Expression of myogenin during embryogenesis is controlled by Six/*sine oculis* homeoproteins through a conserved MEF3 binding site

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ABSTRACT Myogenin, one of the MyoD family of proteins, is expressed early during somitogenesis and is required for myoblast fusion *in vivo*. Previous studies in transgenic mice have shown that a 184-bp myogenin promoter fragment is sufficient to correctly drive expression of a β-galactosidase transgene during embryogenesis. We show here that mutation of one of the DNA motifs present in this region, the MEF3 motif, abolished correct expression of this β-galactosidase transgene. We have found that the proteins that bind to the MEF3 site are homeoproteins of the Six/*sine oculis* family. Antibodies directed specifically against Six1 or Six4 proteins reveal that each of these proteins is present in the embryo when myogenin is activated and constitutes a muscle-specific MEF3-binding activity in adult muscle nuclear extracts. Both of these proteins accumulate in the nucleus of C2C12 myogenic cells, and transient transfection experiments confirm that Six1 and Six4 are able to transactivate a reporter gene containing MEF3 sites. Altogether these results establish Six homeoproteins as a family of transcription factors controlling muscle formation through activation of one of its key regulators, myogenin.

Skeletal muscles are formed through a process involving successive steps of determination of mesodermal precursor cells into myoblasts, fusion of these cells into myofibers, and their maturation. Although the commitment of mesodermal cells into the myogenic lineage depends on the basic helix–loop–helix (bHLH) transcription factors MyoD and Myf-5 (1, 2) and on the paired-homeoprotein Pax3 (3, 4), their differentiation is controlled by the product of another gene of the MyoD bHLH family, named myogenin. In mice with a targeted inactivation of myogenin, no skeletal muscle is formed: the few myotubes that appear die and no secondary fibers are formed (5–7). The genes acting upstream of myogenin and controlling its expression therefore are crucial for the development of vertebrate musculature. The cis-acting regulatory sequences of the myogenin gene have been delineated both *in vitro* and *in vivo*, and it has been shown that the promoter fragment spanning the −184/+18 bp (relative to the transcription start site) was sufficient to confer to a *LacZ* reporter gene a pattern of expression mimicking that of the endogenous myogenin gene during embryogenesis (8–10). Several binding sites for transcription factors are present in this promoter region, and most attention has been focused on the evolutionarily conserved binding sites for the muscle-specific transcription factors MyoD and MEF2. Mutation of either motif had little effect on myogenin promoter expression, but the mutation of

both impaired myogenin activation (8, 9). A third evolutionarily conserved motif present in the −184/+18 fragment is MEF3 (consensus sequence TCAGGTT). MEF3 motifs are found in many other skeletal muscle-specific regulatory regions and have been shown to be involved in the transcriptional regulation of the cardiac troponin C gene (11) and the aldolase A muscle-specific promoter both *in vitro* (12) and *in vivo* (13, 14). In the present study we report that mutation of the MEF3 site present in the myogenin promoter abolishes expression of a myogenin β-galactosidase transgene during embryogenesis and that members of the Six homeoprotein family are able to bind to different MEF3 sites.

MATERIALS AND METHODS

Plasmids and Oligonucleotides. Screening of 10^6 plaque-forming units (pfu) of a mouse adult muscle λgt11 phage library (CLONTECH) with a cDNA spanning the 3′ coding region of *Six4* (corresponding to amino acids 321–776) revealed 25 positive pfu. The isolated cDNA with the longer 5′ end is similar to the published sequence of Six4sk (15). The full-length cDNA was cloned into the pCR3 expression vector (Invitrogen). The *Eco*RI–*Xba*I fragment corresponding to amino acids 414–776 was subcloned into pET28 (Novagen) to allow protein production. Six1 cDNA was obtained by reverse transcription–PCR (RT-PCR) on mRNA from adult muscle by using the Bam-ATG-primer ggatccgccatggggcaggggcgt-gcgtgtg and reverse Not primer gcggccgccccaatatctccccact. The entire 820-bp sequence was compared with the sequence already published (16). One difference was noted at amino acid 46 where Ala gct is replaced by Ala gcc. The full-length Six1 cDNA was further cloned into pET 28 to allow protein production. The 3′ coding region of *Six5* was obtained by RT-PCR on mRNA from mouse adult muscle by using the Not-primer gcggccgcgagtctgatgggaaccccac and reverse Xba-primer tctagaagtggttaaatgcaggca. Both *Six5* and *Six1* were cloned into pCR3. For transfection experiments, the pBLCAT2 plasmid was digested by the restriction enzymes *Hin*dIII and Asp-718, and the thymidine kinase promoter (tk-105)-chloramphenicol acetyltransferase insertion was cloned downstream of a multimerized aldolase A MEF3 site (six repeats of tatgtcaggggcttcaggtttcccta) introduced into pKS+.

Northern Blot. For Northern blot experiments, poly(A)+ mRNA from adult liver (5 μg) or gastrocnemius muscle (1 μg) were separated on a denaturing 1% (wt/vol) agarose gel.

Transferred RNA were hybridized with either Six1, Six4, or Six5 probes. The size of the hybridizing mRNA was estimated by comparison with standard RNA markers.

Protein Purification, Antibodies (Abs), and Western Blot Experiments. Six1 and Six4 proteins were produced in BL21(DE3) pLysS competent bacteria (Novagen). Bacteria were lysed in 6 M urea, 5 mM imidazole, 0.5 M NaCl, 20 mM Tris·HCl, pH 7.9. Target proteins were purified on affinity columns by using pET His Tag systems (Novagen). Fractions containing His-Six1 and His-Six4 recombinant proteins were eluted with 1 M imidazole and further separated on SDS-polyacrylamide gels. Rabbits were immunized (CovalAb, Lyon, France) with, respectively, 1 mg and 800 μg of His-Six1 and His-Six4 proteins in polyacrylamide slices. Abs against Six1 were unable to detect *in vitro*-translated Six4 protein in Western blot experiments. Abs against Six1 nevertheless were purified by immunoaffinity after coupling Six1 protein on Affigel 15 (Bio-Rad). Western blot experiments were done essentially as in ref. 17. In Western blot experiments Six1 Abs were unable to recognize *in vitro*-translated Six4. Although these Abs are directed against the whole Six1 protein (including domains conserved among Six proteins), they were unable to recognize these conserved motifs in the other members of the Six family. Abs against Six4 are directed against the carboxyl-terminal half of the protein, devoid of the conserved motifs. In band shift assays, we never observed cross reactions: Six4 Abs do not crossreact with Six1 or Six5, nor does Six1 Ab crossreact with Six5 or Six4. Both Six1 Ab and Six4 Ab were unable to detect any liver nuclear proteins in the range of 60 to 120 kDa, the expected size of the ubiquitous Six 5 protein (71 kDa, ref. 18).

Preparation of Nuclear Extracts and Embryo Extracts. Nuclear extracts from adult liver and spleen were prepared as in ref. 19 and from adult skeletal muscles or heart as in refs. 14 and 20. Mouse embryos at embryonic day (E) 10.5 were collected and dissected in PBS; head, limbs, heart, and viscera were removed. The remaining trunks of the embryos were frozen in liquid nitrogen. Embryo trunks were resuspended and homogenized in lysis buffer containing 10 mM Hepes (pH 7.6), 100 mM KCl, 3 mM MgCl$_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 0.1% (vol/vol) aprotinin. Total proteins were extracted in the presence of 0.5 M NaCl. After 10 min on ice, chromatin and insoluble proteins were pelleted by centrifugation. Supernatant was used as a source of embryo protein extracts. Gel-mobility shift assays (GMSAs) and transcription-coupled translation were performed as described (21). The sequence of the aldolase A MEF3 site used is tgaatgtcaggggcttcaggtttcccta, and the sequence of the myogenin MEF3 site used is gaggggggctcaggtttctgtggcg.

Generation and Analyses of Transgenic Mice. Introduction of the MEF3 mutation into the myogenin promoter was effected by a two-step PCR strategy, using the wild-type promoter as template and performed with the first couple of oligonucleotides between the *Xho*I mutation and bp -184 for the 5′ sequence and between the *Xho*I mutation and bp $+11$ for the 3′ sequence. After ligation, the mutation was introduced into the pSKTnls-β-galactosidase vector and sequenced. The fragment to be microinjected was isolated on a 1% agarose gel after digestion by *Hin*dIII and *Bam*HI, followed by electroelution and purification on an Elutip column (Schleicher & Schuell). Founder embryos were genotyped by Southern analysis of genomic DNA from placenta. Transgenic mice were generated, identified, and propagated as described (14). For β-galactosidase reactions, embryos were fixed 1 hr at 4°C in PBS containing 0.2% glutaraldehyde, 1% formaldehyde, 2 mM MgCl$_2$. Embryos then were washed three times for 20 min in PBS containing 0.2% Nonidet P-40. β-Galactosidase was detected by overnight incubation at 37°C in PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide,

2 mM MgCl$_2$, and 0.2% X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside).

Cell Culture and Transfection Experiments. One day before transfection, 1.5×10^5 C2C12 myoblasts were plated in 5-cm tissue culture dishes. The cells were transfected as in ref. 21 with 5 μg of test chloramphenicol acetyltransferase (CAT) plasmid, 0.25 μg of pRSVluci and pCR3 containing cDNAs for Six1 or Six4, or just pCR3. Forty-eight hours after transfection, at a stage when C2C12 cells were not yet fused, cells extracts were prepared and assayed for luciferase and CAT activities as reported (21). Chicken primary myocyte transfection was performed as described (14).

RESULTS

Role of the MEF3 Site Present in the Myogenin Promoter During Embryogenesis. We investigated, by using transgenic mice, the importance of this MEF3 motif for myogenin expression during mouse embryogenesis by comparing the expression pattern of a *LacZ* reporter gene driven by the $-184/+11$ promoter region harboring a mutation of the MEF3 motif (transgene $-184mutMEF3$) to that driven by the wild-type $-184/+11$ promoter (transgene $-184nlsLacZ$). Although at E11.5, transgene $-184nlsLacZ$ is very efficiently expressed in 11 of 12 founders in developing muscles (myotome, intercostal, limb, and jaws), the expression of transgene $-184mutMEF3$ is greatly impaired (Table 1): in five of eight lines studied no expression is found from E10.5 to E13.5. In the other three lines, expression of the reporter gene was very weak and was restricted to a limited subset of the wild-type myogenin expression domains: small stripes of cells in the myotomes as well as additional stripes of cells in the ventral aspect of thoracic myotomes at E11.5 (Fig. 1) and developing intercostal muscles at E13.5 (not shown). This pattern is reminiscent of that obtained with mutation of the conserved MEF2 site (8). However, in this latter case, activation in limb muscles was delayed only to E12.5, whereas no expression could be detected as late as E13.5 when the MEF3 sequence is mutated. At no stage (from E9.5 to E13.5) was the $-184mutMEF3$ transgene expression observed in limb or head muscles (Table 1). We conclude from these results that the MEF3 site is crucial for correct myogenin expression, and therefore that MEF3 proteins may play an important role in myogenesis.

Identification of the Proteins Able to Bind to MEF3 Sites. We noticed that the MEF3 motif closely resembles the "*are*" regulatory element of the ubiquitously expressed Na$^+$/K+ ATPase α1 subunit gene. Because proteins of the Six/*sine oculis* family bind the *are* motif (15), we examined whether Six proteins could bind to the MEF3 site. Among the five *Six* genes cloned in mammals (15, 16, 22–25), mRNA of two of them

Table 1. The MEF3 regulatory sequence is required for myogenin expression in transgenic mice

Transgene	Stage	No. of independent transgenes expressed in	
		Myotome/trunk muscles	Limb/jaw muscles
$-184nlsLacZ$	E11.5	11/12	11/12
$-184mutMEF3$*	E10.5	2/6†	0/6
	E11.5	3/8†	0/8
	E13.5	2/6†	0/6

*MEF3 sequence from the murine myogenin promoter ($-90/-100$) was changed from CTCAGGTTTCT to CTCgaGgggCg (consensus MEF3 motif underlined).

†At E10.5 expression of the ß-galactosidase transgene was restricted to small stripes of cells in the myotome that formed a small dorso ventral stripe at the level of the notochord. At E11.5 and E13.5 an additional stripe of cells expressing the transgene was localized in the ventral aspect of the thoracic myotomes.
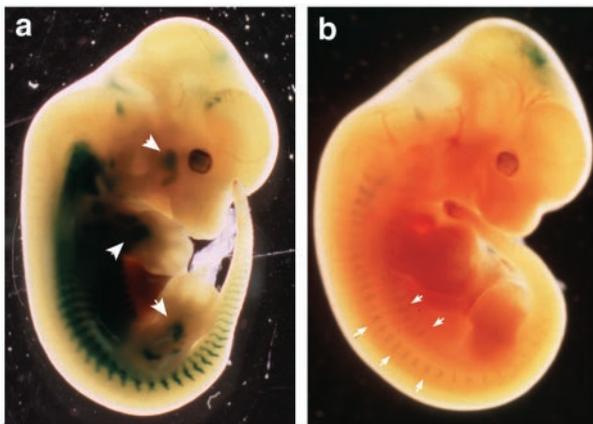
FIG. 1.   The MEF3 motif is crucial for myogenin promoter activation during embryogenesis. One mouse founder embryo carrying the nls-β-galactosidase reporter under the control of a wild-type myogenin −184 promoter (*a*) and one F$_1$ transgenic mouse carrying the β-galactosidase reporter under the control of a mutated MEF3 myogenin −184 promoter (*b*) were sacrificed at E11.5. At E11.5 the control transgene (*a*) gives a β-galactosidase activity in the limbs and in the jaws muscles (arrow heads), which is not observed with the mutated promoter. The transgenic mouse harboring the −184mutMEF3 transgene (*b*) was one of those with a faint expression of the transgene in a small stripe of cells in the myotomes and an additional stripe of cells in the lateral part of the myotome between fore and hind limbs. The ectopic expression in the brain was not reproduced in other independent transgenic lines.

(*Six1* and *Six4*) accumulate specifically in adult skeletal muscle (15, 24), and for one of them (*Six1*) is abundant in somites, as early as E8.5, during mouse embryogenesis (16). Fig. 2*a* shows that recombinant Six1 and Six4 proteins both bind specifically to the myogenin MEF3 site. The same result was observed with the MEF3 site of the aldolase A muscle-specific (pM) promoter (Fig. 2*a*). Fig. 2*b* shows that various mutations of the pM MEF3 site have the same consequences on skeletal muscle MEF3 binding activity and binding of recombinant Six4 protein. Thus, Six4 binds the MEF3 sites with the same relative affinity and specificity as the muscle MEF3 proteins. GMSAs performed with nuclear extracts from various adult tissues, using either the aldolase A or the myogenin MEF3 sites, revealed the formation of an ubiquitous MEF3 complex, as well as the formation of two skeletal muscle-specific complexes migrating, respectively, slower and faster than the ubiquitous one (Fig. 3 *a* and *b*). These three complexes also can be detected with the myogenin MEF3 site when incubated with proteins from E10.5 embryos (Fig. 3*b*). With both adult and embryo extracts the fast migrating complex was suppressed by anti-Six1 Abs, whereas the slowest migrating complex was suppressed by anti-Six4 Abs (Fig. 3). These Abs did not react with the ubiquitous complex (Fig. 3). These data demonstrate that the adult muscle-specific MEF3 complexes are composed of Six1 and Six4 proteins and that Six1 and Six4 DNA binding activities are already present early during embryogenesis at the time of myogenin activation. Northern blot and Western blot analysis showed that *Six1* and *Six4* genes are expressed specifically in skeletal muscles, giving a 44-kDa protein for Six1 and a 98-kDa protein for Six4 (Fig. 4), which fit with the weights predicted from the cDNA sequences (15, 16). In contrast, the *Six5* gene is expressed more ubiquitously (Fig. 4) and is probably responsible for the ubiquitous MEF3 complex (Fig. 3 *a* and *b*), at least in the liver (18). Six5 has been described as a 71-kDa protein, expressed in the liver as well as in other tissues (18); Six5 protein was not detected by the specific Abs against Six1 or Six4 in band-shift assays or in Western blot experiments (Fig. 3 and data not shown).
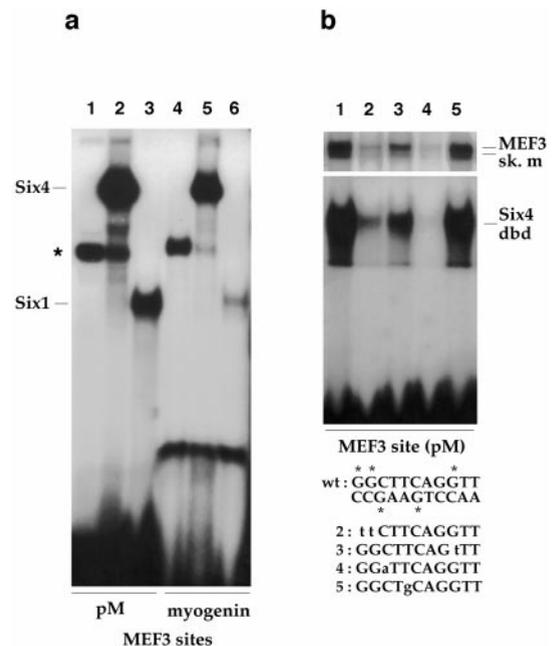

FIG. 2.   Six1 and Six4 are able to bind the myogenin and aldolase A MEF3 motifs. (*a*) GMSAs performed with recombinant full-size Six4 (from *in vitro* T7-synthetized Six4 mRNAs translated in a rabbit reticulocyte lysate) and Six1 proteins (recombinant proteins purified after production in bacteria, see *Materials and Methods*). Mock lysate (lanes 1 and 4), Six4 translation products (lanes 2 and 5), or Six1 recombinant proteins (lanes 3 and 6) were incubated with labeled MEF3 sites from aldolase A (pM) or myogenin genes. ∗ indicates a nonspecific lysate binding activity. (*b*) GMSAs were performed with aldolase A MEF3 site (pM) and with either skeletal muscle (sk.m) nuclear extracts (*Upper*) or recombinant Six4 protein [corresponding to amino acids 1–240 of the AREC protein encompassing the DNA-binding domain (dbd) defined in ref. 15]. In lanes 2–5, 30 ng of double-stranded MEF3 site mutated in different nucleotides was added as competitor (lane 1, no competitor). ∗ indicate the G residues whose methylation inhibits protein binding in a dimethyl sulfate (DMS) interference assay using muscle nuclear extracts (not shown). The mutations were in the bases defined by DMS interference on the MEF3 complex, and the mutated MEF3 sequence site is indicated with mutated bases in small letters. Note that mutation of the T (mut5) in the sequence TCAGG completely abolished the competition, thus showing that this nucleotide is absolutely required for the binding of Six protein to the MEF3 site.

SIX1 and SIX4 Proteins Expression in Myogenic Cells. Immunohistochemical detection of Six1 and Six4 with specific polyclonal antisera (which recognize specifically each protein) revealed that both proteins are present in the nuclei of C2C12 myoblasts and myotubes, with a preferential accumulation of Six1 and Six4 in myotube nuclei as compared with myoblasts (Fig. 5), which fits well with a role for these proteins in controlling myogenin expression. Accordingly, both Six1 and Six4 mRNAs can be detected by Northern blot experiments using C2C12 total RNAs (not shown). To assay the transcriptional activity of Six/MEF3 complexes, we tested whether multimerized MEF3 motifs were able to activate a basal promoter. As shown in Fig. 6*a*, a hexamer of MEF3 sites cis-activates the herpes virus simplex tk-105 in differentiated chicken primary myotubes, but not in myoblasts. Therefore, in this cell culture model, the MEF3 motif acts as a myotube-specific cis-activator. In a second set of experiments, C2C12 myoblasts were transiently cotransfected with the 6xMEF3-tk construct and increasing amounts of Six expression vectors: as shown in Fig. 6*b*, Six4, and more weakly Six1, can activate transcription through MEF3 binding sites in this model. However, al-
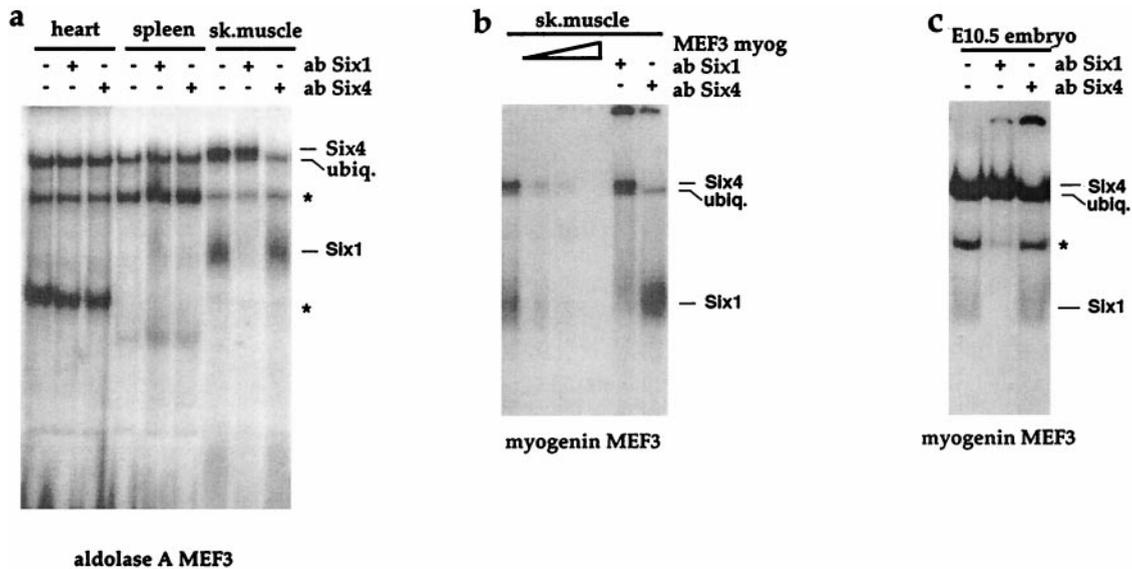
FIG. 3.   Six1 and Six4 are the proteins that form the MEF3 muscle-specific binding activities. (*a*) GMSAs performed with adult nuclear extracts from different tissues on the MEF3 site of aldolase A. Five micrograms of nuclear extracts from heart, spleen, and skeletal muscles from the limb (sk.muscle) were incubated on ice with 0.3 ng of labeled double-stranded MEF3 site (pM). Six1 Ab or Six4 Ab were added subsequently thereafter, and the incubation mix then was kept on ice for 5 min. ∗ indicate nonspecific DNA-protein complexes (faintly competed by an excess of double-stranded MEF3 site). MEF3-binding activity comprises a ubiquitous complex (ubiq. MEF3) detected in each nuclear extract (including liver, not shown) and two muscle-specific complexes that did not form in the presence of anti-Six1 and anti-Six4 sera, respectively. Preimmune sera were not able to displace these complexes (not shown). In contrast, Abs against both Six1 and Six4 abolished the fast and slow migrating bands of the MEF3 DNA-protein complexes, respectively. (*b*) GMSAs performed with adult skeletal muscle (sk.muscle) nuclear extracts in the presence of the myogenin MEF3 site. Five micrograms of nuclear extracts were incubated on ice with 0.3 ng of labeled double-stranded myogenin MEF3 site. Increasing amounts (5, 15, or 50 ng) of myogenin MEF3 site was added in the reaction mix, as competitor. Abs against Six1 (Six1 Ab) or Six4 (Six4 Ab) were added subsequently, and the reaction mix then was kept on ice for 5 min. (*c*) GMSAs performed with protein extracts from embryonic trunks at E10.5 in the presence of the myogenin MEF3 site. Twenty micrograms of E10.5 protein extracts were incubated on ice with 0.3 ng of labeled double-stranded myogenin MEF3 site. Abs against Six1 (Six1 Ab) or Six4 (Six4 Ab) were added subsequently, and the reaction mix then was kept on ice for 5 min. ∗ indicates a nonspecific complex, which is not competed by an excess of MEF3 oligonucleotide and is not reproducibly displaced by Six1ab. ubiq., ubiquitous complex.
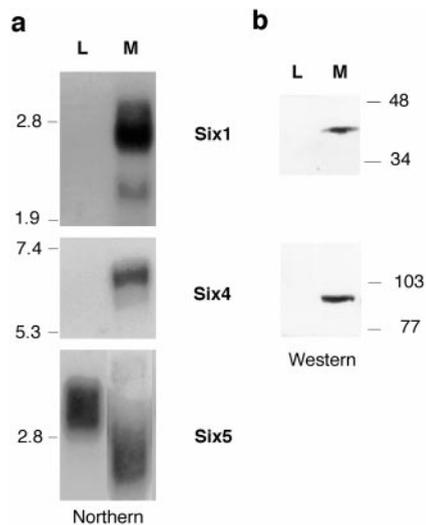
though significant, this activation remains weak (about 2.5-fold for Six4 and 2-fold for Six1).

DISCUSSION

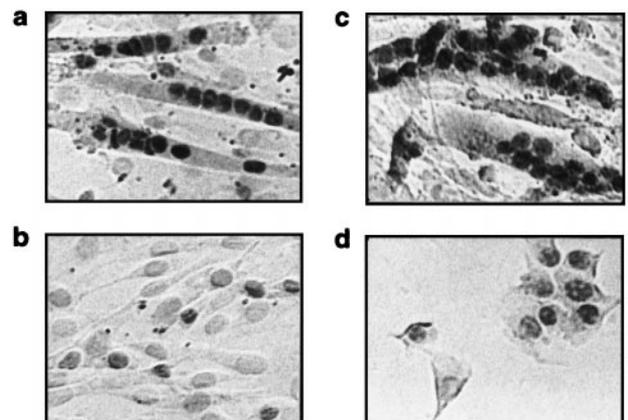We have demonstrated by studies of transgenic mice the importance of the MEF3 motif present in the myogenin

FIG. 4.   Expression of Six genes: analyses by Northern blot and Western blot. (*a*) One microgram of poly(A)+ mRNA from adult limb muscle (M) or 5 μg of poly(A)+ mRNA from adult liver (L) was detected by successive hybridization with specific cDNA probes complementary to *Six1, Six4,* or *Six5*. Size marker positions are indicated on the left in kb. Exposure times were for 6 hr (Six1), 24 hr (Six4 and Six5/L), and 5 days (Six5/M). (*b*) Western blot analyses were performed with 50 μg of nuclear extracts from adult liver (L) or adult skeletal muscle (M). Immunoreactive proteins were revealed with 1/100 dilution of purified antiserum against Six1 or 1/2,000 dilution of antiserum against Six4. The proteins detected with these antisera were not detected with preimmune antisera (not shown). The size of molecular mass standards is indicated in kDa.

FIG. 5.   Nuclear localization of Six1 and Six4 proteins. C2C12 cells (*a* and *c,* differentiated myotubes; *b* and *d,* proliferating myoblasts) were fixed on gelatin-coated slides with 4% formaldehyde at room temperature. Six1 and Six4 proteins were detected by 1:100 dilution of purified antiserum against Six1 (*a* and *b*) or 1:2,000 dilution of antiserum against Six4 (*c* and *d*). Horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) was applied at 1:200 dilution, and then the immunoperoxidase reaction was used to reveal cellular localization of Six proteins. No signal was observed with the corresponding preimmune sera (not shown).
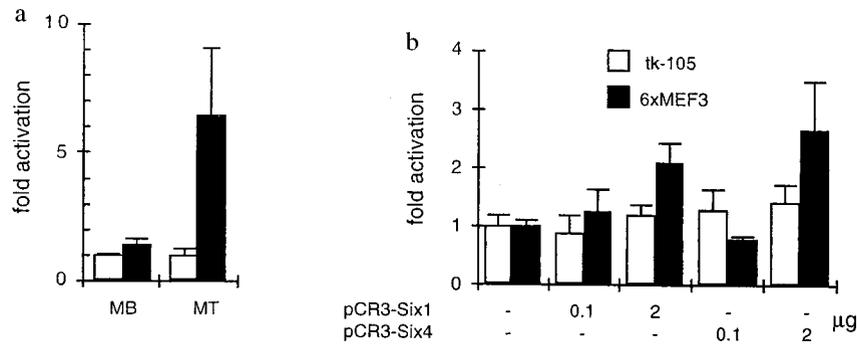
FIG. 6.   Cis- and trans-activating properties of MEF3/Six complexes. (*a*) Activity of the herpes virus simplex tk minimum promoter with (6xMEF3-tk; filled bars) or without (tk-105; empty bars) six multimerized MEF3 motifs, in chicken primary myoblasts (MB) and myotubes after 4 days of differentiation (MT). (*b*) 6xMEF3-tk or tk-105 reporter constructs were transfected with an increasing amount of pCR3 expression vectors containing cDNA for Six1 or Six4 in C2C12 myoblasts. The amount of expression vector used is indicated in μg. Activity was measured 48 hr after transfection.

promoter for its activation and have characterized the MEF3 binding activity as consisting of two skeletal-muscle specific members of the Six family, Six1 and Six4. These two nuclear proteins were shown to transactivate transcription through this DNA motif. Both Six1 and Six4 proteins are present in the embryo at E10.5 and are able to bind to the myogenin MEF3 site. Because Six1 mRNA is expressed as early as E8.5 in somites, before myogenin induction, with whom it is later coexpressed (16), these data are consistent with a major role for the *Six1* gene in the control of early steps of myogenesis. In contrast, Six2, Six3, and Six4 do not seem to be expressed in the somites of mouse embryos during myogenin activation (16, 18, 22). Six1 (and perhaps Six5, whose expression during somitogenesis is not yet documented) seems to be the best candidate to control early activation of myogenin, and thus early steps of myogenesis, in cooperation with MEF2 and myf5/MyoD proteins (8, 9, 26).

In vitro, myogenin also is expressed during myogenic cell differentiation. We show here that MEF3 motifs confer a myotube-specific transcriptional activation on the neutral tk promoter, indicating that the transcriptional activation potential of Six proteins increases concomitantly with myogenin transcription during *in vitro* myogenesis.

Six proteins are the mammalian homologues of *Drosophila sine oculis* (*so*), which is required for the different steps of eye formation (27). Together with eyeless (a protein homologue of vertebrate Pax proteins) and eye absent (*eya,* whose vertebrate homologues Eya have been cloned recently; refs. 28 and 29), *so* has been shown to act within a network of regulators (30, 31), which synergistically drive *Drosophila* eye morphogenesis. In addition to our finding that Six proteins play an important role in the early steps of myogenesis, it has been demonstrated that Pax3 is required to activate somitic myogenesis (3, 4). It is thus possible that Pax, Six, and Eya proteins, all of which are coexpressed during vertebrate somitogenesis, cooperate during vertebrate muscle development, in a manner reminiscent of *eyless*, *so*, and *eya* in *Drosophila* (31). In this developmental context, *so* has been found to interact physically with *eya* (30) through protein motifs conserved between *Drosophila* and mammals. Interestingly, Eya proteins are expressed in mouse somites (29, 32–34): these proteins, which possess a powerful transcription activation domain, but are devoid of any known DNA binding domain (34), could similarly contribute to the transcriptional regulation mediated by Six proteins through MEF3 sites. Such a requirement for a synergistic interaction with Eya may account for the relatively limited reporter gene transactivation by Six4 and Six1 alone in our transfection assays.

In the adult, the requirement for MEF3 binding sites in a muscle promoter expressed in a restricted subset of fast-twitch fibers, aldolase A pM (13, 14) suggests that Six homeoproteins may play an additional role in the specification of myofiber diversity. In this respect, it is of interest to note that in a human muscle disorder (Steinert's dystrophic myotonia), a down-regulation of the human homologue of *Six5* (also named *DMAHP*) has been suspected to account for some of the clinical features observed in dystrophic myotonia patients (35, 36). Interestingly, this syndrome is associated with a selective atrophy/delay of maturation of type I/slow-twitch myofibers and a down-regulation of several genes containing MEF3 motifs in their regulatory elements (cTnC and $Na^+/K+$ ATPase subunit $\alpha 1$) (37, 38).

In conclusion, Six homeoproteins seem to correspond to an upstream level of the hierarchical cascade controlling myogenesis and skeletal muscle development.

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