extract: | MOV-P | - | MEF2CTnT |
| Comp: | MCK, MEF2 | - | + | + | + |

in vitro TNT MEF2C

![Diagram](image)

**B**

| Probe: | βA/T-rich | MCK MEF2 |
|-------|-----------|----------|
| Extract: | MOV-P | - | MEF2CTnT |
| Antibody: | MEF2A-Ab, MEF2B-Ab, MEF2-Ab | - | + | + |

![Diagram](image)

**Fig. 6.** A, EMSA analysis of in vitro transcribed-translated MEF2C protein. Inset, [35S]methionine-labeled MEF2C. The wheat germ lysate system was programmed with 1 μg of linearized mouse MEF2C expression plasmid in the presence of [35S]methionine. The TNT product was resolved on 12% SDS-PAGE and exposed to film. Molecular mass markers (in kilodaltons) are shown on the right. --, represents parallel reaction not programmed with MEF2C expression plasmid. Right panel, competition EMSA of 32P-labeled βA/T-rich and MCK MEF2 probes. 5 μg of MOV-P nuclear extract was incubated with radiolabeled βA/T-rich oligonucleotide (SC, lanes 1–3). Unlabeled competitor oligonucleotides harboring the βA/T-rich (lane 10), MCK MEF2 (lanes 2 and 8), and desmin MEF2 (lanes 3 and 9) binding elements were added at a 100-fold molar excess. Binding assays were also performed using 2 μl of either unprogrammed (–, lanes 4 and 6), or MEF2C cDNA-programmed TNT product (+, lanes 5 and 7–10). Note that MEF2C interacts with the MCK MEF2 oligonucleotide. In contrast, MEF2C does not bind to the βA/T-rich probe as shown by the lack of difference in band pattern between unprogrammed (–) and MEF2C programmed (+) lysate. B, antibody supershift EMSA analysis of MOV-P and MEF2C TNT binding complexes. Antibody EMSAs were performed by preincubation of MOV-P nuclear extract (5 μg) and MEF2C TNT product with 2 μl of either preimmune serum (PI), MEF2A-Ab, MEF2B-Ab, or polyclonal anti-MEF2A, -MEF2B, or -MEF2 antibody for 30 min at room temperature (see “Experimental Procedures”). Only the general anti-MEF2 antibody produced a supershift (SS) of the MEF2C binding complex (lane 10). None of the antibodies disrupted specific binding of the MOV-P factor(s) to the βA/T-rich probe (SC, lanes 3–5).

To assess whether other MEF2 isoforms interact with the human βA/T-rich element, we performed supershift EMSAs using antibodies that specifically recognize either MEF2A or MEF2B, as well as a general MEF2 antibody that recognizes MEF2A, -C, and -D isoforms. Preincubation of MOV-P nuclear extract with either preimmune serum or with any of the MEF2 antibodies (MEF2A-Ab, MEF2B-Ab, MEF2-Ab) did not supershift or immunodeplete the 32P-labeled βA/T-rich binding complex (Fig. 6B, lanes 1–5). The formation of a specific binding complex between in vitro translated MEF2C protein and the 32P-labeled MCK MEF2 probe was not altered by preincubation with preimmune serum or MEF2A- or MEF2B-specific antibodies (Fig. 6B, lane 6 versus lanes 7–9); however, preincubation with MEF2C antibody supershifted the MEF2C 32P-labeled MCK MEF2 probe binding complex (Fig. 6B, lane 10). These data strongly suggest that MEF2 proteins are not likely to be a component of the MOV-P-βA/T-rich complex.

The SRF Does Not Bind the βA/T-Rich Element—The SRE (CC(A/T)TATAAT) is an A/T-rich element previously shown to bind the MADS-box transcription factor, SRF, and to function as a regulator of numerous muscle and nonmuscle genes in response to very diverse stimuli (22). Given the relationship of the consensus SRE to the βA/T-rich element (GGAGATATTT) (Table I), and the observations that the SRF is activated by growth factors, elevated levels of intracellular calcium, and mechanical stretch (three stimuli associated with MOV), we investigated whether the SRF might comprise a component of the enriched MOV-P-binding activity. In competition and supershift EMSA experiments we found that the enriched binding complex formed between the βA/T-rich element and MOV-P nuclear extract was neither competed for by a consensus SRE nor supershifted by SRF antibody (Fig. 7, lanes 1–5). To determine whether SRF could bind to the βA/T-rich element, we performed binding reactions using wheat germ lysate in vitro translated SRF (Fig. 7, inset, [35S]methionine-labeled SRF) and the 32P-labeled βA/T-rich element. A binding complex was not formed when either UL or in vitro translated SRF protein was added to binding reactions containing the 32P-labeled βA/T-rich element (Fig. 7, lanes 6 and 7). When in vitro translated SRF was reacted with a 32P-labeled consensus SRE probe, a complex formed (lanes 8 versus 9) that was inhibited by addition of 100-fold molar excess cold SRE probe, but not βA/T-rich probe (Fig. 7, lane 9 versus lanes 10 and 11). Preincubation of the in vitro translated SRF containing binding reaction with preimmune serum did not alter complex formation, whereas preincubation with SRF antibody resulted in a supershifted binding complex (Fig. 7, lanes 12 versus 13). Thus, these data provide evidence indicating that the SRF is not a component of the MOV-P-binding activity.

The Homeodomain Protein Oct-1 Does Not Bind the βA/T-Rich Element during Skeletal Muscle Hypertrophy—The ubiquitous Oct-1 protein is present in most cell types and is thought to participate in directing B-cell specific immunoglobulin gene transcription through interaction at an octamer (ATGCAAAT) motif (41). In addition to its action in B-cells, several recent findings suggest a regulatory role for Oct-1 in the transcrip-
Supershift (Oct-1) and the 32P-labeled Oct-1 protein was added to binding reactions containing the in vitro translated Oct-1 was reacted with a 32P-labeled consensus Oct-1 probe, a complex formed (lanes 8 versus 9) that was inhibited by addition of 100-fold molar excess cold Oct-1 probe but not βAT-rich probe (Fig. 8, lane 9 versus lanes 10 and 11). Preincubation of the in vitro translated Oct-1 containing binding reaction with preimmune serum did not alter complex formation, whereas preincubation with Oct-1 antibody resulted in a supershifted binding complex (Fig. 8, lane 12 versus 13). These results indicate that Oct-1 is not a component of the enriched binding complex formed between MOV-P nuclear extract and the βAT-rich element.

Biochemical Analysis of MOV-P DNA-binding Factor Interaction at βAT-rich Element—Our EMSA and footprinting analyses support the notion that the human βAT-rich element functions as an MOV element in adult skeletal muscle. Furthermore, the composite nature (5/6 GATA, 8/10 MEF2) of our βAT-rich binding site and its flanking sequence as determined by footprinting analysis suggested the possibility that, under MOV conditions, a multiprotein complex likely forms at this element (Fig. 3, B and C). As an initial inquiry into what factor(s) within the MOV-P nuclear extract interacts with the 32P-labeled βAT-rich probe, we performed two-dimensional UV cross-linking analysis (Fig. 9). A bromodeoxyuridine-substituted βAT-rich probe was incubated with MOV-P nuclear extract, and the enriched binding complex was separated from unbound probe by EMSA. The EMSA gel was then exposed to UV light (312 nm) for 30 min, and both the highly enriched binding complex and the unbound (free) probe were excised from a 5% polyacrylamide gel. This analysis detected two distinct bands of apparent molecular masses of 44 and 48 kDa, thereby indicating that the enriched binding complex formed at the βAT-rich element is comprised of two different proteins whose identities are presently not known (Fig. 9).

When our experimental data herein are considered collectively, it can be tentatively concluded that the βAT-rich element likely functions in vivo as an MOV element involved in βMyHC induction in fast-type II fibers following skeletal muscle overload, and that two distinct proteins are involved in this process.
performed using 0.5 m and considering our findings herein with those of others, it seems into pressure-overloaded adult rat hearts (17). Therefore, when competitor binding complex (\textit{Inset}), \textsuperscript{35}S]methionine-labeled Oct-1 protein. The rabbit reticulocyte lysate system was programmed with 1 \mu g of circular human Oct-1 expression plasmid in the presence of \textsuperscript{35}S]methionine. The radiolabeled TnT product was resolved by 12\% SDS-PAGE and exposed to film. Molecular mass markers (in kilodaltons) are shown on the right. ––, represents reaction not programmed with the Oct-1 expression vector. Right panel, competition and antibody supershift EMSA analysis of radiolabeled \( \beta A/T \)-rich and Oct-1 oligonucleotides. MOV-P nuclear extract (5 \mu g) was incubated with \textsuperscript{32}P-labeled \( \beta A/T \)-rich probe (SC, lanes 1–5). Unlabeled competitor \( \beta A/T \)-rich (lanes 2 and 11) and Oct-1 (lanes 3 and 10) oligonucleotides were added at a 100-fold molar excess. Binding assays were also performed using 0.5 \mu l of either unprogrammed (–, lanes 6 and 8) or Oct-1 CDNA-programmed TnT product (+, lanes 7 and 9–13). Note that Oct-1 protein shows sequence-specific binding to the Oct-1 oligonucleotide but not to the \( \beta A/T \)-rich probe. For antibody supershift EMSA, MOV-P nuclear extract and Oct-1 TnT product were pre-incubated with 2 \mu l of either preimmune (PI, lanes 4 and 12) or polyclonal anti-Oct-1 antibody (lanes 5 and 13) for 30 min at room temperature prior to the addition of the probe. The anti-Oct-1 antibody produced a supershift (SS) of the Oct-1 TnT binding complex (lane 13) but not of the \( \beta A/T \)-rich MOV-P complex (lane 5).

**FIG. 8. EMSA analysis of \textit{in vitro} transcribed-translated Oct-1 protein.** Inset, \textsuperscript{35}S]methionine-labeled Oct-1 protein. The rabbit reticulocyte lysate system was programmed with 1 \mu g of circular human Oct-1 expression plasmid in the presence of \textsuperscript{35}S]methionine. The radiolabeled TnT product was resolved by 12\% SDS-PAGE and exposed to film. Molecular mass markers (in kilodaltons) are shown on the right. ––, represents reaction not programmed with the Oct-1 expression vector. Right panel, competition and antibody supershift EMSA analysis of radiolabeled \( \beta A/T \)-rich and Oct-1 oligonucleotides. MOV-P nuclear extract (5 \mu g) was incubated with \textsuperscript{32}P-labeled \( \beta A/T \)-rich probe (SC, lanes 1–5). Unlabeled competitor \( \beta A/T \)-rich (lanes 2 and 11) and Oct-1 (lanes 3 and 10) oligonucleotides were added at a 100-fold molar excess. Binding assays were also performed using 0.5 \mu l of either unprogrammed (–, lanes 6 and 8) or Oct-1 CDNA-programmed TnT product (+, lanes 7 and 9–13). Note that Oct-1 protein shows sequence-specific binding to the Oct-1 oligonucleotide but not to the \( \beta A/T \)-rich probe. For antibody supershift EMSA, MOV-P nuclear extract and Oct-1 TnT product were pre-incubated with 2 \mu l of either preimmune (PI, lanes 4 and 12) or polyclonal anti-Oct-1 antibody (lanes 5 and 13) for 30 min at room temperature prior to the addition of the probe. The anti-Oct-1 antibody produced a supershift (SS) of the Oct-1 TnT binding complex (lane 13) but not of the \( \beta A/T \)-rich MOV-P complex (lane 5).

**FIG. 9. Two-dimensional UV cross-linking of the DNA-protein complex.** The \( \beta A/T \)-rich MOV-P complex was resolved on a preparative EMSA gel. The wet gel was exposed to UV irradiation (312 nm) for 30 min at 4°C. The cross-linked DNA-protein complex was excised and resolved on a SDS-12\% polyacrylamide gel. Two bands were identified, with approximate molecular masses of 44 and 49 kDa.

**DISCUSSION**

Future characterization of the enriched binding activity we observed between MOV-P nuclear extract and the \( \beta A/T \)-rich element will provide information essential for the mechanistic understanding of the adaptive responses that occur during skeletal muscle hypertrophy. Although phenotypic changes occurring during this process have been extensively described biochemically, there remains a notable information void at the molecular genetic level. One major obstacle toward progress in this area derives from the lack of a myogenic cell line that is capable of maintaining an adult-stage phenotype. In addition, it is currently not possible to emulate in culture the input from integrative systems imposed on a MOV muscle of an intact animal, thus requiring these investigations to make use of transgenic models. In this study, we have advanced the mechanistic understanding of MOV changes in adult skeletal muscle phenotype by providing substantial molecular evidence that the \( \beta A/T \)-rich element located within the 89-bp \( \beta M y H C \) MOV-responsive region functions as a putative MOV element. This finding is important since the induction of \( \beta M y H C \) gene expression is a common MOV response shared by adult-stage rodent cardiac and skeletal muscle, and recently the \( \beta A/T \)-rich element was shown to play a role in the transcriptional activation of rat \( \beta M y H C \)-reporter constructs following direct injection into pressure-overloaded adult rat hearts (17). Therefore, when considering our findings herein with those of others, it seems reasonable to tentatively propose that MOV induction of the \( \beta M y H C \) gene in both adult cardiac and skeletal muscle is conferred in part by a common element; \textit{i.e.} the \( \beta A/T \)-rich site. The authenticity of the \( \beta A/T \)-rich element as an \textit{in vitro} MOV-inducible element in both striated muscle subtypes will require the analysis of chromosomally integrated transgenes carrying wild type and mutant \( \beta A/T \) rich elements, a focus of our ongoing investigations.

GATA-4, SRF, Oct-1, and MEF2 Are Not Components of the Binding Complex Formed at the \( \beta A/T \)-Rich Element during MOV Induction of \( \beta M y H C \) Gene Expression in Adult-stage Skeletal Muscle---An extensive body of literature exists that provide persuasive evidence suggesting that either GATA-4, SRF, Oct-1, or MEF2 may function independently or in combination to regulate \( \beta M y H C \) induction in response to MOV by binding to the \( \beta A/T \)-rich site. For example, several current findings indicate that GATA element(s) and/or protein(s) not only act as mediators of cardiovascular development (43, 44) but are also implicated in the regulation of other cellular responses such as the hypertrophic response in adult rat hearts (17, 45). Nevertheless, our direct, competition, and supershift EMSA experimental results (Figs. 5–8) provide comprehensive evidence that the factor(s) binding the \( \beta A/T \)-rich element in response to mechanical stimuli in skeletal muscle differ from...
that proposed (GATA-4) for cardiac muscle (17). Our experiments revealed that in vitro translated GATA-4 made in both wheat germ and rabbit reticulocyte lysate did not bind the human βA/T-rich element, whereas it did bind to probes harboring α-MyHC, cTnC, and BNP GATA sites in a sequence-specific manner (Fig. 5, A–C). Furthermore, Northern analysis did not detect the expression of GATA-4 transcripts in control or MOV plantaris muscle, eliminating the possibility of load-induced GATA-4 expression in adult skeletal muscle (data not shown). Moreover, the expression of GATA isoproteins as well as the newly identified GATA cofactors, FOG and FOG2 (Friend of GATA), have not been detected within adult-stage skeletal muscle (46–49).

This study also provides ample evidence that rules out other known transcription factors that otherwise might have logically been assumed to activate βMyHC induction during skeletal muscle hypertrophy based on tissue distribution and the nucleotide composition of the βA/T-rich element. Specifically, even though SRF, Oct-1, and MEF2 have been shown to be required for striated muscle expression of a number of contractile protein genes, our findings do not support a role for these transcription factors in the MOV induction of βMyHC expression in adult-stage skeletal muscle. Regardless of the high degree of nucleotide homology shared between the βA/T-rich element and consensus recognition binding sites for SRF, Oct-1, and MEF2 (Table I), our EMSA analysis revealed that the enriched βA/T-rich MOV-P binding complex was neither effectively competed for by these elements nor supershifted/depleted by SRF-, Oct-1-, or MEF2-specific antibodies (Figs. 6–8). The finding that MEF2 was not a component of the highly enriched MOV-P-βA/T-rich binding complex was surprising since it has recently been hypothesized that the activation of a Ca²⁺/calmodulin-dependent calcineurin signaling pathway underlies slow-type I fiber-specific transcription in response to a sustained increase in intracellular calcium induced by slow motor nerve activity (50). Mechanistically, the transcriptional activation of select slow-type I fiber-specific genes was shown to involve both MEF2 and NF-AT proteins (50). The potential applicability of this pathway to MOV is derived from the fact that proposed (GATA-4) for cardiac muscle (17). Our experiments revealed that in vitro translated GATA-4 made in both wheat germ and rabbit reticulocyte lysate did not bind the human βA/T-rich element, whereas it did bind to probes harboring α-MyHC, cTnC, and BNP GATA sites in a sequence-specific manner (Fig. 5, A–C). Furthermore, Northern analysis did not detect the expression of GATA-4 transcripts in control or MOV plantaris muscle, eliminating the possibility of load-induced GATA-4 expression in adult skeletal muscle (data not shown). Moreover, the expression of GATA isoproteins as well as the newly identified GATA cofactors, FOG and FOG2 (Friend of GATA), have not been detected within adult-stage skeletal muscle (46–49).

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An interesting observation gleaned from the MEF2 EMSA experiments that we feel merits discussion is the detection of an additional low migrating binding complex (which was more prominent following longer exposure) that was only visible when using MOV-P nuclear extract (Fig. 6B). In addition to
programs that alter transcription factor expression, activity via post-translational modification, or cofactor(s) availability and/or interaction. Last, the experimental paradigm used herein differs from those typically employed by others in that it utilizes adult-stage skeletal muscle that is undergoing a fast-to-slow fiber-type remodeling induced by MOV. Therefore, it is not unreasonable to suggest that transcription factor type and activity, as well as chromatin structure and/or microenvironment within the nuclear milieu of MOV skeletal muscle differs significantly from that within the cell-types commonly studied.

Our experiments have shown that the β/αT-rich site is a putative MOV element and that the enriched MOV-P binding activity at this site is comprised of two distinct proteins that may be unique to the MOV stimulus. Their identity and relatedness to other α/βT-rich binding factors awaits further investigation, nevertheless, the relative molecular mass of these two proteins (44 and 48 kDa) as determined by UV cross-linking is less than those determined for MEF2 proteins (55–65 kDa (13, 36), Fig. 6A, inset), in vitro translated GATA-4 (Fig. 5A, inset), SRF (67 kDa), and Oct-1 (100 kDa, Fig. 8, inset). Importantly, our experiments are the first to provide evidence at the molecular level indicating that βMyHC induction in adult-stage MOV plantaris muscle likely involves a regulatory program that is distinct from those activated during cardiac hypertrophy and striated muscle development.

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