Synergistic Interactions between Heterologous Upstream Activation Elements and Specific TATA Sequences in a Muscle-Specific Promoter

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Previous investigations have defined three upstream activation elements—CCAC, A/T, and TATA sequences—necessary for muscle-specific transcription of the myoglobin gene. In the present study, we demonstrate that these three sequence elements, prepared as synthetic oligonucleotide cassettes, function synergistically to constitute a cell-type-specific transcription unit. Previously, cognate binding factors that recognize the CCAC and TATA elements were identified. In this study we determine that the A/T element binds two nuclear factors, including myocyte enhancer factor-2 (MEF-2) and an apparently unknown factor we provisionally termed ATF35 (A/T-binding factor, 35 kDa). Mutations that alter in vitro binding of either MEF-2 or ATF35 to this site diminish promoter function in vivo. Functional synergism between factors binding the CCAC and A/T elements is sensitive to subtle mutations in the TATA sequence, recapitulating the unusual preference for specific TATA variants exhibited by the native myoglobin promoter. These results provide new insights into mechanisms that underlie the distinctive pattern of myoglobin gene regulation in mammalian muscle development and lay a foundation for further studies to elucidate general principles of transcriptional control of complex mammalian promoters through combinatorial actions of heterologous transcription factors.

Transcriptional control regions of eukaryotic genes typically include binding sites for several proteins, combinatorial interactions among which provide a basis for exquisitely tuned developmental and physiological regulation. In previous studies of the human myoglobin gene promoter in transgenic mice, we determined that transcriptional control elements sufficient to direct expression selectively in skeletal and cardiac myocytes are contained within the proximal 400 nucleotides upstream from the transcriptional start site (1). A detailed mutational analysis of this region identified two sequence motifs absolutely required for transcriptional activity in both skeletal and cardiac myocytes (1, 2). One of these, termed the CCAC box (see Fig. 1), is similar to elements found in other muscle-specific promoter-enhancer regions (13, 25, 35). CCAC box-binding factors include myocyte nuclear factor (MNF), a novel member of the winged-helix (or forkhead/HNF-3) family of transcription factors expressed in cardiac and skeletal muscle (3); CBF40, which has not been characterized fully (2); and Sp1, which binds the CCAC box with low affinity relative to consensus Sp1-binding sites (39). A second motif required for transcriptional activity of the human myoglobin promoter (2), termed the A/T element, is similar but not identical to consensus binding sites for members of the myocyte-specific enhancer-binding factor 2 (MEF-2)/RSRF family of transcription factors (37, 46). The sequence of the human myoglobin gene promoter and the positions of these transcriptional control elements are illustrated in Fig. 1.

A peculiar feature of transcriptional control elements of the human myoglobin gene is an unusual sensitivity to subtle modifications of sequences within the TATA box. In our initial characterization of upstream activation sequences from this

gene, we noted that muscle-specific enhancer activity was observed after linkage to some, but not all, heterologous core promoter regions. This selectivity was shown to be dependent on the specific sequence of the TATA box (42). A similar dependency for specific TATA sequences has been observed in the *his3* promoter of *Saccharomyces cerevisiae* (22), in E1A stimulated-activation of the human *hsp70* promoter (40), in transcriptional activation of the human immunodeficiency virus long terminal repeat (4), and in muscle-specific transcription of *Xenopus MyoDa* (30).

In the present study, we examined functional interrelationships between the CCAC, A/T, and TATA elements from the human myoglobin gene, posing the following questions. Is the CCAC and/or A/T element sufficient to direct muscle-specific expression of a minimal promoter, or are other as yet unidentified sequence elements from the myoglobin upstream region also required? Is the requirement for specific TATA sequences exhibited by the myoglobin promoter attributable to interactions with proteins that bind the CCAC and/or A/T element? Is the A/T element a nonconsensus binding site for MEF-2 proteins from differentiated skeletal myotubes, or do novel cognate proteins bind this site? These studies were initiated to provide insights into the mechanisms that underlie the distinctive pattern of myoglobin gene regulation in mammalian muscle development (36) and ultimately to elucidate general principles of transcriptional control of complex promoters through combinatorial actions of heterologous transcription factors.

MATERIALS AND METHODS

Cell culture. Monolayers of mouse sol8 (15) myogenic cells were grown in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum. Myotube formation was induced by growing the cells to confluence and switching the medium to differentiation medium (Dulbecco's modified Eagle's medium with 2% horse serum, supplemented with 10 μ g of insulin per ml and 10 μ g of transferrin per ml [Gibco/BRL Research, Gaithersburg, Md.]). Monolayers

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A

AGCTTAGAAACATGACAGGTCCTC -350

TTGGAGGGCTGACCGAGGAGCGTTGGGTTTCAGGCTGCTGGCGTCGG -300

CTTCTGTGGTGCCTTTCTGTGGCTATGAGAGTCCAGACAGTGCCTAAC -250

CTCCTCCCCTTCTTCCACACGCA **CCAC box** CAACACACCCACCCCTGTGG -200

GCTGTCTGCTCGCCCAATGGCA **A/T element** CCTGCCCTAAAAAGCTTCC CATGT -150

GAGGGCTAGAGAAAGGAAAAGATTAGACCTCCCTGGATGAGAGAGAGAA -100

AGTGAAGGAGGGCAGGGGAGGGGACGACGAGCCATTGAGCGATCTTTGT -50

CAAGCATCCCAGAAGG **TATA box** TATAAAAA CGCCTTGGGACGAGCAGCCTCAA -1

B

myoglobin A/T: 5'-CCCTAAAAATAGCTTCC-3'

MCK Mef-2: 5'-CTCTAAAAATAACTCC-3'

RSRF C4: 5'-CTA (A/T) 4 TAG-3'

A/T mut 1: 5'-CCCTAAAAATAGCTTCC-3'

A/T mut 2: 5'-CCCTAAAAATAGTTCC-3'

FIG. 1. (A) Nucleotide sequence of the 373-bp upstream promoter of the human myoglobin gene. The two activation sequences, identified as the CCAC box and A/T element, and a canonical TATA sequence, which have been characterized in previous mutational analyses (2, 16), are boxed. (B) The nucleotide sequence of the myoglobin A/T box is illustrated and compared with the sequence of a consensus MEF-2-binding site from the MCK upstream enhancer (46) and to a sequence selected from among a set of random oligonucleotides as a high-affinity binding site for RSRF C4 (37), a member of the MEF-2 family. As described in the text, the mutation present in the constructs identified as A/T mut1 and A/T mut2 converts the myoglobin A/T box to identity with the MEF-2 and RSRF C4 consensus binding sites, respectively.

of mouse NIH 3T3 fibroblast cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum.

Construction of promoter-reporter plasmids and site-directed mutagenesis of myoglobin promoter sequences. Oligonucleotides representing the CCAC and A/T elements from the human myoglobin gene were designed to include a *Bam*HI overhang at the 5' end, an internal *Bgl*II site, and a 3' *Sma*I blunt end, as previously described (2). The resulting products contained specific sequence cassettes (see Table 1 for nucleotide sequence) in various combinations connected by half of a *Bam*HI site and half of a *Bgl*II site. The synthetic elements were inserted into the *Bam*HI site of pGUPPA, a Bluescript (Stratagene) plasmid which contains in its *Hind*III site the human *hsp70* minimal promoter linked upstream of the firefly luciferase gene (2). pGUPPA includes a simian virus 40 (SV40) polyadenylation signal placed upstream of the transcriptional control elements under analysis. This eliminates expression of luciferase initiated from cryptic promoter elements within the plasmid backbone and reduces background activity observed with minimal promoter or other promoterless constructions.

The TATA element, TATAAAA, present in the human *hsp70* minimal promoter used in these constructions (40) is identical to that found in all known myoglobin genes (6, 7, 45). A complete second set of constructs containing

myoglobin CCAC and/or A/T elements was prepared with a different TATA element, TATTAT, identical to that found in the SV40 early promoter (9). Constructs bearing this variant TATA element were otherwise identical to the original series, including the flanking sequences from the remainder of the *hsp70* minimal promoter. Construction of the plasmid containing the native promoter sequences from the human myoglobin gene (-373 to +7), including the CCAC, A/T, and TATA elements, has been described elsewhere (2).

Constructs bearing site-directed mutations of the A/T region were prepared by incorporation of a thiol-substituted nucleotide into the mutant strand and subsequent selective removal of the nonmutant strand (*In Vitro* mutagenesis system; Amersham Corp., Arlington Heights, Ill.). Single-stranded DNA template for mutagenesis was generated by cloning a *Hind*III fragment containing myoglobin upstream sequences -373 to +7 into the pUC118 phagemid vector. Mutated promoter sequences were subsequently recovered as a *Hind*III fragment and linked to the luciferase gene in a Bluescript plasmid.

Oligonucleotide sequences were synthesized by an Applied Biosystems 394 DNA/RNA synthesizer and are listed in Table 1. The identity of all constructs was confirmed by restriction mapping and dideoxynucleotide chain termination sequencing with Sequenase 2.0 polymerase (United States Biochemical, Cleveland, Ohio).

Transfections and luciferase assay. Transfections and luciferase assays were performed with sol8 and NIH 3T3 cells as described previously (2). Briefly, 10^7 cells were resuspended in 0.5 ml of growth medium and transfected by electroporation (Gene Pulser; Bio-Rad Laboratories, Richmond, Calif.) by the method of Chu et al. (12). Electroporations were performed with 10 μ g of plasmid and 100 μ g of sonicated herring sperm DNA. Following electroporation, the cells were plated onto 60-mm-diameter dishes, incubated for 18 h at 37°C, washed, and overlaid with differentiation medium. Two days after the addition of differentiation medium, the cells were harvested and lysed in 300 μ l of 50 mM Tris-HCl (pH 8)-1% Nonidet P-40-100 μ g of aprotinin per ml. Lysate (50 μ l) was added to 300 μ l of luciferase reaction buffer containing 25 mM glycylglycine (pH 7.8), 15 mM MgCl₂, 2.5 mM ATP, 1 mM dithiothreitol, and 400 μ g of bovine serum albumin per ml and assayed in the presence of 1 mM luciferin (Sigma Co.). Light emissions were integrated for the initial 10 s of emission at 25°C in a Berthold LB9500C luminometer. Reporter gene activity for each construct was determined in four or more independent experiments.

The efficiency of transfection was determined by cotransfection of pCMV-lacZ (from Radek Skoda, Boston, Mass.). β -Galactosidase expression was monitored by adding 30 μ l of a 1:20 dilution of cellular lysate from transfected cells to a final concentration of 0.8 mg of *o*-nitrophenyl- β -D-galactopyranoside per ml in a buffer of 0.1 M sodium phosphate (pH 7.9), 1 mM MgCl₂, and 45 mM β -mercaptoethanol at a final volume of 300 μ l. The samples were incubated at 37°C for 30 min, and the A_{420} was measured with a spectrophotometer. Luciferase expression resulting from transfection of each test construct was normalized to expression of β -galactosidase.

Preparation of nuclear proteins. Nuclear extracts from sol8 myotubes and NIH 3T3 fibroblasts were prepared by washing cells once with cold isotonic buffered saline and then adding to each 150-mm-diameter plate 1 ml of lysis buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.4], 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 20% glycerol, 0.1% Triton X-100, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μ g of leupeptin per ml, 10 μ g of pepstatin per ml, 100 μ g of aprotinin per ml). Cells were harvested by scraping the plate with a rubber policeman and then centrifuging for 1 min at 2,000 rpm in a Sorvall Microspin 24S centrifuge at 4°C. The pellet was resuspended in 5 volumes of lysis buffer containing 500 mM NaCl. The solution was then centrifuged at 12,000 rpm in a Sorvall Microspin 24S centrifuge for 10 min at 4°C. The supernatant was collected on ice and used immediately. The protein concentration was determined by using the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.).

Generation of polyclonal antibodies against MEF-2B and MEF-2D. Plasmids were constructed to express GST-MEF-2B(88-365) and GST-MEF-2D(150-213), which are fusion proteins consisting of glutathione *S*-transferase (GST) linked to selected polypeptides of human MEF-2B and MEF-2D as indicated (8, 46). The purified fusion proteins were injected into New Zealand White rabbits to generate polyclonal antibodies by standard techniques (23). The specificity of the antisera was examined by Western immunoblot analysis and mobility gel shift

TABLE 1. Oligonucleotides used for synthetic elements and mutagenesis

Oligonucleotide	Sequence
(CCAC) _n sense	5'-GATCCAACCAACCCACCCCTGTGGAGATCTCCC-3'
(CCAC) _n antisense.....	5'-GGGAGATCTCCACAGGGGGTGGGGTGGTTG-3'
A/T sense.....	5'-GATCCCTGCCCTAAAAATAGCTTCCAGATCTCCC-3'
A/T antisense.....	5'-GGGAGATCTGGAAGCTATTATAGGCGAGG-3'
TATTAT box sense	5'-AGCTTCTCGAGCGGCTTCCGTGACGACTTATTTATGCCACAGGGGCAAGCGGTCCGGA-3'
TATTAT box antisense.....	5'-AGCTTCCGACCGCTTGGCCCTGGGCATAAATAAGTCGTACGGAGACCCGCTCGAGA-3'
A/T mut1.....	5'-GGCACCTGCCCTAAAAATAGCTTCCATGTGA-3'
A/T mut2.....	5'-GGCACCTGCCCTAAAAATAGTTCCCATGTGAGG-3'

TABLE 2. Oligonucleotides used as probes and competitors

Oligonucleotide ^a	Sequence
A/T sense.....	5'-GATCCTGCCCTAAAATAGCTTCCC-3'
A/T antisense.....	5'-GATCGGGAAGCTATTTTAGGGCAG-3'
MEF-2 sense.....	5'-GATCCTCTAAAAATAACCCT-3'
MEF-2 antisense.....	5'-GATCCAGGGTTATTTTAGAG-3'
A/T mut1 sense.....	5'-GATCCCTAAAAATAGCTTCC-3'
A/T mut1 antisense.....	5'-GATCGGAAGCTATTTTAGG-3'
A/T mut2 sense.....	5'-GATCCCTAAATAGTTCC-3'
A/T mut2 antisense.....	5'-GATCGGAAGCTATTTTAGG-3'
Myo TATA sense.....	5'-GATCAGAAGGTATAAACGCCCT-3'
Myo TATA antisense.....	5'-GATCAGGGCGTTTATACCTTCT-3'
Myo/SV40 TATA sense.....	5'-GATCAGAAGGTATTTATACGCCCT-3'
Myo/SV40 TATA antisense.....	5'-GATCAGGGCGTATAAATACCTTCT-3'

^a Myo, myoglobin.

assays with cell extracts prepared from COS cells expressing MEF-2A, MEF-2B, or MEF-2D (data not shown).

DNA probes and competitors. Both sense and antisense oligonucleotide DNA strands corresponding to the myoglobin A/T region (−174 to −155 bp of the human myoglobin gene) (1, 2), a consensus MEF-2-binding site (−1077 to −1062 bp of the mouse muscle creatine kinase gene) (21), the myoglobin TATA box (−39 to −19 bp of the human myoglobin gene) (42, 45), and a 4-bp substitution within the myoglobin TATA box, rendering it identical to the TATA box from the SV40 early promoter (954 to 961 bp of the SV40 genome) (9), were synthesized with an added GATC nucleotide overhang at the 5' terminus. Complementary strands were annealed and end labelled with Klenow fragment of *Escherichia coli* polymerase I and [α -³²P]dATP (6,000 Ci/mmol) or used as unlabelled competitors in electrophoretic mobility shift analysis. Labelled oligonucleotides were purified by Sephadex G-50 column chromatography (Pharmacia Biotech Inc., Alameda, Calif.). Sequences of oligonucleotides used as probes and competitors are listed in Table 2.

Electrophoretic mobility shift analysis. Mobility shift reaction mixtures were prepared in a final volume of 12 μ l by combining 1 ng of labelled double-stranded DNA (10⁴ cpm) with nuclear extract (~9 μ g) and 4 μ g of sonicated herring sperm DNA (Promega Corp., Madison, Wis.) in a binding buffer containing 25 mM HEPES (pH 7.4), 5 mM MgCl₂, 5 mM dithiothreitol, 100 mM KCl, and 10% glycerol. The amount of unlabelled competitor DNA added is indicated in the figure legends. After 20 min of incubation at room temperature, the reaction mixtures were loaded onto a 4% native polyacrylamide gel equilibrated in 45 mM Tris–45 mM boric acid–1 mM EDTA. After electrophoresis at 10 V/cm for 2 h, the gels were dried and exposed to Hyperfilm-ECL (Amersham Corp., Arlington Heights, Ill.) for 24 h. For supershift experiments, 1 μ l of rabbit polyclonal antiserum was incubated with the nuclear proteins for 20 min before addition of the radiolabelled probe.

UV cross-linking of A/T element to nuclear proteins. DNA-protein complexes were identified by electrophoretic mobility shift analysis and irradiated with UV light via a transilluminator (Fotodyne Incorporated, New Berlin, Wis.) at a wavelength of 312 nm for 1.5 h. The DNA-protein complexes were exposed to a PhosphorImager cassette (Molecular Dynamics, Sunnyvale, Calif.) for 2 h, excised from the gel, placed in buffer consisting of 50 mM Tris-HCl (pH 6.8) and 2% sodium dodecyl sulfate (SDS), and eluted from the acrylamide gel on a rotating wheel overnight. The eluates were concentrated on Micro-Con concentration units (Amicon Inc., Beverly, Mass.), mixed with SDS-polyacrylamide gel sample buffer (100 mM Tris-HCl [pH 6.8], 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol), and loaded onto an SDS–10% polyacrylamide gel. After electrophoresis, the gel was fixed in a solution of 30% methanol and 10% acetic acid, dried on a gel drier (Hoefel Scientific Instruments, San Francisco, Calif.), and exposed to Hyperfilm-ECL (Amersham) for 21 days.

RESULTS

The myoglobin A/T and CCAC elements are sufficient, in combination, to direct muscle-specific transcription from a minimal promoter. Reporter plasmids bearing the firefly luciferase gene were constructed to link synthetic oligonucleotides representing the myoglobin CCAC (−225 to −205) and A/T (−174 to −155) elements (2) to a minimal promoter (−48 to −1) from the human *hsp70* gene (40). After transfection into sol8 myoblasts and subsequent differentiation of these cells into myotubes that express the endogenous myoglobin gene (2), expression of luciferase activity was quantified. Comparisons with the expression of constructs in which the reporter

gene was controlled by the native myoglobin promoter (−373 to +7) or by the minimal *hsp70* promoter alone were made. Luciferase activities were also measured when the same constructs were transfected into NIH 3T3 cells in which the endogenous myoglobin gene is transcriptionally silent.

We observed that the A/T element alone, or up to five copies of the CCAC box in the absence of an A/T element, could direct only low levels of transcription in both sol8 myotubes and 3T3 cells (Fig. 2A). In contrast, the combination of four CCAC and one A/T element linked to the minimal promoter reconstituted a transcription unit that was expressed selectively in myotubes and to levels approximating those directed by the complete myoglobin promoter region (>50-fold above those directed by the minimal promoter alone) (Fig. 2A). Multimerization of A/T elements also increased the activity of the minimal promoter in sol8 myotubes but not in NIH 3T3 cells (Fig. 2B). These results indicate that the CCAC and A/T elements, in the absence of additional protein-binding motifs other than a canonical TATA element, create a muscle-specific transcriptional control region functionally similar to the complete myoglobin promoter. The synthetic enhancer construction based on CCAC and A/T oligonucleotide cassettes was, however, somewhat more active than the native myoglobin promoter/enhancer region in nonmuscle cells, perhaps reflecting the contribution of additional negative control elements to the highly restricted pattern of expression exhibited by the complete myoglobin promoter.

Muscle-specific transcription directed by the synthetic CCAC and A/T elements is sensitive to subtle mutations in the TATA box in a manner similar to that for the complete myoglobin promoter. Previous studies (42) demonstrated that upstream activation sequences from the myoglobin promoter function poorly in combination with certain TATA box sequences that are permissive for the function of other enhancers. Mutation of the canonical TATAAAA sequence present in all known myoglobin genes to TATTTAT (as found in the SV40 early promoter) reduces transcription to >10% of control levels in primary skeletal myotubes derived from chicken embryos. In the present study, we tested whether the synthetic enhancer constructed from oligonucleotides representing the CCAC and A/T boxes would display a similar requirement for specific TATA sequences. We observed that the synergistic effect of combining the CCAC and A/T elements was sensitive to mutation of the TATA box (Fig. 2C). In the (CCAC)₄+(A/T) construction, mutation of the canonical TATA box to the SV40 type (TATAAAA to TATTTAT) reduced transcription in the myotube background by more than 50%, while expression in the fibroblast background was not reduced (Fig. 2C), thereby abolishing the muscle-specific transcription directed by these elements when linked to the TATAAAA core promoter. These results indicate that the TATA specificity that is a characteristic feature of the complete myoglobin promoter is retained in synthetic constructions based on only the CCAC and A/T elements.

The myoglobin A/T region binds nuclear protein factors present in differentiated sol8 myotubes, including both MEF-2 and an unknown factor, ATF35. Electrophoretic mobility shift assays performed with a ³²P-labelled oligonucleotide probe representing the myoglobin A/T sequence and nuclear extracts from sol8 myotubes revealed three protein-DNA complexes (Fig. 3A). Two of these complexes (denoted MEF-2 and ATF35) were abolished by competition with unlabelled oligonucleotide representing the myoglobin A/T motif and thereby include sequence-specific DNA-binding factors. A third com-

FIG. 2. Expression of a luciferase reporter gene under the control of a synthetic minimal promoter containing a canonical TATAAAA box (matching the native myoglobin promoter) (A and B) or a noncanonical TATTTAT box (matching the SV40 early promoter) (C) and linked to selected combinations of oligonucleotides representing the CCAC and A/T boxes from the human myoglobin gene. The sequences included in each oligonucleotide cassette are described in the text. Reporter gene expression was assayed in sol8 myotubes (solid bars) or NIH 3T3 fibroblasts (open bars) and was normalized to expression of a cotransfected pCMV-LacZ plasmid. Results were calculated relative to the activity of the minimal promoter alone and are displayed as mean values ($\pm$ standard error of the mean) from four or more independent observations.

FIG. 3. Electrophoretic mobility shift assays demonstrating specific binding of nuclear factors to the myoglobin A/T box and the MCK MEF-2 motif. (A) DNA protein complexes formed with sol8 myotube nuclear proteins and the myoglobin A/T box as the labelled probe in the presence of various amounts (25, 50, or 100 ng) of unlabelled self (A/T) and nonself (MCK MEF-2) oligonucleotides. A slowly migrating complex (labelled MEF-2) is abolished by both forms of unlabelled competitor DNA, while a rapidly migrating complex (labelled ATF35) is competed only by the self oligonucleotide. A nonspecific (NS) complex of intermediate mobility fails to compete with either unlabelled sequence. (B) DNA-protein complex formed with sol8 myotube nuclear proteins and the MCK MEF-2 motif as the labelled probe. Competition studies were done with either unlabelled A/T and MCK MEF-2 oligonucleotides as described above. The unlabelled MCK MEF-2 sequence is a more potent competitor than the myoglobin A/T box. (C and D) A MEF-2 A/D antibody recognizes proteins in the slowly migrating complex formed with the myoglobin A/T element. sol8 myotube nuclear proteins were incubated with either the myoglobin A/T element (C) or the MCK MEF-2 site (D) as the labelled probe in the presence of 1 μ l of either preimmune serum, antibody recognizing MEF-2 A and D proteins, or antiserum raised against MEF-2B protein.

plex (NS) of intermediate mobility represents a nonspecific DNA binding activity.

Because the myoglobin A/T elements appeared to be similar to previously defined binding sites in other promoters for the MEF-2 family of transcription factors (21, 46), we used several approaches to determine whether MEF-2 proteins bind this site. Cross-competition assays with a synthetic oligonucleotide representing a consensus binding site for MEF-2 from the muscle creatine kinase (MCK) upstream enhancer (21, 25) indicated that MEF-2 proteins bind specifically to the myoglobin A/T element. Unlabelled MCK-MEF-2 oligonucleotide competes avidly with the myoglobin A/T sequence for proteins present in the most slowly migrating complex (Fig. 3A). When the MCK-MEF-2 sequence is labelled as the probe, a complex of similar mobility is observed and the myoglobin A/T element, included as unlabelled competitor, inhibits formation of this complex (Fig. 3B). The myoglobin A/T element, however, appears to be a relatively weak binding site for MEF2, by comparison with the MCK-MEF-2 binding motif.

The presence of MEF-2 in the slowly migrating complex formed with the myoglobin A/T probe was confirmed by the observation that specific antibodies directed against MEF-2 isoforms (A and D) included in the binding reaction alter the mobility of this complex (supershift) (Fig. 3C). A supershift complex was also observed in binding reactions with the MCK-MEF-2 probe with antiserum against MEF-2 A and D isoforms (Fig. 3D). No shift in ATF35 migration was seen in binding reactions with preimmune serum or serum raised against MEF-2B protein; however, a slight decrease in the intensity of the ATF35 complex was observed.

The rapidly migrating, specific binding complex formed between the myoglobin A/T element and nuclear proteins from sol8 myotubes (Fig. 3A, ATF35) includes a factor distinct from MEF-2 by several criteria. This complex was not abolished by competition with unlabelled MCK-MEF-2 oligonucleotide (Fig. 3A) and was not observed when the MCK-MEF-2 oligonucleotide was labelled as the probe (Fig. 3B). Likewise, the mobility of this rapidly migrating complex was not shifted by specific antibodies recognizing MEF-2 isoforms (Fig. 3C). However, the diminution of ATF35 binding by MEF-2 anti-

body allows the possibility that ATF35 is antigenically related to the MEF-2 family.

Finally, photoaffinity cross-linking experiments indicate that this rapidly migrating complex contains an unknown DNA-binding protein that is distinct from MEF-2. After subtraction of the mass attributable to the oligonucleotide (14 kDa), the molecular size of the DNA-binding moiety from this complex was estimated to be 35 kDa (Fig. 4, lane 2), and we termed this protein ATF35 (A/T element-binding factor, 35 kDa). Photoaffinity cross-linking of the slowly migrating complex formed with the myoglobin A/T probe identified proteins of 55 to 65 kDa (lane 4), consistent with the known sizes of the MEF-2A, MEF-2C, and MEF-2D proteins (8, 46) and similar to cross-linked proteins within the major complex formed with the MCK-MEF-2 oligonucleotide probe (lane 1). No distinct pro-

FIG. 4. Nuclear proteins photoaffinity cross-linked to radiolabelled oligonucleotide probes and separated by SDS-polyacrylamide gel electrophoresis. Lanes: M, molecular mass markers; 1, major complex formed with the MCK MEF-2 probe; 2, rapidly migrating complex formed with the myoglobin A/T probe; 3, nonspecific complex formed with the myoglobin A/T probe; 4, slowly migrating complex formed with the myoglobin A/T probe. Predicted molecular masses of the proteins are calculated after subtraction of the approximate size of the oligonucleotide probe, as described in the text.

FIG. 5. Electrophoretic mobility shift assays demonstrating developmental regulation and cell-type-specific expression of nuclear proteins binding the myoglobin A/T element. (A) Complexes formed with nuclear proteins from sol8 myoblasts and myotubes or NIH 3T3 fibroblasts. (B) Complexes formed with nuclear proteins from C2C12 myoblasts and myotubes or sol8 myotubes. Unlabelled competitor oligonucleotides were included as described in the legend to Fig. 3 (50 or 100 ng).

tein bands were observed after photoaffinity cross-linking of the nonspecific binding complex (lane 3).

The DNA-protein complex attributable to ATF35 was not detected in nuclear extracts from NIH 3T3 fibroblasts (Fig. 5A) and became much more abundant following differentiation of both sol8 and C2C12 myoblasts to myotubes (Fig. 5B). DNA-binding activity consistent with MEF-2 was present at low levels in NIH 3T3 cells but was similarly up-regulated during myotube differentiation (Fig. 5A). Thus, the expression and/or DNA-binding activity of proteins binding the myoglobin A/T element, including both ATF35 and MEF-2, is regulated in a differentiation-specific pattern.

Converting the myoglobin A/T element to either of two MEF-2 consensus binding sites compromises promoter function. The myoglobin A/T element binds specifically to MEF-2 proteins but with lower affinity than the MEF-2-binding site from the MCK upstream enhancer (Fig. 3A). To assess the effects on promoter function of converting the weak, nonconsensus MEF-2 site within the myoglobin upstream region to a strong consensus site, we constructed the two A/T mutant promoter-reporter constructions illustrated in Fig. 1B. The sequence present in A/T mut1 converts the myoglobin A/T element to identity with the MEF-2 consensus binding site (46). This mutated sequence bound more avidly than the native sequence to MEF-2 in vitro (Fig. 6B, compare lanes 4 and 7) but was equivalent to the native sequence for binding ATF35 (Fig. 6A). This construction, however, was expressed in the sol8 myotube background to only 30% of the level for the native promoter construct (Fig. 6C).

A second mutant sequence, A/T mut2 (Fig. 1B), was made equivalent to an RSRF C4 (MEF-2) consensus sequence identified by a binding-site selection procedure (37) by the insertion of an additional thymidine nucleotide and the deletion of a cytosine. In contrast to the A/T mut1 sequence, the mutation in A/T mut2 did not appear to alter binding to MEF-2 in vitro (Fig. 6B, compare lanes 4 and 10) but diminished the binding to ATF35 (Fig. 6A, compare lanes 3 and 5 to 7 with lanes 8 to 10). The functional consequence of the lower affinity of this sequence for binding ATF35, in the context of the myoglobin promoter, was reduced expression in the sol8 myotube background (Fig. 6C). Both A/T mut1 and A/T mut2 plasmids, like the parent construct with the native myoglobin upstream se-

quence, were relatively inactive in the fibroblast background (Fig. 6C).

MEF2 binds with different affinities to native and mutant TATA sequences. We reported previously (42) and confirmed in this publication that muscle-specific enhancer activity of the myoglobin upstream region is sensitive to subtle variations in the TATA box sequence. Specifically, we observed that a 3-base substitution to convert the myoglobin TATAAAA element to TATTTAT compromised muscle-specific transcription. Because MEF-2 proteins bind A/T-rich sequence motifs similar to TATA boxes (21), we performed in vitro binding assays with A/T or MEF-2 probes in the presence of TATA AAA or TATTTAT sequences, flanked by identical nucleotides from the myoglobin promoter (Fig. 7). Using either the myoglobin A/T element (Fig. 7A) or the MCK-MEF-2 binding site (Fig. 7B and C) as the probe, we observed that the oligonucleotide containing the TATTTAT sequence competes more avidly than the TATAAAA sequence for binding to MEF-2. These results are similar to findings by Leibham et al. (30) for the *Xenopus MyoDa* promoter. In contrast, proteins within the ATF35 complex did not appear to bind either TATTTAT or TATAAAA oligonucleotides (Fig. 7A).

DISCUSSION

A variety of different DNA sequence motifs function as components of enhancer-promoter regions that are required for muscle-specific gene transcription. These include E box (CANNTG), CARG box [CC(A/T)₄GG], and MCAT (CAT TCCT) elements that serve as binding sites for myogenic basic helix-loop-helix proteins (5, 19), serum response factor (33), and TEF-1 (18, 41), respectively. MEF-2/RSRF proteins (8, 32, 37, 46), which bind a CTA(A/T)₄TAG consensus sequence, represent another set of factors important for muscle-specific gene expression. Additional sequence elements required for transcription of genes expressed selectively in cardiac and skeletal muscle have been defined by mutational analysis of promoter-reporter constructions (26, 31, 34, 35) but await definitive characterization of cognate binding factors.

Our previous studies of transcriptional control elements within the human myoglobin promoter identified two discrete upstream activation sequences (CCAC box and A/T box [Fig.

FIG. 6. (A and B) Electrophoretic mobility shift assays assessing the relative potency of native and mutant A/T sequences in competing for binding of nuclear proteins binding to the myoglobin A/T element (A) or the MCK MEF-2 sequence (B). Oligonucleotide sequences used as competitors, A/T (100 ng), MEF-2 (100 ng), A/T mut1 (25, 50, or 100 ng), and A/T mut2 (25, 50, or 100 ng), are described in the text and illustrated in Fig. 1B. (C) Functional consequences of mutations in the myoglobin A/T box for transcription in the sol8 myotube and fibroblast cell backgrounds. Symbols are as defined in the legend to Fig. 2.

1)) required for transcription in cardiac (1) and skeletal (2) myocytes. In the present study, we demonstrate that these two elements are not only necessary but sufficient, in conjunction with a minimal promoter containing only a TATA box, to constitute a potent muscle-specific transcription unit. This

transcriptional control unit based on CCAC, A/T, and TATA elements leads to reporter gene expression similar to that directed by the native myoglobin upstream region (−373 to +7) in sol8 myotubes. It is, however, somewhat more active than the native sequence in a nonmyocyte background, suggesting that additional negative control elements contributing to muscle specificity may be present within the complete myoglobin promoter.

The combinatorial effects of the CCAC and A/T motifs suggest that functionally synergistic interactions take place among the cognate binding factors that recognize these sequences. An understanding of the nature of this synergism will require identification of the protein factors involved. Efforts in our laboratory to identify CCAC box-binding proteins have led recently to the cloning and characterization of MNF (3), a novel member of the winged-helix (*forkhead*/HNF-3) family (27–29, 43, 44) of transcription factors. MNF is a nuclear phosphoprotein expressed most abundantly in skeletal and cardiac muscle that exhibits sequence-specific DNA-binding activity for the myoglobin CCAC motif. The cDNA we cloned encodes a protein with a predicted molecular size of 66 kDa, although antisera raised against recombinant MNF identify three polypeptides of approximately 65, 68, and 90 kDa. The abundance of both the 65- and 90-kDa forms of MNF-related polypeptides is regulated during differentiation of sol8 myotubes in culture and in response to changes in neural input to intact skeletal muscles in situ, in parallel to increases in transcription of the myoglobin gene. The DNA-binding domain of MNF exhibits 45 to 87% amino acid sequence identity to other *forkhead*/HNF-3 proteins from vertebrate organisms (3). MNF diverges entirely from other known winged-helix proteins outside of this conserved domain but contains a proline-rich transcriptional activation domain in its amino-terminal region.

These recent data provide evidence that MNF may represent the biologically relevant CCAC box-binding factor, but myocytes express at least two other proteins that remain candidates for this role. The myoglobin CCAC box functions in vitro as a weak binding site for the general transcription factor Sp1 (39). In addition, an as yet unidentified 40-kDa protein with CCAC box-binding activity is abundant in myocyte nuclei (2). Studies are under way to determine, unequivocally, the identity of the relevant CCAC box-binding factor, a prerequi-

FIG. 7. Electrophoretic mobility shift assays assessing the potency of different TATA sequences for binding A/T and MEF-2 elements. sol8 myotube nuclear proteins were incubated with labelled probe representing A/T (A) or MCK MEF-2 (B and C) binding site and various amounts (50 or 100 ng) (A, B) or (25, 50, 100, and 200 ng) (C) of unlabeled competitor oligonucleotides representing either the native myoglobin TATAAAA sequence or the TATTTAT sequence found in the SV40 early promoter. The former but not the latter sequence is permissive for muscle-specific transcription controlled by CCAC and A/T elements from the human myoglobin gene (Fig. 2).

site for more-detailed studies of the molecular mechanisms of synergism between CCAC and A/T elements in the myoglobin promoter.

The apparent similarity between the myoglobin A/T element and previously defined binding sites in other genes for members of the MEF-2/RSRF family suggested that one or more of these proteins may represent the biologically relevant ligand for this site. Our current results indicate that the myoglobin A/T element is capable of binding MEF-2 proteins, but this nonconsensus site binds MEF-2 with lower affinity than do consensus sites present in other genes. The myoglobin A/T box, however, also binds *in vitro* to a different protein, termed ATF35, that is distinguishable from known MEF-2 isoforms on the basis of electrophoretic mobility, binding specificity, molecular weight, and recognition by antibodies against MEF-2 protein. Furthermore, modification of the myoglobin A/T sequence to perfect identity with each of two consensus MEF-2/RSRF-binding sites reduced transcriptional activity of the myoglobin promoter, providing additional evidence that a factor distinct from MEF-2 may function in transactivation of this gene *in vivo*. We interpret these data to indicate that ATF35 is distinct from previously defined MEF2 proteins, and current efforts in our laboratory are directed at purification and cloning of this apparently unknown factor. Additional cross-competition binding experiments *in vitro* (data not shown) also indicate nonidentity of this factor with Mhox, another myocyte nuclear protein that binds an A/T-rich sequence motif (14) with similarity to the myoglobin A/T box.

The diminished expression of the A/T mut1 and A/T mut2 constructs relative to the native myoglobin promoter sequence can be interpreted to suggest that a stoichiometric relationship between binding of MEF-2 and ATF35 may be necessary for optimal transactivation. Increasing affinity of the myoglobin A/T element for binding MEF-2, as found in A/T mut1, not only fails to augment promoter strength but also decreases transcriptional activity, perhaps by perturbing the optimal stoichiometry of MEF-2 and ATF35 binding. Decreasing the binding affinity for ATF35, as a result of the sequence variation present in A/T mut2, also compromised promoter function, even though MEF-2 binding *in vitro* was not altered. These data suggest that MEF-2 and ATF35 may bind adjacent or overlapping sites. Perhaps they interact in a manner analogous to the recent description by Chen and Schwartz of interactions between the homeodomain protein Nkx 2.5 and serum response factor (a MADS box protein like MEF-2) in transactivation of the cardiac α -actin promoter (10).

As additional confirmation that synergy between the CCAC and A/T elements observed in synthetic enhancer constructions is relevant to the function of the native promoter, we observed that the enhancing activity of these upstream elements was sensitive to subtle mutations in the TATA sequence in a manner similar to that for the complete promoter (42). We have confirmed the previous observation of Leibham et al. (30) that MEF-2 binds with differential affinities to TATA sequence variants, but the relevance of this binding to the preferential requirement of myoglobin upstream activation elements for specific TATA sequences is uncertain.

Synergism among heterologous transcription factors is emerging as a common theme in gene regulation (11, 17, 20, 24, 38). The molecular mechanisms of synergistic interactions may include positive cooperativity of DNA binding, exponential effects of presenting multiple transactivation domains to the core promoter complex, formation of higher-order heterooligomers that generate unique surfaces required for protein-protein interactions with additional transcription factors, and topological effects on chromatin structure. However, detailed

examination of synergistic mechanisms in mammalian cell systems has been relatively limited. Our observation that two heterologous activation elements function synergistically to confer cell type specificity to a minimal promoter provides an advantageous experimental system for future studies to elucidate the molecular basis of this interaction. Our ability to reconstitute TATA sequence specificity in a simplified synthetic construction also may provide a tractable system in which to explore molecular mechanisms by which enhancer-binding proteins communicate with components of the basal transcriptional apparatus.

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