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# A Novel Complex Regulates *cardiac actin* Gene Expression through Interaction of Emb, a Class VI POU Domain Protein, MEF2D, and the Histone Transacetylase p300

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**Expression of the mouse *cardiac actin* gene depends on a distal enhancer (−7 kbp) which has been shown, in transgenic mice, to direct expression to embryonic skeletal muscle. The presence of this distal sequence is also associated with reproducible expression of *cardiac actin* transgenes. In differentiated skeletal muscle cells, activity of the enhancer is driven by an E box, binding MyoD family members, and by a 3′ AT-rich sequence which is in the location of a DNase I-hypersensitive site. This sequence does not bind MEF2 proteins, or other known muscle transcription factors, directly. Oct1 and Emb, a class VI POU domain protein, bind to consensus sites on the DNA, and it is the binding of Emb which is important for activity. Emb binds as a major complex with MEF2D and the histone transacetylase p300. The form of Emb present in this complex and as a major form in muscle cell extracts is longer (80 kDa) than that previously described. These results demonstrate the importance of this novel complex in the transcriptional regulation of the *cardiac actin* gene and suggest a potential role in chromatin remodeling associated with muscle gene activation.**

Cardiac actin is a major actin isoform not only in the heart but also in developing skeletal muscle (37). Like other genes transcribed specifically in striated muscle, regulation of the *cardiac actin* gene depends not only on a proximal promoter but also on enhancer sequences, which target distinct sites of expression. DNase I-hypersensitive site analysis with cells of the C2 skeletal muscle cell line led to the identification of two such sequences at −7 and −5 kbp upstream of the mouse *cardiac actin* gene (2). In both cases, a DNA fragment of several hundred kilobases around the site was shown to act as an enhancer in differentiated muscle cells. In transgenic mice (3), the proximal promoter can direct reporter gene expression to cardiac and skeletal muscle in the embryo, but transgenic lines tend to show weak or undetectable expression and the adult heart is negative. In contrast, when an upstream genomic sequence which includes both hypersensitive sites is present, high-level, reproducible expression, which resembles that of the endogenous gene, is shown by all transgenic lines analyzed. Transgenic lines with −5 kbp of upstream sequence show expression in embryonic striated muscle and in adult cardiac muscle, but only in about 50% of lines. The −7-kbp enhancer alone, with the proximal promoter, directs reliable transgene expression to embryonic skeletal muscle, suggesting that this sequence acts in vivo as a skeletal muscle enhancer and that its presence facilitates transcription of the transgene at different DNA insertion sites. Displacement of this region of DNA, due to a 5′ duplication of the endogenous *cardiac actin* gene, perturbs its transcription in both skeletal and cardiac muscle of BALB/c mice, which have abnormally low levels of cardiac actin (18, 19). Molecular analysis of the C2 muscle cell line showed that activity depends critically on one of several E boxes, which proved to be the target of myogenic regulatory factors of the MyoD family, conferring skeletal muscle specificity on the enhancer, and on a second 3′ AT-rich region, which did not correspond to a canonical consensus sequence for transcription factors known to be important in myogenesis (2). This sequence is in the location of the DNase I-hypersensitive site and is of interest in regulating access to the locus.

Although other muscle enhancers have been identified on the basis of DNase I hypersensitivity, such as those of the *M-creatine phosphokinase* (*M-CPK*) (24) or *myosin MLC1F/3F* (26) genes, very little is known about sequences which may regulate the accessibility of genes to muscle-specific transcription. A sequence in the vicinity of the mouse *desmin* gene which corresponds to a DNase I-hypersensitive site has been shown to have properties of a locus control region when analyzed by transgenesis, but its molecular regulation is not yet clear (48). If there are almost no examples of bona fide muscle locus control regions comparable to those described for the globin genes (61), there are now a number of reports which implicate muscle transcription factors in chromatin remodeling. The myogenic determination factors Myf5 and MyoD (see reference 5), when transfected into nonmuscle cells, affect the accessibility of muscle genes, such as *M-CPK*, hence facilitating myogenic conversion (20).

MyoD has been shown to interact with p300/CBP (16, 39, 45, 49, 66) and PCAF (47) histone transacetylases, which, by modifying nucleosome structure, render chromatin more accessible to transcription (57). The interaction with p300 involves sequence motifs which overlap with those implicated in the phenomenon described by Gerber et al. (20). In transitory-trans-

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fection experiments, the presence of p300 or PCAF increases the transactivation of a muscle reporter gene by MyoD, in this situation acting as a muscle differentiation factor; inhibitor experiments with muscle cells suggest that PCAF intervenes at the onset of differentiation, while p300/CBP affects the later activation of muscle structural genes and enzymes (44, 47). In addition to a potential effect on chromatin structure, these histone transacetylases directly acetylate MyoD, increasing its transcriptional activity (43, 51). Members of the myocyte enhancer factor 2 (MEF2) family of transcription factors, which play a critical role in the activation of many muscle genes (4), have also been reported to interact with p300/CBP with enhancement of skeletal (50) and cardiac (55) muscle gene transcription in cultured cells.

In contrast to histone transacetylases, histone deacetylases (HDACs) prevent transcription by rendering chromatin inaccessible (59). Class II HDACs, such as HDAC IV, interact with MEF2 transcription factors and render them inactive (15, 29, 31, 38; also see reference 35). This effect is probably also due to repression domains in the HDAC protein. HDAC1 can interact directly with MyoD, and this association, which occurs in undifferentiated myoblasts, renders MyoD transcriptionally inactive (32). The interaction between MEF2 and class II HDACs can be modulated by signaling pathways which phosphorylate the latter, leading to their nuclear export with consequent release of MEF2 for transcriptional activation (35).

Another form of modulation may be exerted by cofactors which facilitate the recruitment of histone modifying enzymes to form complexes with myogenic regulatory factors. An example is provided by GRIP-1 (7), which has intrinsic histone transacetylase activity and also acts as a coactivator by recruiting p300 and PCAF. GRIP-1 can form a complex with MEF2 and with the MyoD family member myogenin to enhance the transcriptional activation of muscle genes during muscle cell differentiation.

Investigation of the 3′ region of the enhancer located at 7 kbp upstream of the mouse α-*cardiac actin* gene led to the identification of a novel protein complex which binds to the sequence. Interference with its binding compromises enhancer activity. The complex contains a longer isoform of the class VI POU factor, Emb (42), which binds to the DNA and is associated with the myogenic factor MEF2D. The histone transacetylase p300 is also present as part of the Emb-MEF2D complex. This finding reveals Emb as a participant in muscle gene regulation. The potential role of this complex in potentiating myogenic expression of the *cardiac actin* gene is discussed.

## MATERIALS AND METHODS

**Cell culture.** C2/7 is a skeletal muscle cell line subcloned from the original C2 cell line derived from C3H mice (64). C2/7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal calf serum at low density and, when approaching confluence, induced to differentiate with DMEM–2% fetal calf serum.

**Transfection experiments.** Cells were grown in 60-mm-diameter dishes to 50% of confluence and transfected by the calcium phosphate method (2) with 10 μg of CAT reporter plasmid and 1 μg of a plasmid where the luciferase reporter gene is under the control of the Rous sarcoma virus promoter (RSV Luciferase) to normalize for transfection efficiency. Precipitates were allowed to form for 30 min at room temperature. Cells were incubated with the DNA-calcium phosphate precipitates overnight (o/n), rinsed twice with DMEM the day after, and induced to differentiate over 48 to 72 h. Cells were then scraped in 100 μl of a solution containing 40 mM Tris (pH 7.5), 150 mM NaCl, and 1 mM EDTA; 30% of the extract was used for the luciferase assay and up to 30% for the CAT assay.

**CAT assay.** Three to four independent transfections were carried out for each point. CAT assays were carried out by the butyryl coenzyme A method (53). The cell extracts were incubated in a 100-μl reaction volume of 250 mM Tris-Cl (pH 7.5) in the presence of 0.53 mM butyryl coenzyme A (B1508; Sigma) and 0.04 μCi of [$^{14}$C]chloramphenicol (50 to 60 mCi/mmol, CFA 754; Amersham) for 1 h at 37°C. The butyrylated products were extracted with 200 μl of tetramethylpentadecane-xylene (2:1), and the extracted radioactivity was measured in an organic counting scintillant. Counts were normalized to the corresponding luciferase assay data.

**Luciferase assay.** Samples were diluted to a 100-μl final volume with LUC buffer (250 mM Tris-phosphate [pH 7.5], 8 mM $MgCl_2$, 1 mM EDTA, 2 mM dithiothreitol). Luciferase activity was measured with a luminometer (LUMAT LB 9501; Berthold), which injects 100 μl of reaction mix (0.2 mM luciferine [sodium] and 4 mM ATP in LUC buffer).

**Mutagenesis.** Point mutations in the AT-rich sequence were obtained by oligonucleotide-mediated mutagenesis with the Transformer TM site-directed mutagenesis kit (Clontech) with the selection primer Trans Oligo TM AflIII/GblII (Clontech). For this purpose, the 3′ PstI fragment (360 bp) from the distal enhancer of the *cardiac actin* gene (2) was cloned into the plasmid vector pBluescript SKII. The selection primer and the 35mer primers carrying the desired mutation in the AT-rich sequence were phosphorylated in vitro, and a 200-fold excess of the primers was annealed to the denatured template DNA, which was then extended by T4 DNA polymerase and ligated with the T4 DNA ligase. The resulting DNA was digested with the restriction enzyme AflIII and transformed in MutS *Escherichia coli* bacterial cells. The plasmid DNA was extracted and analyzed before introduction of the mutated DNA into XL1 Blue *E. coli* cells. The distal enhancer with the point mutations introduced into the AT-rich sequence was excised with HindIII and XbaI from the pBluescript SKII vector and cloned into the HindIII/XbaI sites of the pBLCAT3 plasmid, 5′ to the proximal promoter of the *cardiac actin* gene.

**Nuclear extracts and GMSAs.** C2/7 cells were grown in 15-cm-diameter dishes and taken at the early myoblast stage or at the myotube stage. Nuclear extracts were prepared using a modification of the method of Dignam et al. (12, 17). Nuclear extracts were divided into aliquots and conserved in liquid nitrogen. The protein concentration was measured by using the method of Bradford (Bio-Rad assay kit). The RsaI-PstI AT-rich 3′ fragment (76 bp) of the distal enhancer was cloned into the EcoRV-PstI sites of the Bluescript plasmid vector. To use the AT-rich fragment as a probe in a gel electrophoresis shift assay (GMSA), the DNA fragment was obtained by HindIII-XbaI digestion of the Bluescript plasmid vector and labeled by end filling using [α-$^{32}$P]dTTP and [α-$^{32}$P]dCTP. The IPOCT probe and the Emb binding site were obtained by annealing the complementary in vitro-synthesized oligonucleotides and labeled with [γ-$^{32}$P]ATP, using polynucleotide kinase. The sequences of the sense strand of the IPOCT probe and of the Emb binding site were as follows: IPOCT, 5′-CCTAATCCA CTGCAATAAGCAAATATTTCTATAATGGGAT-3′; Emb/mPOU, 5′-CAG CTCATTATGATAATGAGGGGG-3′ (63). GMSAs were performed in a total volume of 15 μl containing 2 μg of poly(dI-dC), 4 μg of nuclear extract protein, 1.5 μl of gel shift buffer (50 mM Tris [pH 8.0], 5 mM dithiothreitol, 5 mM EDTA, 250 mM NaCl, 10% Ficoll) and 1 ng of labeled DNA probe (6). The assay mixture was incubated at room temperature for 20 min and then run in a 5% nondenaturing polyacrylamide gel (acrylamide/bisacrylamide ratio, 49:1) in 0.5 × Tris-borate-ETDA. After the run was completed, the gel was fixed with a solution containing 10% ethanol and 10% acetic acid for 10 min, dried on a 3MM paper sheet (Whatman), and exposed to MP autoradiography film (Amersham). For the supershift experiments, mPOU (62), Oct1 (52), MEF2A, -B, and -D (22), and HDAC4 (38) antibodies were used. Oct1, MEF2, and HDAC4 antibodies were kind gifts of W. Schaffner, R. Prywes, and T. Kouzarides, respectively. p300 monoclonal antibody (NM11) was supplied by Pharmingen. The competition experiments were performed by diluting the labeled probe with a 50- to 100-fold molar excess of cold competitor double-strand oligonucleotides. The sequences of the sense strands of the oligonucleotides used as competitors were as follows: *muscle creatine phosphokinase (M-CPK)* MEF2 site, 5′-AAGCTCGCTCTAAA AATAACCCTGTCCCTGGT-3′; *M-CPK* Mhox site, 5′-GGTTATAATTAACC CAG-3′; *Troponin I* AT-rich site, 5′-CGCAGTATATTTAGTCTG-3′ (13); *H2B* Oct1 site, 5′-CGAGCTTCACCTTATTTGCATAAGCGATTG-3′ (54).

**Copper-orthophenanthroline footprinting.** To asymmetrically label the coding strand of the AT-rich sequence, the distal enhancer cloned into the HindIII/XbaI sites of pBluescript was first digested with XbaI by end filling and then cut with RsaI, and the labeled fragment was purified by gel electrophoresis, followed by electroelution of the DNA. The noncoding strand was labeled by end filling of the HindIII-SmaI AT-rich fragment. The asymmetrically labeled probes were

incubated with C2 nuclear extracts as for a gel shift experiment, with the binding reactions scaled up threefold. The binding reactions were run in a nondenaturing polyacrylamide gel. After electrophoresis, the gel was equilibrated for 30 min in 300 ml of 50 mM Tris-Cl (pH 8.0). Cleavage of the DNA was initiated by adding 30 ml of 2 mM 1,10-phenanthroline, 0.45 mM copper sulfate, and 30 ml of 58 mM 3-mercaptopropionic acid. After 10 min, cleavage was stopped by adding 30 ml of copper orthophenanthroline "stop" solution (28 mM 2,9-dimethyl 1,10-phenanthroline). After the reaction, the gel was extensively washed in distilled water and then exposed to an autoradiographic film. The bands corresponding to DNA-protein complexes were cut out, and the DNA was eluted in a solution containing 500 mM ammonium acetate, 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), and 10 mM $MgCl_2$ at 37°C o/n. The eluted DNA was precipitated by adding 10 μg of glycogen and two volumes of cold ethanol. The products of the footprinting reaction were loaded onto a 9% denaturing sequencing gel. An A+G Maxam Gilbert sequencing reaction was carried out on the coding and noncoding strands of the AT-rich fragment (34), and the product of the reaction was loaded beside the footprinting reaction. The sequencing gel was dried and quantitatively analyzed using a phosphorimager.

**Biotinylated probe pulldown.** The biotinylated AT-rich probe was obtained by PCR using the following primers (Sigma Genosys): Direct, 5′-ACTTTTCAAT GTTTCCTAATC-3′; Reverse, 5′-GCAGATCTGAACCAGCC-3′. The control probe, obtained by PCR, contains a mutation in this sequence which removes most Emb binding by introducing G residues in the second half of the AT-rich site to give CTGTGATGGG.

The direct primer was biotinylated at the 5′ end. The PCR was conducted with *Pfu* DNA polymerase (Stratagene); after 12 amplification cycles, the product was purified by polyacrylamide gel separation followed by electroelution of the DNA from the gel. Dynabeads M-280 streptavidin beads (Dynal Inc., Lake Success, N.Y.) were prepared by three washes in phosphate-buffered saline (pH 7.4) containing 0.1% bovine serum albumin and two washes with 10 mM Tris (pH 7.5)–1 mM EDTA–1 M NaCl (TEN). After each wash, the beads were collected with a Dynal magnetic particle concentrator. To immobilize the DNA on the beads, 800 μg of biotinylated AT-rich probe was incubated with 250 μl of magnetic beads in 1 ml of TEN at room temperature for 20 min. After three washes with TEN and two washes with the GEMSA buffer containing 0.01% Triton X-100 and 2.5 μg of bovine serum albumin/μl, the beads were incubated with 50 μg of nuclear extract protein in 100 μl of gel shift buffer (see "Nuclear extracts and GMSAs") containing 0.01% Triton X-100, 2.5 μg of bovine serum albumin/μl, and 4 μg of poly(dI-dC) for 20 min at room temperature, under gentle rotation. Proteins bound to the beads were separated with the magnetic particle separator after the unbound proteins had been washed away. The beads were washed three times with 1 ml of 0.5× gel shift buffer containing 0.01% Triton X-100 and 1 μg of poly(dI-dC). The beads were resuspended in 50 μl of 2× SDS gel loading buffer, boiled for 5 min, and centrifuged. The supernatant was analyzed by Western blotting.

**Western blot analysis.** For the analysis of nuclear extracts, 10 μg of nuclear proteins obtained from proliferating or differentiated C2 cells were diluted in 1× SDS gel loading buffer (50 mM Tris-Cl [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and run in an SDS–8% polyacrylamide gel (5% to detect p300). After electrophoresis the proteins were transferred to a polyvinylidene difluoride membrane (Amersham). Filters were blocked by incubation with PBST (phosphate-buffered saline containing 0.1% Tween 20), with added 5% lowfat dry milk, for 1 h and then incubated with the primary antibodies (1:1,000) for 2 h. The following antibodies were used: polyclonal anti-mPOU antibody and the corresponding preimmune serum, polyclonal anti-MEF2D, polyclonal anti-HDAC4, and monoclonal NM11 P300 (Pharmingen). Filters were extensively washed in PBST and then incubated with horseradish peroxidase-conjugated secondary antirabbit and antimouse antibody (Amersham) (1:5,000) for 1 h. After further extensive washes with PBST, antigen-antibody complexes were visualized with the enhanced chemiluminescence kit (ECL Plus) from Amersham.

**ChIP assay.** The chromatin immunoprecipitation (ChIP) protocol was adapted from the method of Soutoglou et al. (56). C2C7 muscle cells ($3 \times 10^8$) at different stages of differentiation were fixed with 1% formaldehyde at room temperature for 10 min. The cross-linking was stopped by addition of glycine at a 0.125 M final concentration for 5 min. Cells were detached in phosphate-buffered saline and pelleted by centrifugation at 1,000 rpm for 5 min at 4°C in a Sigma 4-15 centrifuge. The cells were resuspended in 5 volumes of lysis buffer (0.3 M sucrose, 10 mM HEPES-KOH [pH 7.9], 10 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM dithiothreitol, 0.3% NP-40, 0.5 mM phenylmethylsulfonyl fluoride) and lysed with a Dounce homogenizer. Nuclei were pelleted by centrifugation at 2,000 rpm for 5 min at 4°C in a Sigma 4-15 centrifuge and resuspended in 4 ml of sonication buffer (50 mM HEPES-KOH [pH 7.9], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, 0.5 mM phenylmethylsulfonyl fluoride, and a protease inhibitors tablet [Complete; Sigma]). Nuclei were sonicated for 4 min at 4°C to reduce the DNA length to between 500 and 2,000 bp. After the sonication, the chromatin was separated from nuclear debris by centrifugation at 14,000 rpm for 30 min at 4°C in a Sigma 4-15 centrifuge, and 10 optical density units at 260 nm were used for the immunoprecipitation step. Prior to immunoprecipitation, the chromatin was precleared for 1 h at 4°C with 5 μl of preimmune antibody and a 1/10 volume of unblocked protein A-Sepharose (Sigma). The precleared chromatin was incubated o/n with 5 μl of primary antibody. Immune complexes were immunoprecipitated with a 1/10 volume of protein A-Sepharose previously blocked with 0.1 mg of sonicated salmon sperm DNA/ml and 1 mg of bovine serum albumin/ml and then washed twice with sonication buffer, twice with sonication buffer containing 500 mM NaCl, twice with buffer B (20 mM Tris-HCl [pH 8.0], 250 mM LiCL, 1 mM EDTA, 0.5% Na-deoxycholate, 0.5% NP-40, 0.5 mM phenylmethylsulfonyl fluoride) and twice with TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) buffer. The immunoprecipitated DNA-protein complexes were eluted in 400 μl of elution buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% SDS) at 65°C for 20 min. NaCl to a final concentration of 200 mM was added to the eluted DNA-protein complexes, and the cross-linking was reversed by incubation at 68°C o/n. Proteins were digested by addition of 100 μl of 5× proteinase K buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 5 mM EDTA, 5% SDS) and 0.1 mg of proteinase K/ml at 37°C o/n. Following a phenol-chloroform (1:1) extraction, the DNA was precipitated with 0.3 M Na acetate (pH 5.2) and 2.5 volumes of ethanol at −20°C o/n. The immunoprecipitated DNA was dissolved in 100 μl of distilled water and digested with 3 μg of DNase-free RNase at 37°C for 30 min.

The immunoprecipitated chromatin (12 μl) was analyzed by real-time PCR using the SYBR GREEN system (Abiprism 7000; Applied Biosystems). The chromatin was subjected to 45 amplification cycles. For the analysis, the following oligonucleotides were used to amplify the AT-rich sequence of the distal enhancer of the *cardiac actin* gene: 5′-ACCAGCCTACATCCCATTATAGAA A-3′, act. forward; 5′-TCCTAATCCACTGCAATAAGC-3′, act. reverse.

To quantify the product, a series of dilutions of the corresponding input DNA was amplified with the same oligonucleotides. Enrichment in the ChIP experiments was estimated as the quantity of actin DNA immunoprecipitated in the presence of mPOU (Emb), p300, or MEF2D antibodies. Controls were carried out with unrelated antibodies, to β-galactosidase, to hemoagglutinin, or to the CAAT box binding factor NFYB.

As a further control, the precipitated chromatin was amplified with two oligonucleotides that amplify an enhancer region of the liver-specific *phenylalanine hydroxylase* (PAH) gene (45). The sequence of the oligonucleotides used to amplify the PAH sequence are the following:5′-CAAAATGGTGCTGTATC TCTGATATTC-3′, PAH forward; 5′-GGCACCAACTTCCTCTTTGAGT-3′, PAH reverse.

The p300 antibody used for the immunoprecipitation was from Santa Cruz (sc 585X). The NFYB antibody used as a control was a gift from R. Mantovani. Polyclonal β-galactosidase antibody was from Molecular Probes, and polyclonal hemoagglutinin antibody was from Sigma.

The chromatin immunoprecipitated from proliferating C2 cells has been also subjected to standard PCR with the same oligonucleotides used to amplify the actin sequence in the real-time PCR. The reaction was carried out with 1/10 of the immunoprecipitation product, 0.5 μM (each) primer, 100 μM deoxynucleoside triphosphates, 0.4 μl of Advantage *Taq* polymerase (Clontech), and 1 μl of [$^{32}$P]dATP (Amersham) in a 25-μl final reaction volume. After 30 cycles of amplification, all the reaction was separated on a 10% polyacrylamide gel. The gel has been dried and subjected to autoradiography.

## RESULTS

**Protein complexes binding to the 3′ AT-rich fragment from the distal enhancer of the cardiac actin gene.** As shown previously, the activity of the distal enhancer of the mouse *cardiac actin* gene in skeletal muscle cells depends on the presence of an E box which binds myogenic factors and on an AT-rich sequence (76 bp) located at the 3′ end (2, 3). The MEF2 family of transcription factors, involved in muscle gene regulation, bind to a C/TTA(A/T)$_4$TAG/A consensus sequence (21; also see reference 4). This is not present as such in the 3′ AT-rich region. There is a consensus binding site for Oct1 (23, 57) with one mismatch in the central octamer core (AAGCA

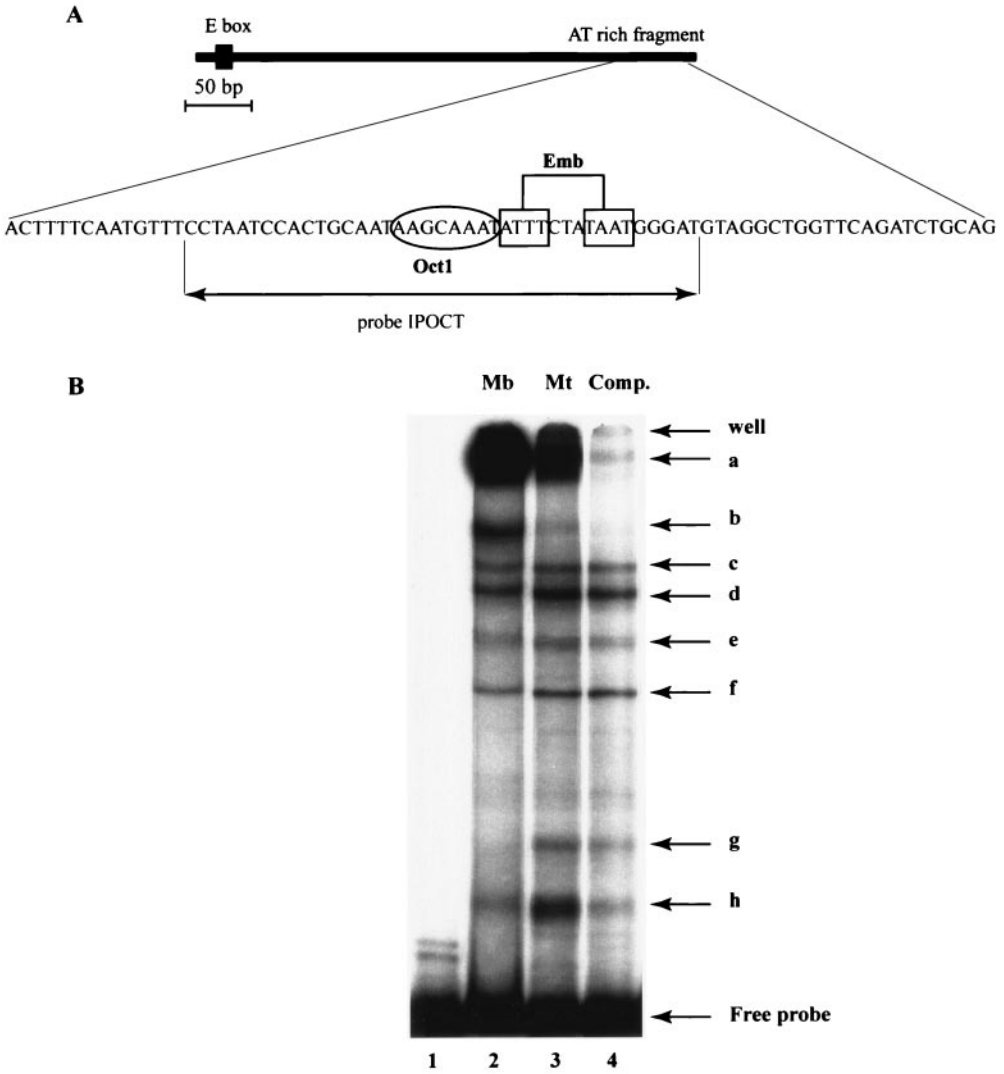


FIG. 1. (A) Schematic representation of the distal enhancer of the *cardiac actin* gene, showing the E box, which is essential for activity and the sequence of the AT-rich 3′ region. This contains consensus sites for Oct1 and Emb, as indicated. The shorter probe, IPOCT, used in subsequent experiments, is shown. (B) Gel shift experiment using the entire AT-rich fragment (76 bp) as probe. The nuclear extracts used were from C2 myoblasts (lanes 2 and 4) and myotubes (lane 3). The main DNA-protein complexes are indicated by arrows and letters (a to h). A self-competition experiment is shown, where a 50-fold excess of cold probe was added to the binding reaction (lane 4). In lane 1 the migration of the free probe is shown.

AAT instead of ATGCAAAT) and conserved core flanking sequences. Downstream of the Oct1 site there is a second AT-rich sequence ($ATTT(N)_3TAAT$) which is similar to a homeodomain transcription factor binding site, shown to be optimal for human mPOU, another POU domain protein (63), identified as Emb in the mouse (42). These consensus sites are indicated in Fig. 1A.

In order to identify binding activities with the 3′ AT-rich fragment, we performed gel shift experiments using nuclear extracts from the C2 skeletal muscle cell line. Incubation of the 76-bp fragment with myoblast or myotube nuclear extracts led to the formation of several DNA-protein complexes (a to h; Fig. 1B). The complexes a and b were clearly competed by a 50-fold excess of cold probe but not by an equivalent molar excess of an unrelated binding site (not shown). These complexes were seen with extracts from muscle cell lines and embryonic muscle (not shown).

**Characterization of the DNA-protein contacts in complexes a and b by DNA footprinting.** To further analyze the complexes a and b, we examined the nucleotides contacted by the DNA binding activities in C2 nuclear extracts. The copper orthophenanthroline in situ footprinting strategy was used, since in the presence of several DNA-protein complexes it allows analysis of DNA-protein contacts in the complex of interest. Figure 2 shows the footprinting profiles obtained with the labeled coding and noncoding strands, respectively, of the 3′ AT-rich fragment involved in complex a. The nucleotides contacted in complex b are identical (not shown), suggesting that the bigger complex a may be an aggregate of b or involve additional proteins which do not affect the sites of DNA contact. A schematic

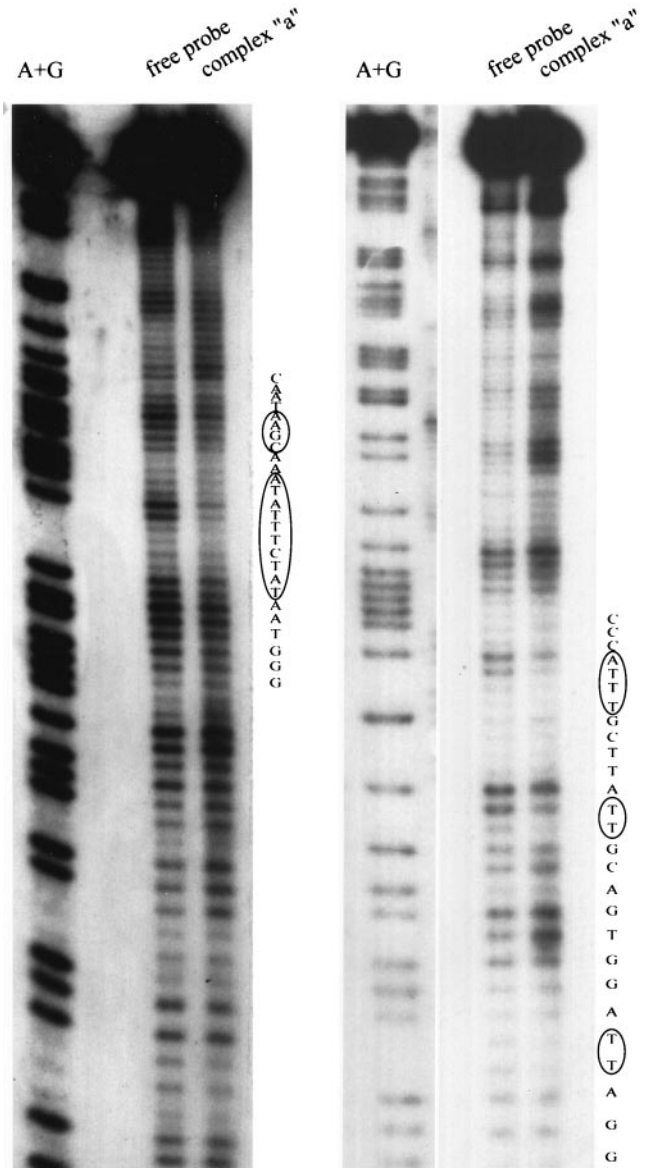


FIG. 2. An example of a footprinting experiment using the copper orthophenanthroline method. The analysis shown is with the DNA-protein complex a, obtained on incubation of C2 myoblast nuclear extracts with the AT-rich fragment; the same profile of protection was obtained with complex b (not shown). Both the coding strands and the noncoding strands were analyzed. To identify precisely the protected nucleotides, a sequencing reaction with the same fragment obtained by a Maxam Gilbert reaction for nucleotides A and G (A+G) was run at the same time. The sequence in the region of the protected area is shown, and protected nucleotides are encircled.

representation based on phosphorimager quantification of the footprint is shown in Fig. 3A. The putative Emb binding site is contacted on the coding strand. A protection is observed on the 5′ side of the Oct1 site and on the 5′ Emb half-site, ATTT, extending more weakly with this consensus. Hypersensitivity is observed on the other side of the Emb site on the coding strand and is also clearly evident on the noncoding strand, where protection is seen on the 3′ end of the Oct1 consensus.

**Characterization of *cis*-acting elements which affect activity of the 3′ AT-rich element.** The 3′ AT-rich fragment by itself, as a single copy or in multimerized form, is not able to enhance the activity of the *cardiac actin* promoter in driving reporter gene expression (data not shown). We therefore tested the effect of mutations, based on the results of the footprinting experiment, in the context of the entire distal enhancer (360 bp), with the proximal promoter (680 bp) of the mouse *cardiac actin* gene. Results of reporter CAT activity, shown in Fig. 3B, are expressed relative to the construction with the wild type (WT) enhancer. Mutation of the 3′ part of the putative Emb site (Mut6) results in a significant reduction (70%) of activity. Mutating other nucleotides did not lead to significant decreases in the activity of the enhancer, with the possible exception of Mut3, which affects the 5′ part of the Emb site and consistently resulted in a minor reduction. Mutating the putative Oct1 site to a perfect Oct1 consensus (Mut7), if anything, resulted in a small increase in activity, and mutations which destroy the Oct1 consensus (Mut2) had no significant effect. Mutations in the region of hypersensitivity 3′ to the Emb consensus (Mut4 and Mut5) and in a putative PEA3 site (Mut1) had no effect.

**The influence of mutations which affect distal enhancer activity on the binding of protein complexes.** The 3′ AT-rich fragment is involved in the formation of several DNA-protein complexes, as seen in gel shift experiments with nuclear extracts from the C2 muscle cell line. We tested whether the presence of the mutations in the 3′ fragment which influence the activity of the distal enhancer affects these complexes (Fig. 4). The mutation (Mut6) which reduces the activity by 70% leads to a marked reduction in band a and loss of band b, compared with effects of WT sequence or Mut5. Band d is also somewhat reduced. Mut3, which has a small negative effect on activity, also results in a diminution of bands a and b. These mutations affect the Emb consensus sequence and suggest that the Emb POU domain protein is involved in complexes a and b. Mut7, which gives a minor increase in activity, results in increased intensity of bands c and d, which may be associated with Oct1 binding since this mutation improves the Oct1 consensus.

**Characterization of the proteins present in the complexes binding to the 3′ AT-rich fragment.** In order to establish which proteins are present in the complexes formed with the 3′ AT-rich fragment, competition experiments were performed with oligonucleotides to the consensus binding sites and with antibodies to transcriptional factors suspected of binding to the fragment. Since Emb is a candidate, the presence of the protein was verified in C2 muscle cell extracts by Western blot analysis, using an antibody directed against the human homologue, mPOU (62). This reveals a protein of about 80 kDa, which is not recognized by the preimmune serum (Fig. 5A). It is present in both myoblast and myotube extracts. An oligonucleotide containing the proposed binding site for Emb/mPOU (ATTA. . .TAAT) (59) was used as a probe in gel shift experiments with myotube nuclear extracts. This forms two complexes, one of which is supershifted by an Oct1 antibody (52) and the other of which is disrupted by the mPOU antibody (Fig. 5B). Oct1 is abundant in C2 extracts, as seen on Western blots (data not shown) (also see reference 10); the band shift results show that Emb is present, but at a lower level, as indi-

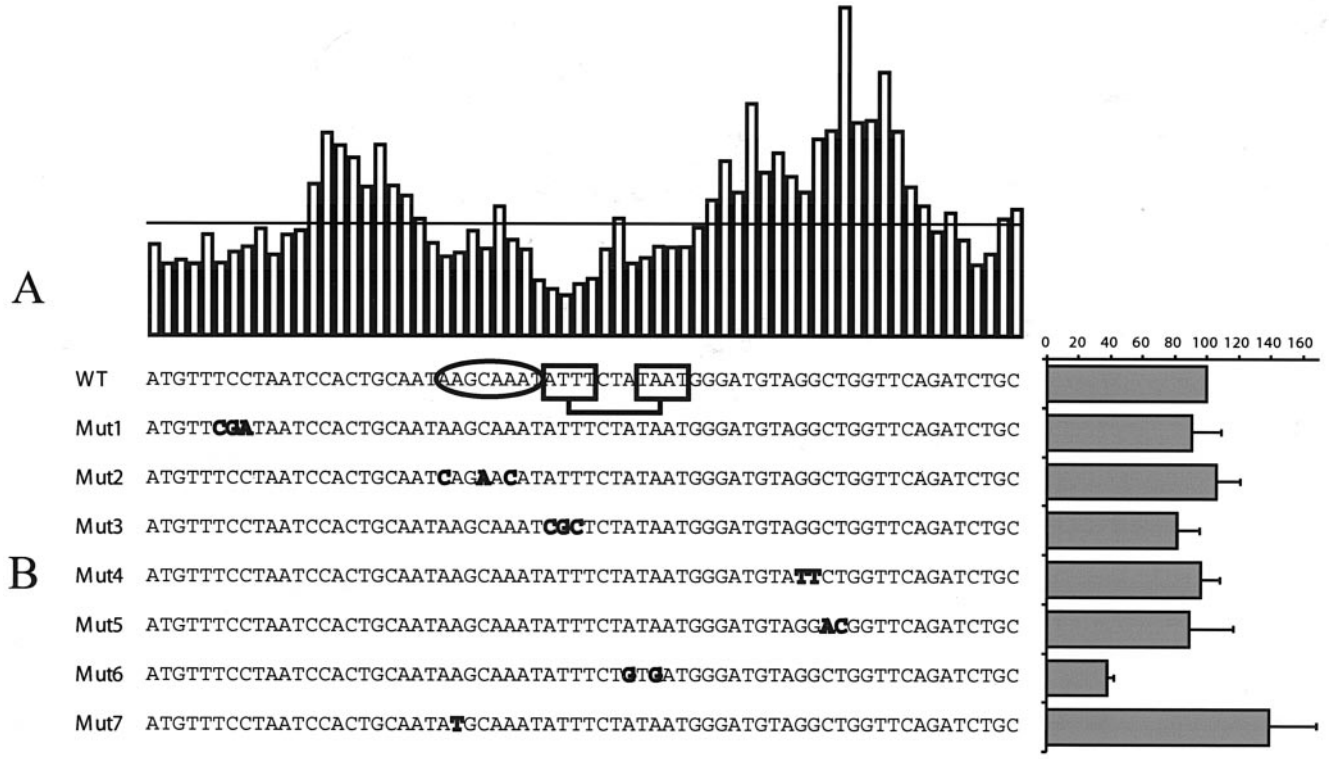


FIG. 3. Mutational analysis of the 3′ AT-rich sequence. (A) Schematic representation of the protection profile obtained for the coding strand of the 3′ AT-rich sequence in the footprinting experiment, based on phosphorimager quantification of the autoradiograph. The baseline corresponds to the intensity of the signal obtained for each nucleotide of the free probe. Each column represents the signal obtained for each nucleotide in the complex. The protected areas correspond to the signals below the baseline. Both 5′ and 3′ to the protected region there are hypersensitive areas. (B) The sequence of the AT-rich fragment as WT or containing mutations (Mut) is shown. The point mutations are in bold; the nucleotides to be mutated were chosen on the basis of the footprinting profile and potential transcription factor binding sites. The functional analysis of each point mutation is reported on the right. The effect of the WT or mutated enhancer (360 bp, including the active E box) with the promoter (680 bp) of the *cardiac actin* gene on the expression of a CAT reporter gene was measured in transient transfections in C2 myotubes. The activity of the WT enhancer was considered to be 100%.

cated by the relative intensities of binding to the Emb/mPOU sequence, which also has affinity for Oct1 (1).

In gel shift experiments with C2 muscle cell extracts and the 3′ AT-rich fragment, the mPOU antibody disrupts complexes a and b (Fig. 6A). In competition experiments with a 50-fold excess of the Emb/mPOU consensus sequence (ATTA. . . TAAT), bands a and b are considerably reduced (Fig. 6B). These results show that Emb binds to the 3′ AT-rich fragment in complexes a and b. There is also competition with band d; an Oct1 antibody reduced band d (Fig. 6A), which was competed with an Oct1 consensus binding site (ATGCAAAT) (Fig. 6B). In this experiment, band c, which varies between extracts, was not visible. The presence of other proteins able to bind AT-rich regions was also tested. The MEF2 family of transcription factors (4) bind to an AT-rich consensus. A MEF2 consensus oligonucleotide, as present in the enhancer of the *M-CPK* gene (21), does not compete with complexes a and b (Fig. 6B) or indeed interfere with other bands seen with the 3′ AT-rich fragment. However, an antibody against the MEF2D isoform (22) specifically disrupts band b, while antibodies to MEF2A and MEF2B have a more minor effect on this band (Fig. 6A). The Mhox protein binds to an AT-rich consensus (TTATAA TTAA) which has been shown also to bind MEF2 and Oct1 (10); again there is no competition of bands a and b and a minor effect on band d (Fig. 6B). This is also the case for the AT-rich sequence (TATATTTA) in the *Troponin I* promoter, which has been shown to bind MEF2A and Oct1 (13). Since we are using muscle cell extracts, the presence of myogenic regulatory factors, MyoD, myogenin, and Myf5, in the complexes was also checked. There is no E-box consensus (CANNTG) in the 3′ AT-rich fragment, but these factors can form complexes with MEF2 (4) without binding directly to DNA. Antibodies to these proteins had no effect on the DNA-protein complexes seen on gel shift (results not shown), and we conclude that this family of myogenic regulatory factors is not involved.

Further gel shift experiments were carried out with a shorter 40-bp fragment (IPOCT; Fig. 1A), which contains the Oct1 and Emb sites. This gives one major band migrating at the level of band d, which is now competed by the Oct1 and Emb consensus sequences (Fig. 6C). MHox also competes Oct1 binding, as observed previously (10). The Oct1 antibody shifts the major complex, which is also partially disrupted by an mPOU antibody. We conclude that this band corresponds to Oct1 binding. In excess, the Emb consensus will compete for Oct1 (see Fig. 5B), as seen also for band d in Fig. 6B. There is also some interaction between the mPOU antibody and Oct1 (see Fig. 5B). The complex which migrates at the level of band b is affected only by competition with the Emb consensus or

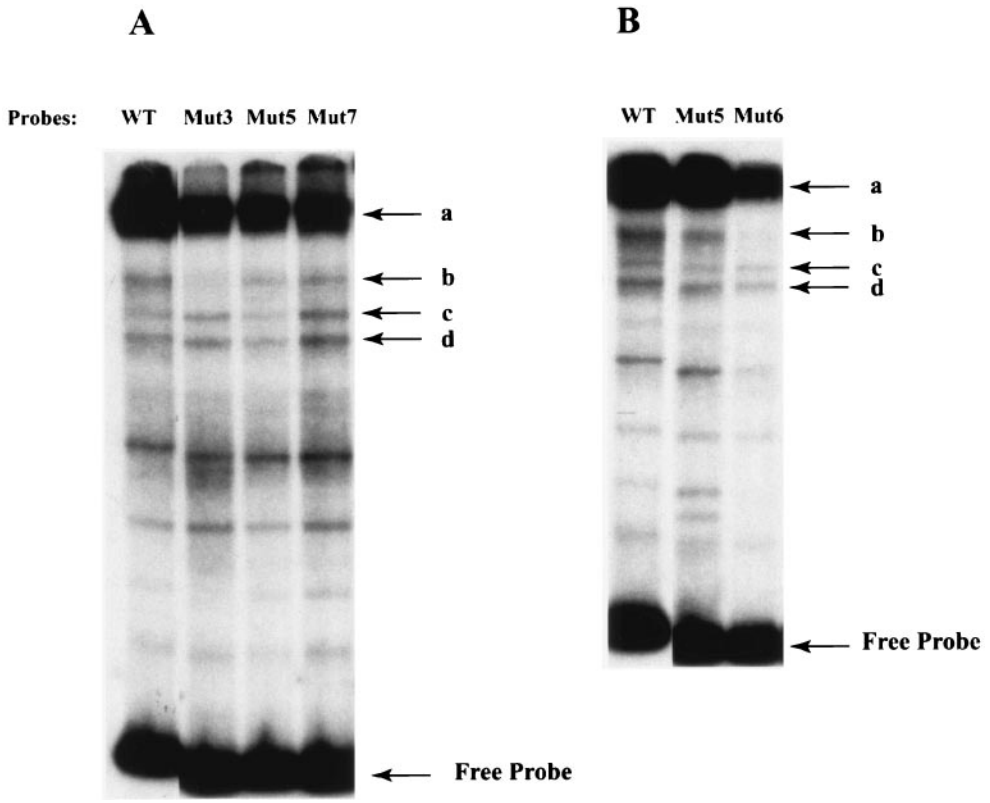


FIG. 4. Gel shift experiments with C2 myoblast nuclear extracts using the AT-rich 3′ fragment as a probe (WT) or the same fragment mutated in the positions indicated in Fig. 3. The WT DNA fragment and the mutated DNA fragments were cloned into different restriction enzyme sites of the polylinker of the cloning vector pBluescript, so they have slightly different sizes. Complexes indicated by letters and arrows are as shown in Fig. 1.

incubation with the mPOU antibody, confirming that it contains Emb but not Oct1. As expected, the MEF2 consensus does not compete complex b, but it is disrupted by the MEF2D antibody. We conclude that complex b contains Emb and MEF2D and complex d contains Oct1. The large complex a does not form, under gel shift assay conditions, in the absence of the region lying 3′ to the Emb site. Under these conditions, Oct1 binding to give complex d predominates.

**The proteins present in complex a.** The data presented in Fig. 6 show that complex a contains Emb. It may also contain MEF2D, like complex b, but unless there is disruption of the complex this is difficult to assess at this level in the gel. The size of complex a would suggest that other large proteins may be associated with it. It has been shown that MEF2 can interact with HDACs and also with the histone transacetylase CBP/p300 (35). We therefore investigated whether these proteins are present. Preincubation of C2 muscle cell nuclear extracts with a p300 antibody resulted in a partial disruption of complex a in a gel shift experiment (Fig. 7A). No disruption was seen when an HDAC4 antibody was used. In order to characterize better the nature of the complex, we adopted an alternative strategy, based on the use of streptavidin-coated magnetic beads to which the biotinylated 3′ AT-rich sequence had been bound. C2 nuclear extracts were incubated with the beads with or without DNA. Gel shift analysis of the flow-through fraction showed that in the presence of DNA, complexes (a to h) were no longer present (Fig. 7B, lane 2), whereas with the control beads (Fig. 7B, lane 3), without DNA, the gel shift profile was similar to that with the nuclear extracts before passage over the beads (Fig. 7B, lane 1). The eluted proteins were separated on a denaturing polyacrylamide gel and analyzed by Western blotting. Figure 7C (lane 2) shows that MEF2D, p300, and mPOU antibodies detected bands, whereas no HDAC4 band was detectable. Figure 7D confirms that the complex is specifically retained by the AT-rich sequence (lane 2), since when this is mutated it no longer binds (lane 3). The amount of MEF2D, as well as p300, indicates that it is also present in the main complex a, as well as in the minor complex b, as seen in band shift experiments. Relatively low levels of Emb were detected by the mPOU antibody in this experiment, possibly due to degradation during the elution procedure, reflecting its relative instability (S. Molinari and M. Buckingham, unpublished data) compared to MEF2D/p300. Alternatively, since the total disruption of complex a, when Emb binding is prevented by an antibody (Fig. 6A), and the footprint (Fig. 2) would suggest that DNA binding of MEF2D and p300 to the 3′ AT-rich fragment goes through Emb, this protein may have remained partially bound to the DNA and thus been less efficiently eluted.

**ChIP.** In order to verify that the complex containing Emb, MEF2D, and p300 also exists on the enhancer sequence of the endogenous *cardiac actin* gene, we performed ChIP experiments with antibodies to these proteins on sonicated chromatin from muscle cells. The genomic DNA in the precipitated

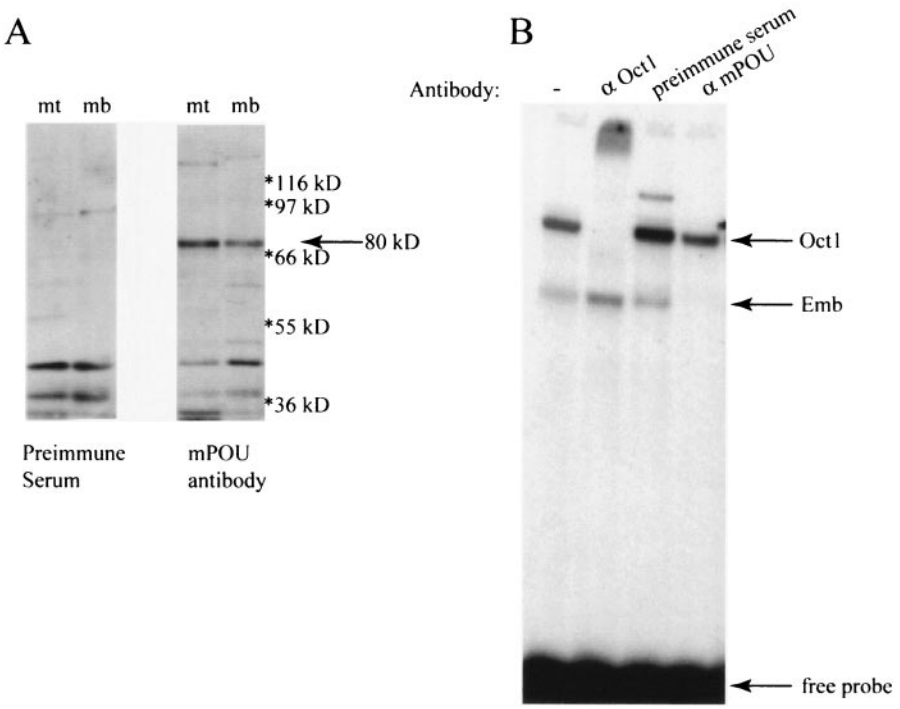


FIG. 5. Identification of a protein contained in the nuclear extracts of C2 myoblasts and myotubes that is related to Emb. (A) Western blot analysis in which 10 μg of protein was loaded in each lane. After migration, the proteins were transferred to filters and probed with the antibody directed against mPOU (the human Emb homologue) or the corresponding preimmune serum. A protein corresponding to a molecular mass of 80 kDa is detected specifically by the mPOU antibody in both myoblast and myotube nuclear extracts. The position of protein markers, run in parallel, are indicated with an asterisk. (B) The published mPOU consensus site (CAGCTCATTATGATAATGAGGGGG) (62) was used as a probe in gel shift experiments with nuclear extracts from C2 myotubes. Two major DNA-protein complexes were observed; the upper one contains Oct1 and the lower Emb, as indicated by the interaction with these antibodies. α mPOU, anti-mPOU antibody; α Oct1, anti-Oct1 antibody.

complex was analyzed by real-time PCR, using primers to amplify the enhancer site. The results of such an experiment with myoblast nuclear extract are shown in Fig. 8A, with the *cardiac actin* regulatory sequence and with a control sequence from the PAH gene, which is not expressed in skeletal muscle. All three antibodies to mPOU, which recognizes Emb, MEF2D, and p300, immunoprecipitate a complex with the *cardiac actin* sequence, which is detectable at a lower amplification frequency than the background level seen with the control sequence. In Fig. 8B, the sequence amplified by PCR after chromatin immunoprecipitation is visualized by autoradiography after gel electrophoresis, again demonstrating the formation of a complex with Emb, MEF2D, and p300. Histograms of the results of such experiments, where the amplification with the *cardiac actin* sequence has been corrected relative to the control sequence (Fig. 8C), demonstrate that Emb, MEF2D, and p300 form a complex on the site in the enhancer sequence of the endogenous *cardiac actin* gene in myoblasts, confluent cultures, and differentiated myotubes. It is striking that p300 gives much more enrichment of the α-cardiac sequence in myotubes when the α-cardiac actin gene is actively transcribed. This may reflect the involvement of p300 in complexes at other sites, such as the E box, on the enhancer sequence.

## DISCUSSION

Analysis of the proteins which bind to the 3′ AT-rich sequence of the skeletal muscle enhancer, located 7 kbp upstream of the mouse *cardiac actin* gene, revealed a novel complex which is important for activity. Interaction with DNA depends on the presence of a novel longer form of Emb, a POU domain protein belonging to the POU VI subclass (42). The myogenic factor MEF2D and the histone transacetylase p300 form a complex with Emb. Another POU domain protein, Oct1, binds to an adjacent site.

The role of Oct1 in muscle gene regulation is not clear. It is widely expressed, including in striated muscle, and it has been identified as having a binding activity for a number of AT-rich regulatory regions implicated in the expression of muscle genes. The skeletal muscle enhancer of the *M-CPK* gene has an AT-rich sequence, in addition to the consensus MEF2 site, which binds the Mhox homeodomain protein. Oct1 and MEF2 also bind to this sequence. Mutagenesis, which disrupted each of these binding activities separately, established that only MEF2 is functionally necessary for reporter gene expression in C2 myotubes (10). Both Oct1 and MEF2 bind to two regulatory sites in the promoter region of the *IIB myosin heavy chain* gene (28). Binding appears to be cooperative, but the functional requirement for Oct1, in addition to MEF2, was not established. The *cardiac Troponin 1* promoter also has an AT-rich site, required for expression in cardiomyocytes. Here again, MEF2 and Oct1 bind as separate protein-DNA complexes; the role of the latter, relative to MEF2, which was shown to transactivate the promoter, is not clear (13). In the case of the AT-rich sequence of the *cardiac actin* enhancer,

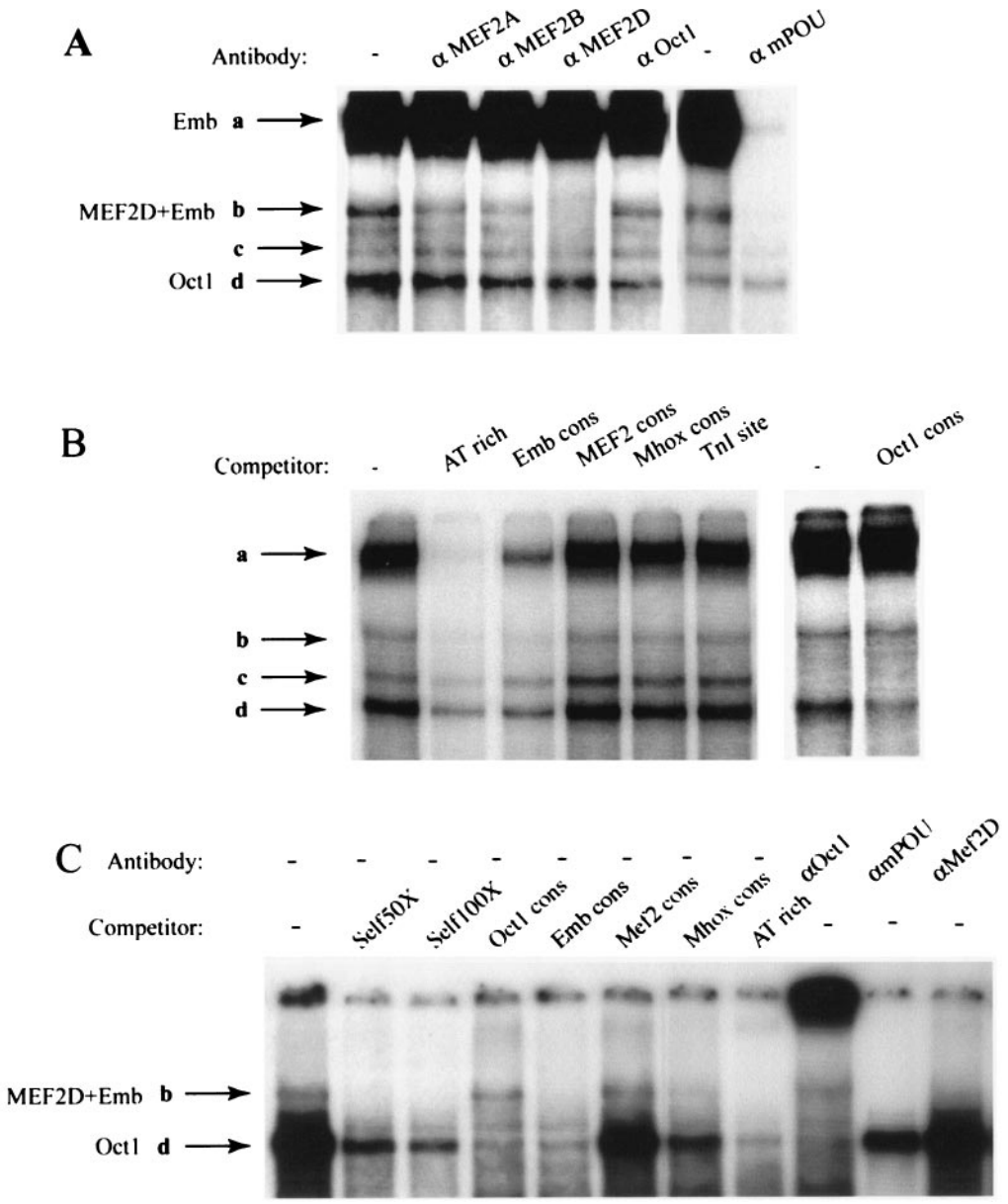


FIG. 6. Characterization of the DNA-protein complexes obtained on gel shift analysis with the 3′ AT-rich DNA fragment as a probe and C2 myoblast nuclear extracts. (A) The addition of antibodies directed against three of the four MEF2 isoforms (A, B, and D), Oct1, and mPOU, the human homologue of the POU protein Emb, revealed the presence of these proteins as indicated. (B) Competition experiments using an excess of the 3′ AT-rich sequence itself (AT-rich), the consensus site for Emb, the MEF2 binding site from the *M-CPK* enhancer, the Oct1 consensus site from the *H2B histone* gene promoter, the so-called Mhox site from the *M-CPK* enhancer, and the MEF2 consensus site from the *Troponin I* gene (TnI site). The consensus sites for the transcription factor PEA3, like that for Sp1, did not compete any of the DNA-protein complexes in gel shifts (data not shown). (C) Gel shift experiment using as probe the IPOCT sequence (see Fig. 1A for the sequence), which shows one minor and one major binding activity with myoblast nuclear extracts. These correspond to protein complexes b and d, respectively. The two complexes are competed by preincubating the binding reaction with a 50- or 100-fold excess of cold IPOCT probe (Self50X, Self 100X) or with the entire AT-rich 3′ fragment (AT rich). Complex d is competed by the Oct1 consensus site, while both the complexes are competed by an excess of the Emb consensus site. Competition with the *M-CPK* MEF2 site (MEF2 cons) does not change the binding profile. Competition with the MHox sequence mainly affects Oct1 binding, as expected. The addition of antibodies to Oct1 (αOct1) mainly shifts complex d, whereas the Emb antibody (αmPOU) mainly affects band b. An antibody to MEF2D (αMEF2D) also eliminates band b.

Oct1 binding to the site is weak, but this is probably due to interference by Emb, since when Emb binding (as complex a) is compromised with the shorter AT-rich sequence, Oct1 binds strongly. Mutations which affect binding of Oct1 to the site did not have an effect, although activity was slightly higher with a perfect Oct1 site. This may reflect a positive effect of Oct1 on the recruitment of other proteins to the site (41), although complex b, for example, did not notably increase with this mutant or with the shorter AT-rich sequence. There is promiscuity in the binding of Oct1 and Emb to their consensus se-

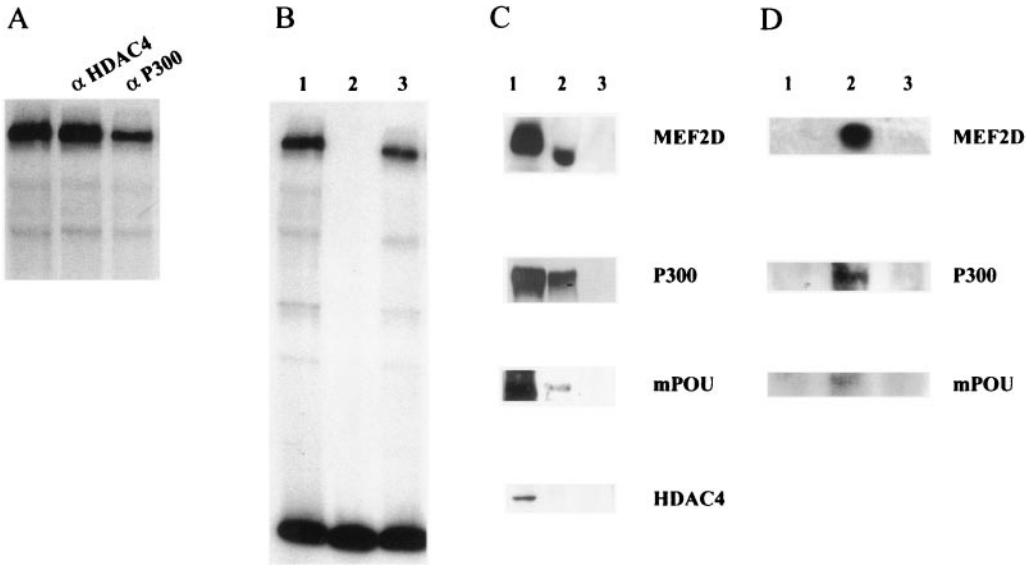


FIG. 7. Further characterization of complex A. (A) Gel shift experiment with the labeled 3′ AT-rich sequence incubated with a C2 myotube nuclear extract, where antibodies (α) to HDAC4 or p300 were added as indicated to the binding reaction. (B) Gel shift experiment with this probe and a C2 myoblast nuclear extract before (lane 1) or after (lane 2) passage over magnetic beads to which the same biotinylated sequence had been bound. Lane 3 shows the gel shift given by an extract after the passage over magnetic beads without bound DNA. (C) Extracts before passage over the beads (lane 1), after elution from beads to which the biotinylated sequence had been bound (lane 2), or after elution from the beads in the absence of bound DNA (lane 3) were analyzed by Western blotting, using antibodies to MEF2D, p300, mPOU (Emb), and HDAC4. Bovine serum albumin was added as a competitor for nonspecific protein binding in the bead experiment. It has a molecular weight similar to MEF2D's, distorting the MEF2D band on the Western blot (lane 2). (D) Extracts after elution from beads, in the absence of DNA (lane 1), in the presence of the AT-rich biotinylated sequence (lane 2), or in the presence of a biotinylated AT-rich sequence mutated to abolish Emb binding (lane 3) were analyzed by Western blotting with MEF2D, p300, and mPOU (Emb) antibodies, as in panel C.

quence (e.g., Fig. 5B; also see reference 42), so that it is possible that Emb may also be implicated in some of the other Oct1/MEF2 sites described.

Binding of MEF2 to the AT-rich sequence described here appears to be indirect, via interaction with Emb. In fact, we could not identify any consensus MEF2 site, although as seen for the Mhox site in the *M-CPK* enhancer (10), MEF2 can bind to some other AT-rich sequences. However, there is also no competition with known MEF2 binding sites, and we therefore conclude that MEF2 is present in a complex with Emb, which renders it inaccessible for DNA binding. The DNA footprint also points to DNA binding through Emb. No direct interaction, as monitored by the yeast two-hybrid system or coimmunoprecipitation assays, was detectable between Emb and MEF2D (data not shown), indicating that the interaction may require DNA. Complexes a and b are seen with both myoblast and myotube extracts. MEF2D is present already in dividing myoblasts (4, 33), and in gel shift experiments with MEF2 antibodies, it is the antibody to this isoform which completely shifts band b. Because of the position of band a at the top of the gel, it is difficult to distinguish a shift; however, the bead experiment, where MEF2D is as abundant as p300 in the eluate, strongly suggests that it is also present in the large complex a. This is also indicated by the ChIP experiment. The 3′ AT-rich sequence alone does not enhance reporter gene transcription with the proximal promoter. Functional assays were carried out in the context of the larger enhancer sequence, which includes the E box, which is essential for activity and binds myogenic factors of the MyoD family. These activate muscle gene transcription in differentiating muscle cells only, rendering the enhancer inactive in myoblasts. There is a MEF2D isoform, which as a result of differential splicing has a muscle-specific exon (33). In the absence of specific antibodies, it was not possible to see if this is the form present in the complex with Emb in myotubes. If this is so, then, like the more ubiquitous MEF2D form present in myoblasts, it is not capable of transactivating the enhancer alone. Other MEF2 isoforms, such as MEF2C, accumulate in myotubes. MEF2C is also a major isoform in cardiac muscle, and indeed, *cardiac actin* gene expression is notably downregulated in the hearts of $MEF2C^{-/-}$ mutant mice (30), probably due to an essential MEF2C binding site in a more proximal cardiac enhancer 5′ to this gene (M. Lemonnier and M. Buckingham, unpublished data).

MEF2 does not appear to play a primary role as a transcriptional transactivator of the *cardiac actin* enhancer but may rather be instrumental at the level of chromatin remodeling. MEF2 is known to interact with class II HDACs (35), and one possibility was that a MEF2D/HDAC complex might prevent premature expression of the *cardiac actin* gene in myoblasts or in other cell types where MEF2D is expressed (4). However, this is not the case, and it is the histone transacetylase p300 which is present in the large complex a, in both myoblasts and myotubes. In vitro experiments have shown that p300 interacts with MEF2C through the MADS box domain and that this stimulates transactivation by this factor of skeletal muscle gene regulatory sequences, such as the *M-CPK* enhancer (50). Such MEF2-p300 interaction may also exert an effect through the stabilization of essential MyoD/p300 complexes on adjacent E-box sites (16). The increased immunoprecipitation by p300

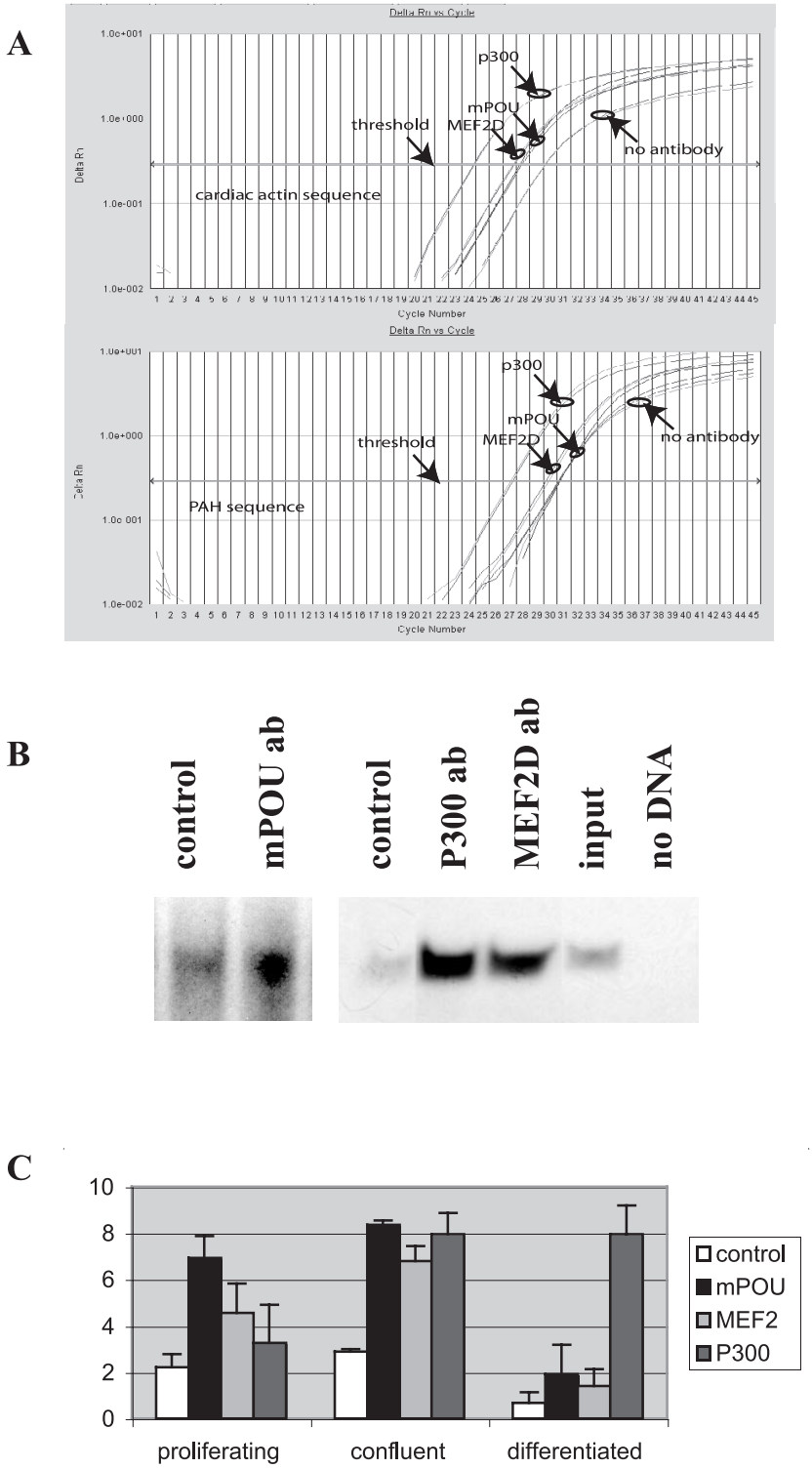


FIG. 8. Chromatin immunoprecipitation of protein bound to the 3′ AT-rich sequence of the *cardiac actin* enhancer. (A) This shows the results of a real-time PCR experiment with the *cardiac actin* sequence and a neutral sequence from the PAH gene, not expressed in skeletal muscle, amplified after chromatin immunoprecipitation with antibodies to mPOU, which recognizes Emb, MEF2D, and p300. (B) Autoradiography of a gel in which the amplified products of the *cardiac actin* sequence have been separated. The control shown adjacent to the lane with the mPOU antibody is without antibody. The control in the second series with p300 and MEF2D antibodies is the hemoagglutinin antibody, which gave results similar to those with the control with no antibody. The lane marked input is the result obtained with chromatin after fixation and before immunoprecipitation. (C) Summary of real-time PCR experiments with extracts from muscle cells at different stages, as proliferating myoblasts, confluent cultures which are beginning to differentiate, and differentiated myotubes. The results are corrected with respect to the figures obtained in the same experiment with the neutral PAH sequence. The controls in this series of experiments were β-galactosidase or NFYB antibodies, which gave results similar to those with the no-antibody control. The results are the average of at least three estimations.

in the ChIP experiment, with extracts from differentiated myotubes, probably reflects such a MyoD/p300 complex acting through the essential E box. In the 3′ sequence of the *cardiac actin* enhancer, it is clear that MyoD, which can interact with MEF2 in the absence of an E box (40), is not involved in the MEF2/p300 complex. In cardiomyocytes, MEF2D has been shown to interact with p300, and the MEF2 site where this interaction takes place in the *skeletal actin* gene is essential for expression both in cardiac cells in culture and in the heart (55). As in other examples, p300 increases the transcriptional transactivation exerted by MEF2D. Interestingly, however, only MEF2D, and not other MEF2 isoforms, appears to be capable of interaction with p300 on MEF2 regulatory elements from a number of muscle genes in this cardiac context. When overexpressed, MEF2 isoforms appear to function similarly, as in the case of interaction with HDACs shown for MEF2A (29, 38) or MEF2C (31, 60), and it is not clear how the isoforms differ functionally. Both the results of Slepak et al. (55) and those reported here for the *cardiac actin* sequence suggest a preferential interaction of p300 with MEF2D in both cardiac and skeletal muscle cells. It has been shown that inhibition of p300/CBP in skeletal muscle cell cultures prevents the expression of genes encoding structural muscle proteins, such as myosins and actins (44), but does not affect the first stages of differentiation, such as the p21-mediated block to cell cycling or activation of the myogenic regulatory factor myogenin, which depend on another histone acetyltransferase, PCAF (47). The PCAF gene is not an essential gene in mice, but in embryos with a null mutation in the gene for the related histone acetyltransferase, GCN5, skeletal muscle formation is compromised (65). *p300*$^{-/-}$ mutant mice die during embryogenesis due to severe cardiac defects (67).

The Emb protein is a component of complexes a and b. DNA footprinting and competition experiments show that it binds to the AT-rich sequence, to a motif, TATTTCTATA ATG, which is similar to the central motif TATTA TAAT described for the rat protein Brn5 (49) or ATTA$(N)_3$TAAT for the human mPOU protein (63). Mutation of the 3′ TAAT part of the motif, in particular, affects activity of the enhancer. Mutation of the 5′ ATTT has less effect, but this may be because there are AT-rich sequences immediately upstream; AAAT$(N)_7$TAAT and TAAT$(N)_7$TAAT are also preferred binding sites (63). Given the conservation of POU specific and POU homeo domains, it is probable that Emb, like Oct1 (58), contacts the AT-rich motifs through these two protein domains, which are connected by a flexible linker sequence. Brn5 (Emb), again like Oct1, probably binds as a homodimer; the extended DNA binding site, typical for POU proteins, makes it likely that Emb can adopt multiple conformations (50). Indeed, the two complexes seen with the *cardiac actin* AT-rich sequence, namely, the minor complex Emb-MEF2D (b) and Emb-MEF2D-p300 (a), may reflect this phenomenon. The Emb protein identified in C2 muscle cell extracts and, after binding to the 3′ AT-rich sequence, on Western blots using an mPOU antibody, migrates at about 80 kDa. This is twice the size predicted on the basis of sequences generated from the single *Emb* gene (42). The human mPOU (58) and rat Brn5 (1) mRNA sequences, as previously described, would also code for a protein of about 40 kDa. In zebrafish, a longer transcript, encoding a 64-kDa form, has been identified (25). We have carried out 5′ rapid amplification of cDNA ends experiments from the first exon of mouse *Emb* and found additional transcripts, including a major species with an open reading frame encoding a protein of about 80 kDa (48a). This protein contains the POU domain characteristic of the POU VI class to which Emb belongs, but it also has homology in the NH2 part to bromodomain-containing proteins. This domain, which is found in a number of transcriptional regulators involved in chromatin remodeling, specifically binds to acetylated lysine residues (11). These are generated by acetylation of histones, and it is therefore possible that Emb, as well as p300 itself, which has a bromodomain, interacts with histone tails, possibly recognizing different features of the histone acetylation code (58). Histone transacetylases may themselves be acetylated (27), providing a possible mechanism for Emb-p300 interactions. Tests of function with the shorter form of Emb have given contradictory results, and its potential transcriptional targets are ill defined. In vitro experiments with rat Brn5 (1) and human TCFβ1/mPOU (36) suggest that it may act as a transcriptional activator, while other experiments with human mPOU might suggest repression, possibly by competition with Oct1 (62). Based on our observations, we would suggest that it is involved in transcriptional activation, via chromatin remodeling, and that this probably involves the NH2 domain, absent from the shorter forms tested. *Emb*/*Brn5*/*mPOU* are expressed in a number of adult tissues, including brain and striated muscle (1, 36, 42, 62). Brn5 is high in postmitotic neurons, and overexpression of the 40-kDa form has a negative effect on proliferation, although it does not affect differentiation (8, 9). It has also been detected in adult myelinating Schwann cells, where its transcription is regulated by neuregulin (64). In the mouse, in situ hybridization with an Emb probe detects transcripts in the postimplantation embryo, especially in the central nervous system (42). In another report (14), based on RNase protection, *Emb* expression was found to be higher in embryonic and fetal muscle and lower in the developing heart and brain; in myogenin$^{-/-}$ mutant embryos, Emb levels were not affected, as might be expected given its presence in myoblasts. In both adult and embryonic tissues where Emb transcripts have been detected, the 80-kDa protein is accumulated (48a). It is potentially interesting that MEF2, like Emb, is high in brain and striated muscle, although MEF2D is detected in many cell types (4).

The distal enhancer of the mouse *cardiac actin* gene is active in embryonic skeletal muscle in vitro and in differentiating skeletal muscle cell cultures (2, 3). However, in addition, the presence of this upstream region renders transgene expression more reproducible; indeed, in conjunction with the sequence which also corresponds to a DNase I-hypersensitive site at −5 kbp from the gene, 100% of transgenes express correctly in heart and skeletal muscle (3). In addition, displacement of the distal enhancer by insertion of a partial duplication of the gene results in low levels of cardiac actin in the heart as well as in skeletal muscle (19). The DNase I-hypersensitive site at kbp −7 maps to the 3′ end of the enhancer, where the AT-rich sequence is located. Activation of the *cardiac actin* gene in striated muscle may depend on an initial opening of chromatin around the locus through the Emb-MEF2D-p300 complex, which would already function in myoblasts, followed by activation of this and other enhancers by cardiac or skeletal muscle

transcription factors as the cells differentiate. It is not clear in this model, at present, how the initial chromatin remodeling is restricted to striated muscle, since Emb, MEF2D, and p300 are present in other tissues. However, it may depend on events such as phosphorylation of MEF2D (35) or Emb (1) by convergent signaling pathways, which would build in a tissue-specific response. Such remodeling of chromatin is also likely to depend on a number of effectors, including, in skeletal muscle precursors, the myogenic determination factors Myf5 and MyoD (20). Other types of chromatin remodeling activities, such as methylases and Swi/Snf complexes (58), are also likely to be involved in rendering muscle genes susceptible to transcription; the Emb-MEF2D-p300 complex described here is one potential player in this complex process.

## ACKNOWLEDGMENTS

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