MEF-2 and Oct-1 Bind to Two Homologous Promoter Sequence Elements and Participate in the Expression of a Skeletal Muscle-specific Gene*

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The murine adult IIB myosin heavy chain (IIB MyHC) gene is expressed only in certain skeletal muscle fibers. Within the proximal promoter are two A + T-rich motifs, mAT1 and mAT2, which greatly enhance muscle-specific transcription; myogenic cells contain proteins that bind to these sequences. MEF-2 binds to both mAT1 and mAT2; a mutation abolishing its binding to mAT1 greatly diminishes the activity of the promoter. Both mAT motifs also form complexes with a protein requiring a target sequence typical of POU domain proteins, which migrate in electrophoretic mobility shift assays to the same position as a complex containing purified Oct-1 and which are supershifted by an antibody specific to Oct-1; this protein is therefore probably Oct-1. Footprinting experiments demonstrate that mAT1 is preferentially occupied by MEF-2 and mAT2 by Oct-1 and that these two proteins appear to bind cooperatively to their respective sites. Although the two mAT motifs have sequences that are very similar, they nonetheless exhibit distinct behaviors and perform differently in the activation of the promoter. The contribution of the IIB MyHC gene to specification of the myogenic phenotype is thus at least in part regulated by MEF-2 and Oct-1.

Skeletal muscle represents an excellent model to examine tissue-specific controls on transcription, since most of the major muscle structural proteins have several isoforms that often have characteristic spatiotemporal patterns of expression (reviewed in Ref. 1). The mouse adult IIB myosin heavy chain (IIB MyHC) gene, for example, is expressed only at a mature stage of development and only in the fast-twitch glycolytic fibers of differentiated skeletal muscle (2–4). Transcriptional control is thought to be a primary level of regulation for many of the genes coding for these different isoforms (5–7). Among the transcription factors important for muscle gene expression are the myogenic basic helix-loop-helix (bHLH) regulatory factors (myf-5, MyoD, myogenin, and MRF4), which are able to induce muscle differentiation in non-myogenic cells (reviewed in Ref. 8). The MEF-2 proteins (summarized in Ref. 9) are also often required for the transcription of muscle genes. Many genes require interactions among several promoter-bound proteins, both tissue-specific and non-tissue-specific, to be expressed in a tissue-specific manner. We have previously characterized numerous potential binding sites for known transcriptional activators within the 5'-flanking region of the IIB MyHC gene (10–12). In particular, two regions rich in the nucleotides A and T, situated between −140 and −190 bp, enhance the transcriptional activity of those constructions that contain them. This activation is found only in differentiated myotubes and not in undifferentiated myoblasts, implying that these sites contribute to the restriction of the expression of the IIB MyHC gene to mature muscle tissue.

There are numerous DNA-binding proteins that recognize and bind to sequences that are predominantly composed of A and T nucleotides. Among these are the ubiquitous SRF (13) and the “related-to-SRF” proteins that share with SRF the existence of an amino-terminal MADS domain (13, 14); the nuclear protein MEF-2 belongs to the related-to-SRF family. There are at least four different mef2 genes, each of which may be alternatively spliced to produce several isoforms of the MEF-2 protein, some of which are selectively expressed in muscle and brain (9, 15–17). Proteins containing a homeodomain also generally have at least a short AT-rich motif (often TAAT) as part of their target sequence (reviewed in Ref. 18); among the homeodomain proteins are the POU domain proteins, such as Oct-1 and Pit-1 (19).

The sequences of the two related AT-rich motifs found in the proximal region of the IIB MyHC are conserved among numerous distinct skeletal muscle MyHC genes of several vertebrate species (11), suggesting an important role for these “mAT” (myosin AT-rich) sites in the regulation of this family of genes. This observation led us to examine in detail the interactions among the two mAT sites and the DNA-binding proteins that presumably associate with them in vivo to effect transcriptional activation. In this study we demonstrate that Oct-1, bound to mAT2, and a MEF-2 protein, bound to the more downstream mAT1, are both involved in the regulation of the activity of the promoter. Muscle-specific transcription of this gene may thus be achieved by combinations of widely and narrowly expressed factors, and the interactions among them

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The abbreviations used are: IIB MyHC, mouse adult IIB myosin heavy chain; EMSA, electrophoretic mobility shift assay; bHLH, basic helix-loop-helix; WT, wild type; mut., mutant; CAT, chloramphenicol acetyltransferase; MADS, MCM1-agamous-deficiens-serum response factor family of DNA-binding proteins.
appear to be important for the activation of the IIB MyHC gene.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—All the deletion constructions of the 5'-flanking sequence of the murine IIB MyHC gene (inserted into the pCAT-Basic vector) used in this study were based on those described by Takeda et al. (22). The constructions mutated in the mAT and palindrome regions were prepared by polymerase chain reaction, and the sequence of the mutations was verified experimentally.

**Transfections and CAT Assays**—Murine C2C12 myoblasts (20, 21) were maintained in Dulbecco's modified Eagle's medium with 20% fetal calf serum at 37 °C in an atmosphere of 7% CO₂. One day before transfection, 4 × 10⁶ cells were plated in 25 cm² tissue culture flasks; the next day, transfection was performed with 2 μg of the deletion construction in the presence of the cationic lipid dioctadecylamidoglycyl spermine (22), as described previously (12). The activity of the CAT reporter gene in the cellular extract was measured according to the method of Seed and Sheen (23).

**Nuclear Extract Preparation**—To prepare extracts of nuclear proteins from cultured cells, we used a method based on that of Dignam et al. (24). The method used for the preparation of nuclear proteins from hindlimb muscle of rats (1 week to 10 days old) or mice (2 weeks old) was that of Mar and Ordahl (25).

**DNA Binding Assays**—Segments of DNA (100–400 bp) used as probes for DNase I footprinting assays were isolated from plasmids containing the promoter region of the IIB MyHC gene (12). The probes were labeled with [³²P]dXTP and the Klenow fragment of DNA polymerase I and purified on a 3.5–5% polyacrylamide gel. Oligonucleotides used as probes were gel-purified and labeled with T4 polynucleotide kinase and 1 μM [γ-³²P]ATP. The sequences of the upper strand of the double-stranded oligonucleotides used as probes and competitors are given in Table I.

The electrophoretic mobility shift assay (EMSA) binding reactions for the proteins from the nuclear extracts, or for Oct-1, that bind to the mAT sites were carried out under the following conditions. The nuclear extracts (0.4 to 10 μg of total protein, determined by a Bio-Rad protein assay) were incubated at 4 °C for 30 min with poly(dI-dC)·poly(dI-dC) (1 to 2 μg) in 33 mM KCl, 20 mM HEPES, 1 mM EDTA, 7% glycerol, 1 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, and 1 mM Na₂HPO₄. The competitors (at 50 mol per mol of probe) and the probe (1 ng) were added, and the reaction was continued at room temperature for 20 min before being loaded onto a 6% non-denaturing acrylamide gel in 0.25 × TBE (except for the gel in Fig. 2A, which was 4.5% acrylamide in 0.5 × TBE and run in 1 × TBE). In experiments designed to produce a supershift of the Oct-1 probe complex using an antibody specific for Oct-1, the antibody was added to the binding reactions which were then further incubated for 1 h before being loaded onto the gel. The proportion of the probe that was free of bound proteins was always in great excess.

For certain gels, the radioactive signals were quantified with a PhosphorImager apparatus (Molecular Dynamics) and ImageQuant software. These values are those that are used in the Fig. 2C and Table II. Even though each experiment was carried out several times, in general only one or two of the representative gels were analyzed via the PhosphorImager, so that readers could more easily see the results instead of only one or two of the representative gels were analyzed via the PhosphorImager apparatus (Molecular Dynamics) and ImageQuant software. For certain gels, the radioactive signals were quantified with a PhosphorImager apparatus (Molecular Dynamics) and ImageQuant software. These values are those that are used in the Fig. 2C and Table II. Even though each experiment was carried out several times, in general only one or two of the representative gels were analyzed via the PhosphorImager, so that readers could more easily see the results instead of only one or two of the representative gels were analyzed via the PhosphorImager, so that readers could more easily see the results instead of only one or two of the representative gels were analyzed.

**RESULTS**

**Nuclear Proteins from Myogenic Cells Bind the mAT1 and mAT2 Elements in Vitro and Are Not All Present at the Same Levels in Different Cell Types**—To identify nuclear proteins that bind specifically to the mAT1 and mAT2 sequence motifs of the IIB MyHC promoter, we performed electrophoretic mobility shift assays (EMSA) with oligonucleotide probes containing the sequences of these sites (refer to Table I for the sequences of all oligonucleotides used). By using nuclear extracts from myotubes of the murine skeletal muscle C2 cell line, we observed the formation of three main complexes with a probe (entire mAT1) containing the 23-bp mAT1 site (Fig. 1A, lane 3), and two main complexes with a probe (mAT2) containing the 17-bp mAT2 site (Fig. 1A, lane 4). Further experiments suggested that the binding of these proteins is mutually exclusive.

Complexes 1 and 3 were formed with nuclear extracts from several types of differentiated myogenic cells (C2 (murine) and quail skeletal muscle myotubes, and adult mouse hindlimb muscle) but were either very weak or undetectable with extracts from undifferentiated myogenic cells or precursors (C2 and quail myoblasts, 10T1/2 fibroblasts) (Fig. 1A and B). The mobilities of the complexes formed with the mouse and the quail extracts were similar, implying that the mouse and the quail proteins involved were approximately the same size. The complex 1 produced with quail myotube extracts was relatively less intense than that produced with mouse myotube extracts, with respect to the total quantity of nuclear proteins. Since complex 3 was formed by the same nuclear protein as is the larger complex 1, there was a possibility that the protein producing it was merely a degradation product of the complex 1 protein. However, when C2 myotube nuclear extracts were heated to 25 °C for 6 min and then 37 °C for 10 min, there was no change in the intensity of either of the complexes (data not shown).

Complex 2 was seen in EMSAs carried out with all cell types examined as follows: 10T1/2 fibroblasts, C2 myoblasts and myotubes, quail myoblasts and myotubes, and mouse hindlimb muscle (Fig. 1A and B). C2 myoblasts had approximately twice as much of the complex 2 protein as did C2 myotubes and three times as much as 10T1/2 fibroblasts (determined by comparing the intensities of the complex 2 bands with a PhosphorImager and normalizing to the amount of total nuclear protein loaded in each lane). Quail cells appeared to have less of this protein than did C2 cells.

In addition to these three complexes seen with differentiated muscle tissue, there were two other bands produced in EMSAs with a mAT1 probe, which migrated immediately ahead of complex 3. These complexes, 4a and 4b, were prominent in EMSAs with 10T1/2 fibroblasts, less prominent in myoblasts, and practically non-existent in myotubes (Fig. 1A). The pattern of their appearance over development was thus roughly the opposite of that of complex 1.

**The Protein That Forms Complex 1 Binds to MEF-2-like Recognition Sequences within the mAT1 and mAT2 Sites**—A sequence with homology to the consensus binding site for the MEF-2 protein ((C/T)(A/G)ATT(A/T)AAATA(A/G); see Ref. 27) is found within both of the mAT sites (mAT1 = ATTCTTAAT-TATATCCATTCA; mAT2 = TGTCCAATTATTTATAG). The mobility in an EMSA of the complex 1 formed with an oligonucleotide probe of either the mAT1 or the mAT2 sites was identical to that of a complex formed with an oligonucleotide probe containing the MEF-2-binding site from the mouse mck enhancer (Fig. 2A). In addition, the formation of the complex with the mAT1 or mAT2 probes could be inhibited by an excess of the mck oligonucleotide, although not by an excess of the mAT1 oligonucleotide mutated to prevent binding of MEF-2 (mutant 2 mAT1; Fig. 2B, lanes 4 versus 3). The protein had approximately the same affinity for the mck MEF-2 and IIB MyHC mAT1 sites but a slightly reduced affinity for the IIB MyHC mAT2 site (Fig. 2C). From EMSAs in which a series of competing oligonucleotides was used, it was evident that the
protein that forms complex 1 bound to the CTAAATTATAT sequence within the mAT1 site and the CTATAAATAA sequence (lower strand) within the mAT2 site (Figs. 2B and 3A, and additional data), both of which fit the established MEF-2 consensus sequence. Based on its recognition sequence and tissue distribution, the protein that bound to the mAT1 and mAT2 sites to produce the abundant complex 1 therefore appears to be MEF-2.

**TABLE I**

| Oligonucleotide probes and competitors | Sequence, 5'-3' | Position |
|---------------------------------------|----------------|----------|
| Original mAT1                         | AGCTTATGACAAAGAATTTTCTAATTATATCCATTC | -172/-138 |
| mAT1 downstream half                  | TATTTCTAATTATATCCATTC | -161/-140 |
| mAT1 downstream half mutant 1         | TATTTCTAATTATATCCATTC | -161/-140 |
| mAT1 upstream half                    | AGTACAAGAAATTATCTATAT | -172/-152 |
| Entire mAT1                           | GACAGAAATTATCTAATTATATCCATTC | -170/-138 |
| Mutant 1 mAT1                         | GACAGAAATTATCTAATTATATCCATTC | -170/-138 |
| Mutant 2 mAT1                         | GACAGAAATTATCTAATTATATCCATTC | -170/-138 |
| Mutant 3 mAT1                         | GACAGAAATTATCTAATTATATCCATTC | -170/-138 |
| Mutant 4 mAT1                         | GACAGAAATTATCTAATTATATCCATTC | -170/-138 |
| Embryonic rat mAT1                    | CTGCCATTTATATATCTAATTATTCCTTG | -167/-139 |
| Embryonic rat mAT2                    | ATACCCCATTTATATATCTAATTATTC | -417/-383 |
| mAT2                                  | CCCCCTGCAATTTATATCTAATTATTC | -192/-167 |
| mAT2 MEF-2 mutant                     | CCCCCTGCAATTTATATCTAATTATTC | -192/-167 |
| mAT2 Oct-1 mutant                     | CCCCCTGCAATTTATATCTAATTATTC | -192/-167 |
| mAT2 double mutant                    | CCCCCTGCAATTTATATCTAATTATTC | -192/-167 |
| mAT2 → palindrome                     | CCCCCTGCAATTTATATCTAATTATTC | -192/-167 |
| Palindrome                            | GACAGAAATTATCTAATTATTC | -170/-152 |
| mAT3 palindrome                       | GACAGAAATTATCTAATTATTC | -170/-152 |
| Palindrome G → T                      | GACAGAAATTATCTAATTATTC | -170/-152 |
| Interrupted palindrome                | GACAGAAATTATCTAATTATTC | -170/-152 |
| 1/2 palindrome                        | GACAGAAATTATCTAATTATTC | -170/-152 |
| Distal A/T 1                          | TGCAATTTTTAAAGATAGATAGATTATTTACC | -1121/-1091 |
| Distal A/T 2                          | TGCAATTTTTAAAGATAGATAGATTATTTACC | -1121/-1091 |
| pMyoD                                 | AAGTAGGTCCGCAGCTAGTCGCTGCTGCTGTGT | -586/-46 |
| dMyoD                                 | TCTATCTCAGCAACAGTCTGTCTGCTATAGAC | -960/-929 |
| mck MEF-2 control                     | GCTGCCCTAATTTATATCTAATTATTC | -1083/-1055 |
| mck MHox control                      | GCTGCCCTAATTTATATCTAATTATTC | -1083/-1055 |
| MHox control, modified                | GCTGCCCTAATTTATATCTAATTATTC | -1083/-1055 |
| Heptamer control                      | CGATCTGCTGCTGATACATAC | IgH promoter |
| Pit control 1                         | CCTTATTATATATATATCTAATTATTCAGA | Rat prl, -65/-42 |
| Pit control 2                         | CCTTATTATATATATATCTAATTATTCAGA | Rat prl, -65/-42 |
| Oct control 1                         | AGCAAAACACCACCCTGGGTAATTTC | IgH enhancer |
| Oct control 2                         | AGCAAAACACCACCCTGGGTAATTTC | IgH enhancer |

**Fig. 1.** EMSAs show that the relative amounts of the four major complexes formed between nuclear extracts and the entire mAT1 oligonucleotide probe (−170/−138 bp) change over the course of myogenic differentiation and that only two complexes are formed between myocyte nuclear extracts and a mAT2 oligonucleotide probe. The proteins forming complexes 1 and 3 are present only in differentiated myogenic cells, that forming complex 2 is present at all stages, and those forming complexes 4a and 4b disappear with differentiation. The total amount (m) of nuclear proteins loaded per lane is indicated in parentheses. All gels were exposed for 4 h except for lane B5. A, lane 1, 10T1/2 fibroblasts (Fb) (6.6); lane 2, C2 myoblasts (Mb) (2.0); lane 3, C2 myotubes (Mt) (4.5); and lane 4, C2 myotubes (Mt) but with the mAT2 oligonucleotide probe (−192/−167 bp). B, lane 1, quail myoblasts (Qb) (8.1); lane 2, quail myotubes (Qt) (9.5); lane 3, C2 myotubes (4.5); lane 4, mouse (M) leg muscle (4.1, 65 h exposure); lane 5, mouse leg muscle (4.1).
FIG. 2. The protein in C2 myotube extracts (9 μg of total nuclear protein in A, 4.5 μg in B) forming complex 1 is MEF-2. A. EMSAs show that the complex 1 band migrates to the same position with oligonucleotide probes from the mck enhancer MEF-2 site (lane 1), the mAT2 site (−187 to −167 bp; short mAT2) (lane 2), and the mAT1 site (−172 to −138 bp; original mAT1) (lane 3). (The low percentage of acrylamide of this gel does not permit the distinction of complexes 1 and 2.) B, complex 1 cannot form when the potential MEF-2-binding site within mAT1 is mutated to prevent binding of MEF-2. Lane 1, no competitor (no comp) added to the binding reaction; lane 2, a nonspecific competitor (non-spec) (the pMyoD oligonucleotide); lane 3, the mutant 2 mAT1 competitor, unable to bind MEF-2; and lane 5, the mck enhancer MEF-2 site competitor. C, the affinity of MEF-2 for the mAT1 site is slightly less than for the mck enhancer MEF-2 site and slightly greater than for the mAT2 site. Various oligonucleotide competitors were used in EMSAs at different molar ratios of competitor to probe, and the intensities of the bands were measured using a PhosphorImager.

The Protein That Forms Complex 2 Requires a Target Sequence Containing a ATAAAT, a TAAT/T(A) or an ATGAAAA Motif—Complex 2, whose presence was independent of the state of myogenic differentiation of the cells, migrated immediately ahead of complex 1 in EMSAs; indeed, the two could only be resolved on gels containing an elevated concentration of acrylamide and run for a long period. For the purposes of this study, we designated the protein that formed complex 2 as “2BB2,” since in an EMSA with probes of the IIB (2B) MyoHC promoter it formed the second band (B2). Based on competition EMSAs, 2BB2 appeared to require a typical homeodomain protein TAAT target sequence (18, 28), TAAT or TAAT(T/A), in its binding site (Figs. 2B [compare lanes 3 and 4] and 34 and Table II). In addition, the formation of the 2BB2-mAT1 complex was also inhibited by a second group of oligonucleotide competitors whose sequences do not conform to a homeodomain consensus binding sequence but instead have in common an ATTGAAAA motif (two Pit-1 binding sequences (29), two octamer binding sequences (30, 31), and the heptamer sequence (32)). This motif is homologous to part of the consensus binding sequence for the POU domain transcription factor Pit-1, AGT-NATA(T/A) (29, 33).

These results therefore suggested that 2BB2 could be related to the POU family of transcription factors, which have two DNA-binding structures, a POU homeodomain and a POU-specific domain (reviewed in Ref. 19). Both domains of the POU domain protein Oct-1 are required for DNA binding to the ATGAAAAAT octamer motif, with the POU-specific domain recognizing the 5' end of the motif, and the homeodomain the more A/T-rich 3' end (34). Thus, 2BB2, which could bind to two different classes of sequences, may belong to the POU family of DNA-binding proteins. Although 2BB2 recognized sequences similar to the consensus binding sequence of MHox (TA/ T)AAAT(T/A)A, a widely distributed homeodomain protein which can bind to the mck enhancer (35, 36), the pattern of migration of MHox in published EMSAs with respect to MEF-2 does not resemble that of 2BB2.

2BB2 Is Oct-1 or an Oct-1-related Protein—Since 2BB2 is widely expressed, and since it has a binding specificity which resembles that of a POU domain factor, we considered that it could be the DNA-binding POU domain protein Oct-1, which is expressed in many types of mammalian cells (19, 37). When an EMSA was performed with nuclear extracts from C2 myoblasts or myotubes and an oligonucleotide probe of the mAT2 site (mAT2 mutant MEF-2, mutated to selectively prevent binding of MEF-2 and thus render the 2BB2 band easier to distinguish), the 2BB2 complex could be supershifted by an antibody that specifically recognizes Oct-1 (Fig. 3B, lanes 1–4). This same complex was formed with a nuclear protein found in CV-1 cells (lanes 5 and 6). In addition, when the same mAT2 probe was used in an EMSA with purified Oct-1 protein, the band produced migrated to the same position as the 2BB2 band (lanes 7 and 8). We believe that the prominent, rapidly migrating complex seen in the Oct-1 lanes was due to a proteolytic

| Competitor | % nonspecific competitor signal remaining at 50 mol competitor: 1 mol probe |
|------------|--------------------------------------------------------------------------------|
| Oct control 1 | 0 |   |
| mAT1 | 4 | 4 |
| mAT2 | 5 | 10 |
| Oct control 2 | 8 | 8 |
| Pit control 2 | 10 | 12 |
| Heptamer control | 12 | 16 |
| Rat embryonic mAT2 | 19 | 36 |
| mAT1 mutant 3 | 36 | 36 |
| mAT2 | 37 | 37 |
| Distal A/T 1 | 60 | 60 |
| Distal A/T 2 | 80 | 80 |
| Rat embryonic mAT1 | 83 | 83 |
| pMyoD | 93 | 93 |
| Inverted palindrome | 100 | 100 |
| MCK MEF2 control | 142 | 162, 181 |
| mAT3 palindrome | 162 | 181 |
| Palindrome | 181 | 181 |

*The percent of the complex in an EMSA remaining in the presence of 50 mol of competitor per 1 mol of probe, normalized to the percent of signal remaining in the presence of 50 mol of nonspecific competitor (indicated in the two columns) per 1 mol of probe.*
mAT1 Is Preferentially Occupied by MEF-2 and mAT2 by Oct-1 in Differentiated Myogenic Cells—Proteins present in nuclear extracts from both myoblasts and myotubes (C2 and quail) produced a similar footprint over the mAT1–mAT2 region (Fig. 5A, lanes 1 versus 2). Oct-1 in myogenic differentiation. This footprint was abolished by competition with an oligonucleotide (entire mAT1) containing the mAT1 site mutated to prevent binding of MEF-2 but not Oct-1 (Fig. 4B). The protein or proteins that formed these complexes therefore probably required a TAAT motif.

These complexes migrated in an EMSA to approximately the same position as does the MHox complex, with respect to the MEF-2 complex (35), and the proteins required target sequences similar to the consensus binding sequence of MHox. However, the amount of the proteins forming complexes 4a and 4b (as detected by EMSAs) declined drastically as myoblasts differentiated into myotubes (Fig. 4C), whereas that of MHox (as inferred from its mRNA detected by Northern blots), in contrast, declines only very slightly, if at all, during this transition (35).

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Since MEF-2 and Oct-1 can bind to both mAT sites, it was of interest to investigate whether each mAT site is preferentially occupied by one or the other factor. Off-rate EMSAs carried out to determine the relative dissociation rates of MEF-2, Oct-1, and the Pal-BP from the mAT1 site demonstrated that the binding of MEF-2 was the most stable of the three (data not shown). In order to determine which proteins are responsible for particular regions of the footprint, we carried out DNase I footprinting experiments using numerous competing oligonucleotides containing binding sites for different proteins (as previously determined from the results of EMSAs). From Fig. 5A, it is clear that the mAT1 region of the footprint was more effectively abolished by competition with an oligonucleotide that could bind MEF-2 (mck MEF-2 control (lanes 5 and 6) or 

2 M. Salminen, personal communication.
FIG. 4. A, the protein responsible for complex 3 binds to the palindromic sequence AGAAATTTTCT located between mAT1 and mAT2.

EMSA using C2 myoblast extracts (4.5 μg of total nuclear protein) and the entire mAT1 oligonucleotide probe (170/138 bp). Lane 1, no competition (no comp.); lanes 2–9, with oligonucleotide competitors: lane 2, entire mAT1; lane 3, the upstream half of mAT1, including the entire palindromic sequence; lane 4, palindromic half (1/2 pal.); lane 6, interrupted palindromic (int. pal.), with an extra T inserted in the middle; lane 7, palindrome G → T (pal. G-T), with the G mutated to a T; lane 8, mAT3 palindrome (mAT3 pal.) (which has a palindromic motif with the 5’ and 3’ halves inverted; lane 9, mutant 1 mAT1 (mut. mAT1). B, the protein(s) responsible for complexes 4a and 4b disappear during the course of myogenic differentiation and require an A/T-rich target sequence. The total amount (μg) of nuclear proteins loaded per lane is indicated in parentheses. Lanes 1–4, 10T1/2 fibroblasts (Fb) (6.6); lanes 5–8, C2 myoblasts (Mb) (2); lanes 9–12, C2 myotubes (Mt) (4.5). Lanes 1, 5, and 9, no competitors; lanes 2, 6, and 10, MHox control oligonucleotide competitor; lanes 3, 7, and 11, mutant 1 mAT1 oligonucleotide competitor; lanes 4, 8, and 12, E box oligonucleotide competitor (pMyoD). All competitors present at 50:1 mol/mol with respect to the probe. C, quantitative presentation of the disappearance of complexes 4a and 4b, based on EMSA data. Fb, 10T1/2 fibroblasts; Mb, C2 myoblasts; Mt, C2 myotubes; Qb, quail myoblasts; and Qt, quail myotubes. The intensity of the bands produced with the C2 myoblast and myotube extracts (corrected for total nuclear protein loaded per lane) are normalized to that of complex 4b produced with the fibroblast extract, whereas the intensity of the bands produced with the quail myoblast extract is normalized independently to that of complex 4a produced with the quail myoblast extract.

mAT1 downstream half (lane 9) than by one that could bind only Oct-1 (mutant 1 mAT1, lanes 3, 4, and 10). This suggested that the mAT1 site was primarily occupied by MEF-2. The converse, that the oligonucleotide which could bind Oct-1 preferentially abolished the mAT2 footprint, was also true (Fig. 5A, compare the mAT2 region of lanes 4 versus 6 and lanes 9 versus 10; note in lanes 4 and 10 that the mAT2 region bands are stronger relative to the mAT1 region bands with respect to lanes 6 and 9). This implied that the mAT2 site appeared to be occupied by Oct-1, rather than by MEF-2. The footprint attributable to the palindromic-binding protein was limited to a single A (indicated by a dot between lanes 11 and 12).

FIG. 5. DNase I footprints created by C2 myoblast nuclear extracts (20 μg of total nuclear protein) over the mAT1 and mAT2 sites on a probe of the MyoHC IIB promoter region (29S/13 bp) coding strand. A, lanes 1 and 2 the probe without proteins; lanes 2 and 11, no competitors; lanes 3–10, with competitors; lanes 3, 4, and 10, mutant 1 mAT1; lanes 5 and 6, mck MEF-2 control; lanes 7 and 8, palindrome; lane 9, mAT1 downstream half, lane 13, G + A chemical sequence; lane 14, T + C chemical sequence. The molar ratio of competitor to probe is 50:1 for lanes 3, 5, and 7 and 500:1 for lanes 4, 6, and 8–10. The A nucleotide protected by the palindromic-binding protein is indicated by the dot next to lane 8. B, lanes 1 and 14, T + C chemical sequence; lanes 2 and 13, the probe without proteins; lanes 3–12, the probe with proteins; lanes 3 and 12, no competitor; lanes 4–11, with oligonucleotide competitors; lanes 4 and 5, entire mAT1; lanes 6 and 7, mutant 1 mAT1; lanes 8 and 9, mAT1 downstream half; and lanes 10 and 11, mAT1 downstream half mutant 1. The molar ratio of competitor to probe is 38:1 for lanes 4, 6, and 8, 380:1 for lanes 5, 7, 9, and 11. The A nucleotide protected by the palindromic-binding protein is indicated by the dot between lanes 11 and 12.

These experiments provided evidence that MEF-2 (at the mAT1 site) and Oct-1 (at the mAT2 site) could bind cooperatively to the proximal promoter sequences. When the mutant 1
mAT1 oligonucleotide was used as a competitor in DNase footprinting experiments, it abolished both the mAT2 (Oct-1) and the mAT1 (MEF-2) regions of the footprint, even though it could not bind MEF-2 (e.g. Fig. 5B, lane 7 (mutant) versus lane 12 (no competitor)). However, the mAT1 footprint was abolished at a much higher concentration of the competing oligonucleotides than that necessary to abolish the mAT2 footprint (Fig. 5B, compare the lanes using the mutated competitors (lanes 6 and 7 and lanes 10 and 11) with those using the equivalent wild type competitors (lanes 4 and 5 and lanes 8 and 9)). Thus it seems that after removal of Oct-1 from mAT2, MEF-2 was no longer able to bind as well. As demonstrated above, the binding of Oct-1 was also somewhat dependent on that of MEF-2, since an oligonucleotide specific for MEF-2 led to some restoration of the bands to the footprint over the mAT2 site.

Transcriptional Activity of the IIB MyHC Promoter Requires MEF-2 Binding to mAT1 and Oct-1 or MEF-2 Binding to mAT2—We have previously demonstrated that the presence of the mAT1 site or the mAT1 plus mAT2 sites leads to an increased level of transcriptional activation when included in reporter gene plasmid constructions used for transient transfection assays in quail myotubes (12) and C2 myotubes (10). (Note that in order for the IIB MyHC promoter to be active in C2 myogenic cells, co-transfection with an expression vector for one of the myogenic bHLH factors is necessary; this system has been analyzed and validated in our laboratory (10, 39).) The 192-bp construct (−192 bp/+13 bp of the IIB MyHC promoter immediately upstream of the CAT reporter gene), containing both mAT sites, is the most active of all the promoter constructs, even including those which are much larger (e.g. to −2330 bp).

A 192-bp construction containing a mutation in the mAT1 site which abolished MEF-2 binding (as established by EMSAs; mutant 1 mAT1) was much less active than the non-mutated construction in transient transfection experiments (Fig. 6A, the mAT1 MEF2 mut. lanes). This mutant construct produced a CAT activity approximately equal to that of the construction containing only the first 40 bp of the promoter, which we consider the basal promoter (not shown). Therefore, MEF-2 most probably does bind to the mAT1 site in vivo and is involved in the transactivation relay which is dependent on this site.

A different mutation was introduced into the mAT1 site of a 192-bp transfection construct that abolished the binding of Oct-1 yet still permitted the binding of MEF-2. The resulting CAT activity was equivalent to or slightly higher than that of the wild type construct (Fig. 6A, the mAT1 Oct-1 mutant lanes). This implied that Oct-1 does not bind to the mAT1 site in vivo, consistent with the suggestion provided by the DNase I footprinting experiments that mAT1 is occupied by MEF-2. Note that mutating the palindromic-binding protein target site had no effect on the activity of the promoter as determined in these transient transfection assays (Fig. 6A, the mAT1 pal. mut. lanes).

A mutation in the mAT2 site that eliminated the binding of MEF-2 yet did not affect the binding of Oct-1 (based on EMSA results) did not decrease the activity of the 192-bp construction in transfection experiments (Fig. 6B, the mAT2 MEF2 mut. lanes). Furthermore, the mutation in the mAT2 site which totally abolished Oct-1 binding (but increased that of MEF-2) also did not decrease and actually increased the transcriptional activity of the 192-bp construction (Fig. 6B, the mAT2 Oct-1 mutant lanes). Only a double mutation in the mAT2 site, eliminating the binding of MEF-2 and reducing by one-half that of Oct-1, decreased the activity of the 192-bp promoter construct (containing mAT1 and mAT2) to approximately that of the 159-bp construct which contains only mAT1. Thus it is only when neither protein is able to bind to the mAT2 site, and therefore cooperative binding of MEF-2 to the mAT1 site is lost, that the presence of the mAT2 site becomes irrelevant. This suggests that for the promoter to be active in the C2 myotube culture system the mAT2 site must be occupied by either Oct-1 or MEF-2.

To analyze further the role of the mAT1 and mAT2 elements, we created chimeric promoter constructions that contained either the mAT1 (−166/−136) or the mAT2 (−193/−168) region directly in front of the basal IIB MyHC promoter (−40/+13). Consistent with the above results, the mAT1–40IB construction is active only in myotubes (although relatively inactive in the absence of an excess of exogenous myogenic bHLH protein), whereas the mAT2–40IB construction is active in many types of cells (CV1, fibroblasts, undifferentiated myoblasts, differentiated myotubes), paralleling the wide distribution of the Oct-1 protein (data not shown).

Further evidence that MEF-2 binds to mAT1 and participates in the activation of the IIB MyHC promoter came from co-transfection experiments with different promoter constructions and expression vectors for MEF-2C and myogenin. MEF-2C alone activates both the 192 WT and the mAT1–40IB constructions, whereas the mutation that prevents MEF-2 from binding to the mAT1 motif completely prohibits this activation (Fig. 6C). Furthermore, for the 192 WT construction, there was a clear synergy in the activation due to MEF-2C and myogenin.

When isolated from the rest of the promoter in the mAT2-IIB construction, the mAT2 element clearly activates transcription through a bound Oct-1-related protein. A mutation in the mAT2 site that abolishes the binding of Oct-1 (mAT2–40 Oct-1 mut.) abolishes activity of the promoter in CV1 cells and also significantly reduces the activity in myoblasts and myotubes (to about 33% of the wild type activity) (Fig. 6D). When MEF-2 is in turn prohibited from binding by a mutation in mAT2 which also halves the affinity of Oct-1 for mAT2 (mAT2–40 double mutant), the chimeric construction is less active than is the wild type in CV1 cells (40% wild type activity) and in myoblasts and myotubes (75%).

DISCUSSION

Both a Muscle-specific and a Ubiquitous Factor Are Involved in the Muscle-specific Expression of the IIB MyHC Gene—Combinations of tissue-restricted and widely expressed factors are not infrequently used to mediate muscle-specific expression (e.g. Refs. 35, 40, and 41–46). The muscle-specific expression of the IIB MyHC gene studied here is at least partially controlled by the factors MEF-2 and Oct-1 binding to the mAT1 and mAT2 motifs. The MEF-2 isoform that activates the IIB MyHC promoter is restricted to differentiated muscle cells (this study and Ref. 12), whereas Oct-1 is present in a wide variety of tissues (19, 37). These two proteins contain distinct DNA-binding domains: MEF-2 (a MADS box) and Oct-1 (a POU domain, which itself contains both a homeodomain and a POU-specific domain). There are several known MADS domain-homeodomain protein pairs that are important for transcriptional activation. Unlike the MCM1/MATa2 (47) and SRF/Phox1 (48) pairs, where the homeodomain partner contributes the tissue specificity, in the MEF-2/Oct-1 pair on the IIB MyHC promoter it is rather the MADS domain partner that is specific to muscle.

MEF-2C Activates the IIB MyHC Promoter through mAT1—The four vertebrate myf2 genes give rise via alternative splicing to numerous MEF-2 proteins (15–17). MEF-2A (9) and MEF-2C (15, 16) are specific to differentiated muscle tissue and to nerve cells. MEF-2D is present at the myoblast stage, and MEF-2A appears almost immediately after myoblasts are
FIG. 6. CAT assays of transiently transfected C2 myotubes demonstrate that within mAT1 the MEF-2-binding site is required and that within mAT2 a functional site for either MEF-2 or Oct-1 is required. Legends indicate the expression vector co-transfected with the IIB MyHC deletion construction. No DNA, no expression vector; pEM, the pEMSVscribe expression vector; pEM-myf5, the pEMSVscribe vector expressing myf5; pEM-MyoD, the pEMSVscribe vector expressing MyoD; pEM-myogenin, the pEMSVscribe vector expressing myogenin; and pEM-MRF4, the pEMSVcribe vector expressing MRF4; pEM-MEF2C, the pEMSVscribe vector expressing MEF2C. The diagram below B shows which proteins (indicated by plus signs) are able to bind to the motifs found in the constructions. A, mutations in the mAT1 site: pCAT, the pCAT-Basic vector alone without any promoter sequences inserted; 192 WT, 192pCAT (~192 bp/15 bp of the promoter inserted into pCAT-Basic; this construction contains mAT1 and mAT2), mAT1 MEF-2 mut, 192pCAT with a mutation in the mAT1 site which abolishes only MEF-2 binding (CTAATTATAT to CTAATTTATTT), mAT1 Oct-1 mut, 192pCAT with a mutation in the mAT1 site which abolishes only Oct-1 binding (CTAAATATAT to CTAAATAATAT), and mAT1 pal. mut., 192pCAT with a mutation in the palindrome motif which abolishes only Pal-BP binding (AGAAATATTTCT to AGAAAGTTTCT). The latter mutant serves as a control that binds both MEF-2 and Oct-1. B, mutations in the mAT2 site: 192 WT, as in A; mAT2 MEF-2 mut., 192pCAT with a mutation in the mAT2 site that abolishes only MEF-2 binding (CTAATTATAT to CTAATTTATTT); mAT2 Oct-1 mut, 192pCAT with a mutation in the mAT2 site that abolishes Oct-1 binding but increases MEF-2 binding (CTAAATATATATAGA to CAAATTATTTCGCGA); mAT2 double mut., 192pCAT with a mutation in the mAT2 site that abolishes MEF-2 and reduces by half Oct-1 binding (CTAAATATATATAGA to CAAATCGCTTATAGA). C, mutations in the mAT1 site: 40 WT, ~40 bp/13 bp inserted into pCAT-Basic; mAT1–40 WT, the mAT1 site inserted immediately upstream of ~40 bp in 40 WT; mAT1–40 mut.4, mAT1–40 WT but with a mutation in mAT1 that abolishes binding of MEF-2 (CTAAATATAT to CTACGCATAT); 192 WT, as in A; 192 mut.4, 192 WT but with the same MEF-2 mutation in mAT1. D, mutations in the mAT2 site: 40 WT, mAT2–40 WT, as in C; mAT2–40 WT, the mAT2 site inserted immediately upstream of ~40 bp in 40 WT; mAT2–40 double mut., mAT2–40 WT but with the same mutation as the mAT2 double mut. in B; mAT2–40 Oct-1 mut., mAT2–40 WT but with the same mutation as the mAT2 Oct-1 mut. in B.
transferred to differentiation medium, whereas MEF-2C is expressed only after several days of differentiation. The myotubes used here to prepare the nuclear extracts have spent 3 to 4 days in differentiation medium. The endogenous IIB MyHC gene is expressed at a significant level in C2 cells only after several days in differentiation medium (6). The MEF-2C mRNA levels are extremely low in C2C12 myotubes (39), and overexpression of MEF-2C activates the IIB MyHC promoter. These observations strongly argue in favor of the importance of the MEF-2C isoform.

Oct-1 or a Closely Related Protein Binds the mAT2 Site in Vivo—We have shown that the protein 2BB2 has binding requirements similar to those of a protein containing not only a homeodomain but also a POU-specific domain and that it is bound by an antibody against Oct-1. Moreover, we have shown using chimeric constructs in transfection experiments that the mAT2 element cis-activates the minimal IIB MyHC promoter in muscle and non-muscle cells. There is strong reason to believe that the widely expressed POU domain protein Oct-1, or a closely related protein, is bound in vivo to the mAT2 element.

This protein is present in undifferentiated cells, in determined but not yet differentiated myogenic cells, in differentiated myogenic cells, in mature muscle fibers from rodent muscle, and in CV-1 cells. In addition to Oct-1, which is widely expressed among mammalian cells (19, 37), the alternatively spliced isoform Oct-1B has the same wide tissue distribution as Oct-1 (49). There is also evidence for other alternatively spliced isoforms of Oct-1, which exhibit some degree of tissue-restricted expression (50). Oct-1, despite its wide expression pattern, is involved in the cell-specific activation of several genes (51–56). Although we cannot formally attribute a function to the binding of Oct-1 to the mAT2 motif (discussed below), we suggest that Oct-1 could be involved in the specification of the myogenic phenotype, in conjunction with muscle-specific factors.

The Homologous mAT1 and mAT2 Motifs Are Functionally Different—Although our transfection data with a construct containing both mAT sites together support the idea of cooperative binding between the proteins interacting with these two sites, and clearly show that the mAT1 motif is the target of MEF-2, they do not allow us to tell whether Oct-1 or MEF-2 recognizes the mAT2 motif in mature myotubes. However, although in the context of the entire 192-bp of 5′-flanking sequence the respective roles of Oct-1 and MEF-2 were not sufficiently clear, isolation of the elements allowed us to conclude that, while MEF-2 can bind to mAT2, it is unlikely that MEF-2 occupies this site, at least up to the stage of early myotubes. The activities of the chimeric construct in non-mygogenic and myogenic cells argue in favor of Oct-1 being bound to this element. Indeed, Oct-1 may normally out-compete MEF-2 for the mAT2 site in vivo. However, when Oct-1 is prevented from binding by the Oct-1 mutation, MEF-2 usurps its place and is actually better able to activate the promoter in the context of cultured myotubes.

Thus the two strikingly similar mAT motifs have distinct and separate binding proteins, MEF-2 on mAT1 and Oct-1 on mAT2. However, although our data with the entire IIB MyHC promoter clearly show that MEF-2 acts as an activator through the mAT1 site, we cannot rigorously determine the role of Oct-1. We suggest that the possibility of modulating the protein binding to the mAT2 site is essential for the tissue and/or temporal specificity of the transcription of the IIB MyHC gene. In non-muscle tissue or myoblasts, where the IIB MyHC gene is not expressed, the mAT sites could be occupied by the MIBox-like proteins or by Oct-1; it is unlikely they are occupied by the more precociously expressed isoform MEF-2D, since there was no corresponding complex in EMSAs with myoblast nuclear proteins. In mature skeletal muscle, on the other hand, these sites are probably bound by Oct-1 and MEF-2. Our co-transfection experiments with MEF-2C and myogenin suggest that MEF-2 activates this muscle-specific promoter perhaps by interacting with a myogenic bHLH protein which would provide a functional activation domain, as in the model suggested by Molkentin et al. (57). MEF-2, bound to mAT1, could recruit or help Oct-1 bind to mAT2 (47, 58). Oct-1 itself has been shown to activate transcription by aiding the binding of other factors (54, 59). Oct-1 bound to mAT2 could thus improve the binding, interaction, and/or activation properties of MEF-2 or of other transcription factors bound elsewhere on the promoter, e.g. the CARG box at −100 bp. The proteins SRF and NF1 bind to adjacent sites centered at −100 bp on the IIB MyHC promoter and induce a bend in the DNA (data not shown), which may in turn facilitate the physical rapprochement of the proteins of the mAT sites with those in the vicinity of the TATA box.

The basis for tissue-specific, developmentally regulated control of the expression of the murine IIB MyHC gene is thus at least in part dependent on the elements of its proximal promoter region. The identity and the activity of the protein bound to the mAT2 site during the course of myogenic maturation may be controlled by the relative concentration of several factors (e.g. different homeodomain and MEF-2 proteins) and by the post-transcriptional modifications they undergo (e.g. splicing and phosphorylation). Because of the considerable evolutionary conservation of several motifs in the proximal promoter region (11), our findings on the cross-talk between homeodomain and MADS domain proteins bound to similar sequence elements may well have bearing on many members of the MyHC multigene family (60, 61).

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