Six proteins regulate the activation of *Myf5* expression in embryonic mouse limbs

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Edited by Eric N. Olson, University of Texas Southwestern Medical Center, Dallas, TX, and approved May 24, 2007 (received for review December 19, 2006)

Myf5, a member of the myogenic regulatory factor family, plays a major role in determining myogenic cell fate at the onset of skeletal muscle formation in the embryo. Spatiotemporal control of its expression during development requires multiple enhancer elements spread over >100 kb at the *Myf5* locus. Transcription in embryonic limbs is regulated by a 145-bp element located at −57.5 kb from the *Myf5* gene. In the present study we show that *Myf5* expression is severely impaired in the limb buds of *Six1*$^{-/-}$ and *Six1*$^{-/-}$*Six4*$^{-/+}$ mouse mutants despite the presence of myogenic progenitor cells. The 145-bp regulatory element contains a sequence that binds Six1 and Six4 in electromobility shift assays *in vitro* and in chromatin immunoprecipitation assays with embryonic extracts. We further show that Six1 is able to transactivate a reporter gene under the control of this sequence. *In vivo* functionality of the Six binding site is demonstrated by transgenic analysis. Mutation of this site impairs reporter gene expression in the limbs and in mature somites where the 145-bp regulatory element is also active. Six1/4 therefore regulate *Myf5* transcription, together with Pax3, which was previously shown to be required for the activity of the 145-bp element. Six homeoproteins, which also directly regulate the myogenic differentiation gene *Myogenin* and lie genetically upstream of *Pax3*, thus control hypaxial myogenesis at multiple levels.

embryonic mouse limb muscle | Pax3 | Six1

Skeletal muscles arise from myogenic progenitor cells present in the somites of the embryo. At the interlimb level, cells delaminate from the edges or lips of the epithelial dermomyotome, the dorsal part of the somite (1), to form the subjacent postmitotic myotome, which gives rise to trunk muscles. At the limb level, cells from the hypaxial (ventrolateral) lips of the dermomyotome delaminate and migrate into the limb bud to form the musculature of the limbs. This process depends on Pax3, a transcription factor present in the dermomyotome, which marks myogenic progenitor cells. Entry of progenitor cells into the myogenic program depends on the myogenic determination factors Myf5, MyoD, and MRF4, of which Myf5 and MyoD are expressed in the limbs, whereas differentiation of these cells into postmitotic muscle fibers in the limb bud is under the control of Myogenin, the fourth member of this family (see ref. 2). Myf5 is the first of the myogenic regulatory factors to be expressed in the mouse embryo, starting at embryonic day 8 (E8) in the epaxial (dorsomedial) lip of the dermomyotome, followed by its expression in the hypaxial dermomyotome and all subsequent skeletal muscles (3). At the limb bud level, it has been shown that myogenic progenitor cells do not activate *Myf5* until they reach the limb (4). During limb bud development *Myf5* is expressed before *MyoD* (5, 6), and in *Myf5*$^{-/-}$ embryos *MyoD* expression is delayed (7).

Mice and humans have six *Six* genes (Sine oculis homeobox genes, *Six1* to *Six6*) in their genome. These homeogenes are expressed in several cell types during embryonic and adult life (see ref. 8) and are involved in different types of organogenesis (9–13). In the mouse, *Six1*, *Six4*, and *Six5* genes are expressed from E8 in overlapping expression patterns in somites, limb buds, dorsal root ganglia, and branchial arches (14). Four *Eya* genes have been cloned in mice, humans, and chicks (15). Physical interactions between Six and Eya proteins, first described in *Drosophila*, are conserved in vertebrates and allow transcriptional synergy of the Six–Eya complex (16–19). *Six1* is expressed throughout muscle development, from E8 in the mouse embryo to adult skeletal muscle (19). *Six1*$^{-/-}$ fetuses die at birth and display severe but selective muscle hypoplasia in the diaphragm, forelimb, distal ventral hindlimb, and abdomen (20). *MyoD* and *Myogenin* expression is delayed in forelimbs and hindlimbs of *Six1*$^{-/-}$ embryos, whereas their expression pattern in the trunk is reduced ventrally (20). *Myogenin* expression is directly controlled by Six proteins through a MEF3 site present in its promoter (21). *Six1*$^{-/-}$*Six4*$^{-/-}$ embryos show an aggravation of the *Six1*$^{-/-}$ muscular phenotype. Notably, these double mutant embryos no longer have myogenic progenitor cells in their limb buds, resulting in muscle-less legs (22). We have shown that in both *Six1*$^{-/-}$*Six4*$^{-/-}$ and *Eya1*$^{-/-}$*Eya2*$^{-/-}$ double mutants, *Pax3* expression in the hypaxial dermomyotome is lost, leading to cell misrouting and cell death, preventing muscle progenitor cell migration into the limbs (18, 22). We have thus established that these *Six* and *Eya* genes lie upstream of *Pax3* in the genetic hierarchy of hypaxial myogenesis. In the trunk, *Six1* and *Six4* genes have been shown to control the expression of *Mrf4*, and *Six1*$^{-/-}$*Six4*$^{-/-}$ embryos also have a reduced and delayed expression of *MyoD*, *Myogenin*, and myotomal markers, whereas the early activation of *Myf5* in the epaxial somite still takes place (22).

The spatiotemporal expression pattern of the *Myf5* gene is driven by multiple DNA elements dispersed throughout its locus. The onset of *Myf5* expression in the limb bud depends on a DNA sequence located between −58 and −48 kb from the gene (23, 24). Within this sequence, a shorter region is both necessary and sufficient to drive the expression of a *lacZ* transgene in the limbs (23, 25). A 145-bp element within this region has been shown to drive *Myf5* expression in the limb buds, and this sequence is directly under the control of the Pax3 homeodomain and paired domain protein (26). We show here that Six homeoproteins are also involved in the expression of *Myf5* in limb buds through direct binding to a conserved MEF3 binding site present in the 145-bp regulatory sequence of *Myf5*, adjacent to the Pax3 binding

Author contributions: J.G. and L.B. contributed equally to this work; J.G., L.B., P.D., M.B., and P.M. designed research; J.G., L.B., J.D., and P.D. performed research; J.G., L.B., J.D., P.D., M.B., and P.M. analyzed data; and J.G., M.B., and P.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Abbreviation: E*n*, embryonic day *n*.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0611299104/DC1.

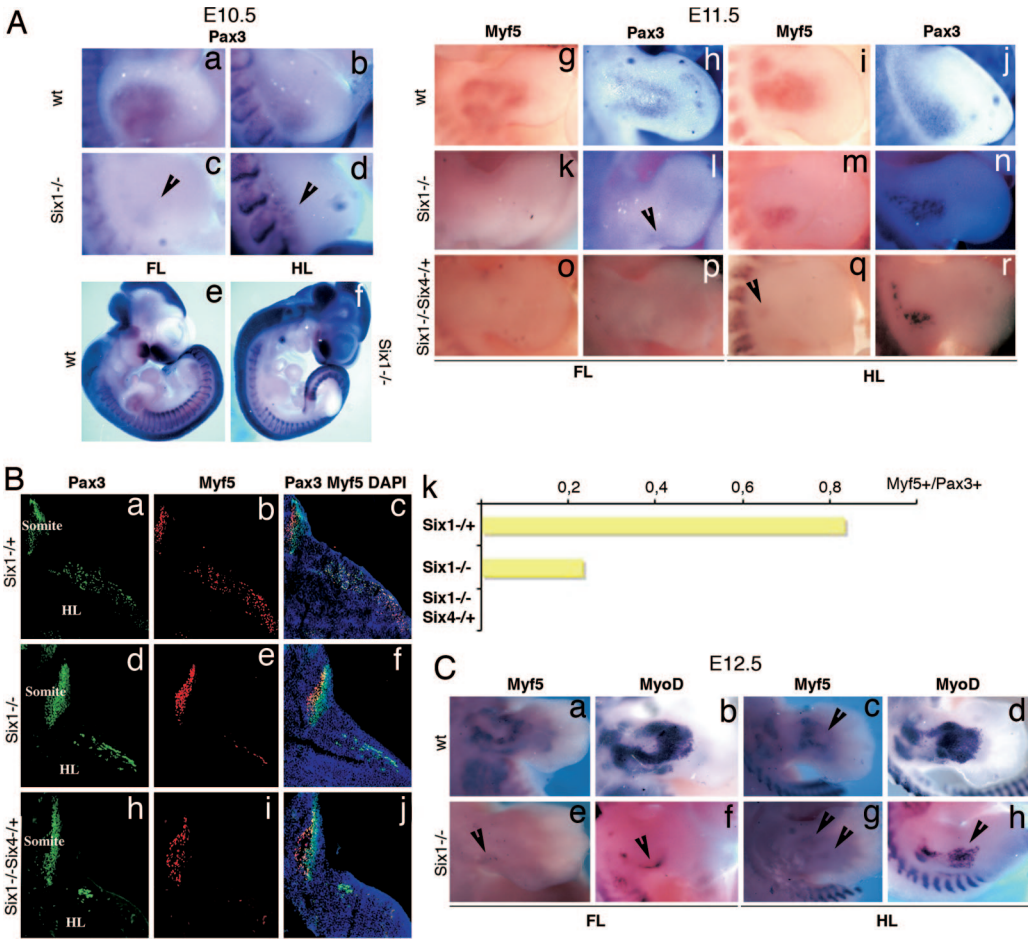

Fig. 1. Whole-mount *in situ* hybridization with *Myf5*, *MyoD*, and *Pax3* probes and immunocytochemistry data performed with Myf5 and Pax3 antibodies. (*A*) Whole-mount *in situ* hybridization with *Myf5* and *Pax3* probes at E10.5 (*a–f*) and E11.5 (*g–r*) on WT (*a*, *b*, *e*, and *g–j*), $Six1^{-/-}$ (*c*, *d*, *f*, and *k–n*), and $Six1^{-/-}Six4^{-/+}$ (*o–r*) embryos. FL, forelimb; HL, hindlimb. Arrowheads in *c* and *d* show the reduced number of myogenic progenitors expressing *Pax3* migrating into the limbs of $Six1^{-/-}$ embryos at E10.5. The arrowhead in *l* shows the few posterior cells with *Pax3* transcripts observed in the $Six1^{-/-}$ forelimb, and the arrowhead in *q* shows the few cells with *Myf5* transcripts present in the $Six1^{-/-}Six4^{-/+}$ hindlimb. (*B*) Coimmunocytochemistry on transverse sections at the level of the hindlimbs of $Six1^{+/-}$ (*a–c*), $Six1^{-/-}$ (*d–f*), and $Six1^{-/-}Six4^{-/+}$ (*h–j*) embryos at E11.5 using antibodies against Pax3 (green in *a*, *c*, *d*, *f*, *h*, and *j*) and Myf5 (red in *b*, *c*, *e*, *f*, *i*, and *j*). Nuclei are shown by DAPI staining (*c*, *f*, and *j*). Histograms indicate the number of $Myf5^{+}$ cells compared with the number of $Pax3^{+}$ cells in hindlimbs from $Six1^{-/+}$, $Six1^{-/-}$, or $Six1^{-/-}Six4^{-/+}$ embryos at E11.5 (*k*). (*C*) WT (*a–d*) and $Six1^{-/-}$ (*e–h*) embryos at E12.5 were hybridized with probes for *Myf5* (*a*, *c*, *e*, and *g*) or *MyoD* (*b*, *d*, *f*, and *h*). Enlargements at the forelimb and hindlimb levels are shown. The arrowhead in *c* shows *Myf5* expression in the dorsal region of WT hindlimb. The arrowheads in *e* and *f* show restriction of *MyoD* and *Myf5* expression to the posterior domain of the forelimb. The arrowheads in *g* point to the reduced expression of *Myf5* in the dorsal part of the hindlimb, compared with the expression of *MyoD* in this structure indicated by the arrowhead in *h*.

site. Mutation of this MEF3 site abrogates the activity of this regulatory sequence without interfering with Pax3 binding. These results show that Six1/4 and Pax3 are involved in *Myf5* transcription in the limb and further highlight the multiple steps controlled by Six proteins during muscle development.

Results

***Myf5* Expression Is Altered in the Limbs of *Six1* Mutant Embryos.** To address the requirement for Six1 in the control of *Myf5* expression in the limb buds, we analyzed its expression in relation to *Pax3*, which marks myogenic progenitor cells. Although substantially reduced, *Pax3*-positive cells are still detectable within the forelimb bud and entering the hindlimb bud from the hypaxial dermomyotome of adjacent somite at E10.5 (Fig. 1*A a–f*). At this stage, *Myf5* transcripts are not detectable in $Six1^{-/-}$ embryos (data not shown), whereas in controls they are already seen in the forelimb (4). Analysis of *Myf5* expression by *in situ* hybridization at E11.5 [supporting information (SI) Fig. 6*I*], when Pax3-positive progenitor cells present in the forelimb and hindlimb buds have engaged the myogenic program in WT embryos (Fig. 1*A g–j*), shows that transcripts are undetectable in the few *Pax3*-positive cells of the forelimb and are reduced in the *Pax3*-positive cells of the hindlimb in $Six1^{-/-}$ embryos (Fig. 1*A k–n*). In $Six1^{-/-}$ $Six4^{-/+}$ embryos, no Pax3-positive cells are detectable in the forelimb; however, at this stage a few Pax3-positive cells can be seen in the hindlimb, with barely detectable *Myf5* hybridization (Fig. 1*A o–r*). To quantify the proportion of $Myf5^{+}/Pax3^{+}$ cells at E11.5, we performed serial vibratome sections through the limb buds of embryos in which *in toto in situ* hybridization had been performed with *Pax3* and *Myf5* probes (SI Fig. 6*II*). We evaluated the extent of the $Myf5^{+}$ region compared with the $Pax3^{+}$ region. From 115% in the hindlimbs of WT embryos, this ratio drops to 35% in $Six1^{-/-}$ hindlimbs and to 25% in $Six1^{-/-}Six4^{-/+}$ hindlimbs, suggesting that Six4 can partially compensate for the absence of Six1 in controlling *Myf5* transcription in the limb. We also performed immunocytochemistry for Pax3 and Myf5 on sections of forelimb and hindlimb buds at E11.5. In $Six1^{+/-}$ embryos, most Pax3-positive cells are also Myf5-positive in the hindlimb (Fig. 1*B a–c*), whereas in the absence of Six1 most Pax3-positive cells do not express Myf5

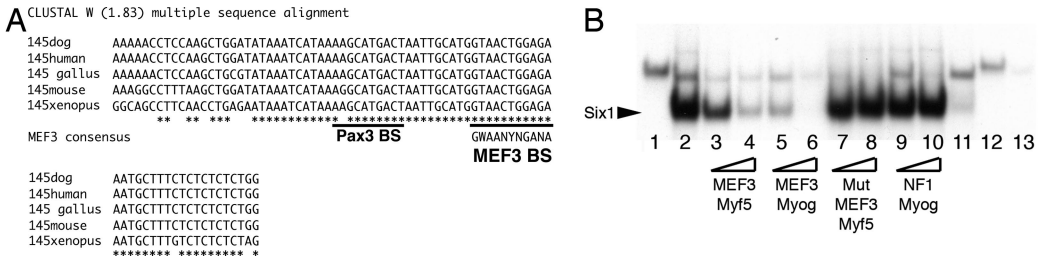

Fig. 2. The MEF3/Six binding site present in the 145-bp sequence is conserved between species and binds Six1 and Six4 proteins. (*A*) DNA sequence alignments, from different species, of the 145-bp *Myf5* element (26), showing 100% sequence conservation of the putative MEF3 sites. MEF3 and Pax3 sequences are underlined. The MEF3 consensus is shown below the MEF3 box site. (*B*) Electromobility shift assays with a 30-bp oligonucleotide containing the Myf5 MEF3 site (lanes 1–11) or a mutant MEF3 site (lanes 12 and 13), incubated with recombinant Six1 protein (lanes 2–11 and 13) or with crude reticulocyte lysate (lanes 1 and 12). Six1 antibodies (lane 11) or a 66- or 200-fold excess of MEF3-Myf5 (lanes 3 and 4), MEF3-Myogenin (lanes 5 and 6), mutant MEF3-Myf5 (lanes 7 and 8), or NF1-Myogenin (lanes 9 and 10) unlabeled competitors was added in the mix. Note that Six1 is unable to recognize the mutant MEF3 oligonucleotide.

(Fig. 1*B d–f*), and in the *Six1*$^{-/-}$ *Six4*$^{+/-}$ embryos Myf5-positive cells are absent (Fig. 1*B h–j*). These results are quantified in Fig. 1*Bk* for the hindlimb; by this approach, Myf5 was also detectable in a few Pax3-positive cells of *Six1*$^{-/-}$ forelimb (data not shown). At E12.5, *Myf5* and *Myod* transcripts are present in the posterior region of *Six1*$^{-/-}$ forelimb but not in the forelimbs of *Six1*$^{-/-}$ *Six4*$^{-/+}$ embryos, where no Pax3-positive cells can be detected (data not shown). Expression of *Myf5* is severely reduced in the hindlimbs of *Six1*$^{-/-}$ embryos at E12.5, as compared with *MyoD*, which is still expressed in the dorsal part of the limb (Fig. 1*C g* and *h* and SI Fig. 6*III*).

A Conserved MEF3 Binding Site Lies Within the 145-bp Sequence at −57.5 kb from the *Myf5* Gene. *Myf5* expression in the embryonic limb has been shown to depend on a 145-bp element located at −57.5 kb from the *Myf5* transcription start site (26). Examination of the 145-bp sequence revealed the presence of a GTAACTGGAGA motif, matching the MEF3 site consensus sequence, GWAANYNGANA, recognized by Six proteins (21, 27). Multiple sequence alignments of this site from several species show that the MEF3 motif at this position is 100% conserved (Fig. 2*A*), suggesting that it is functionally important. To test the binding of the Six1 protein to this enhancer, we performed gel electromobility shift assays using a 30-bp oligonucleotide containing this putative MEF3 site as a probe and *in vitro* translated Six1 or Six4 protein. Both Six1 and Six4 bind to the sequence (Fig. 2*B* and SI Fig. 7*A*). Antibodies raised against Six1 disrupt the formation of the Six1 DNA–protein complex (Fig. 2*B*). DNA competition experiments with the MEF3 site from the *Myf5* regulatory region or from the previously characterized MEF3 site from the promoter of the *Myogenin* gene (21) showed the specificity of the binding; the *Myf5* sequence with the MEF3 site mutated or a non-MEF3-containing sequence showed no competition (Fig. 2*B*). We next verified that the MEF3 mutation did not modify Pax3 binding to the adjacent Pax3 binding site that is essential for the activity of this *Myf5* regulatory sequence (26). Individually each protein binds to an oligonucleotide containing both sites, and, using competition experiments, Pax3 was shown to bind equally well when the Six site is mutated (SI Fig. 7*B*). Mutation in this site therefore reflects a role for Six1, independent of Pax3, in activating the *Myf5* enhancer. When Pax3 and Six1 are present together, a larger complex is formed and may correspond to both proteins binding to the same fragment (SI Fig. 7*B*).

Forced Six1 Expression Triggers the Transcriptional Activity of the 145-bp Enhancer. We next tested whether Six1 transactivates the 145-bp *Myf5* element through its MEF3 site. We placed the WT 145-bp sequence or the sequence with its MEF3 site mutated upstream of a minimal TATA box and a luciferase reporter and cotransfected these plasmids into primary chick embryonic myoblasts, with increasing concentrations of Six1 expression vector. Luciferase activity was significantly increased by cotransfection with the Six1 vector (Fig. 3). In contrast, cotransfection of the vector with the MEF3 site mutated did not result in an increase in luciferase activity. Thus, the Six1 protein is able to transactivate the 145-bp element through binding to the MEF3 site in myogenic cells, suggesting that Six proteins may regulate its activity *in vivo*.

The Six1 Protein Is Loaded on the MEF3 Site of the 145-bp *Myf5* Element *in Vivo*. Six1 and Six4 are present in the nuclei of Pax3^{+} muscle progenitor cells of the limb buds as shown by coimmunochemistry (Fig. 4*A* and data not shown), allowing Six and Pax homeoproteins to activate *Myf5* gene expression. To test whether Six binds to the *Myf5* limb enhancer in cells, we first prepared chromatin from myogenic cells of the C2 skeletal muscle cell line

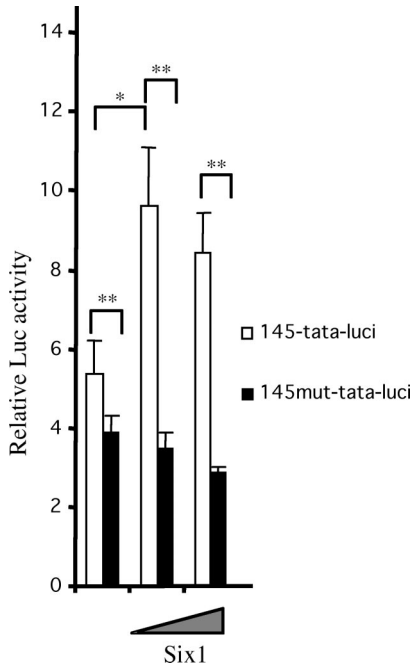

Fig. 3. The 145-bp *Myf5* element can be activated by forced Six1 expression. Primary chick myoblasts were cotransfected with a 145-tata-luci or a 145mut-tata-luci in the presence of increasing amounts of expression vectors coding for Six1. Relative luciferase activity was measured by normalizing the luciferase (Luc) 145-bp-dependent activity to the *Renilla* activity. *, $P < 0.05$; **, $P < 0.01$.

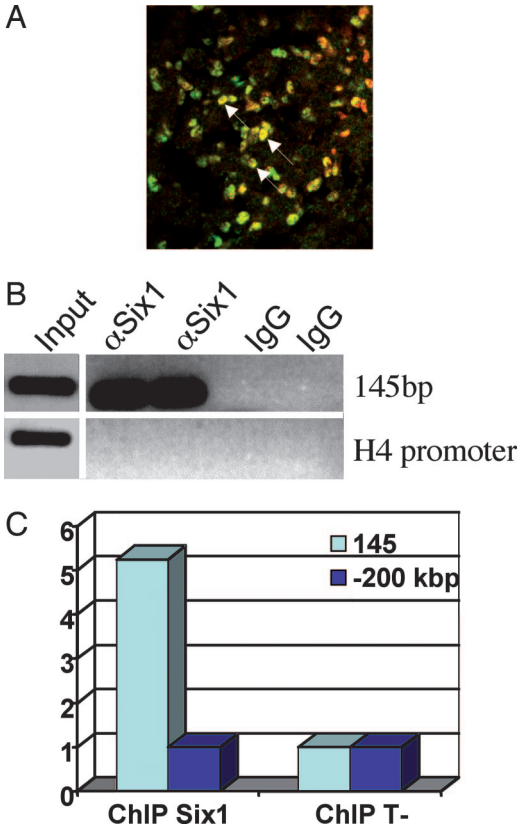

Fig. 4. Six1 is bound to the 145-bp *Myf5* element *in vivo*. (*A*) a double immunohistochemistry experiment performed on a section of a forelimb from a WT embryo at E10.5 revealing coexpression of Six1 (green) and Pax3 (red) in nuclei of myogenic progenitor cells. Cells expressing both proteins show a yellow nucleus (arrows). (*B*) ChIP assays with nuclear extracts from C2C12 myotubes. ChIP assays were performed with Six1 antibodies or IgG from preimmune goat serum. Immunoprecipitated chromatin was amplified by semiquantitative PCR to test for the selective enrichment of the 145-bp element compared with the *Histone H4* promoter, used as negative control. An aliquot of input chromatin (before ChIP) was also amplified by the same primer pairs to ensure PCR efficiency. Input DNA underwent 22 cycles of PCR amplification, whereas ChIP samples underwent 35 cycles of PCR amplification. (*C*) ChIP assays using nuclear extracts from WT embryos at E11.5. ChIP was performed with anti-Six1 antibodies or IgGs from preimmune goat serum (T−). ChIP samples were challenged for the selective enrichment of the 145-bp enhancer vs. an irrelevant sequence located 200 kb upstream of the 145-bp sequence of the *Myf5* gene by quantitative PCR. 145-bp quantification is normalized to that of the −200 kb amplicon.

and looked for selective enrichment, after immunoprecipitation with Six1 antibody, of the 145-bp element versus a control DNA sequence by semiquantitative PCR experiments. As shown in Fig. 4*B*, Six1 antibodies specifically immunoprecipitate the 145-bp sequence but not the *histone H4* promoter sequence, showing that, in these cells, the MEF3 site in the *Myf5* element is loaded with Six1 proteins. Chromatin was then prepared from E11.5 WT embryos, and selective enrichment of the 145-bp enhancer versus a control DNA sequence, located at 200 kb upstream of the 145-bp enhancer element, was assessed by quantitative PCR (Fig. 4*C*). We detected a 5-fold enrichment of the 145-bp sequence versus the sequence at −200 kb from the *Myf5* gene, showing that Six1 proteins are bound to the 145-bp element *in vivo*. This enrichment is specific for Six1, because it was not seen when chromatin was immunoprecipitated with preimmune serum.

Mutation of the MEF3 Site in the 145-bp Element Impairs Its Activity *in Vivo*. We next determined the importance of the MEF3 site in the 145-bp *Myf5* element by transgenic analysis *in vivo*. We had

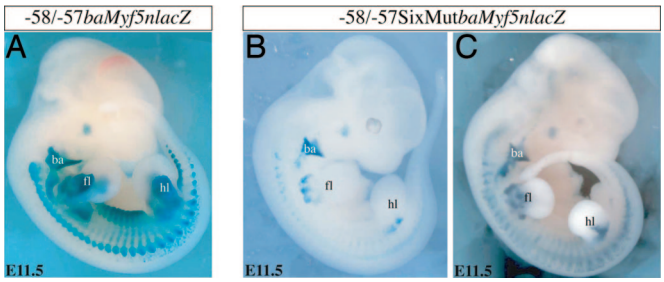

Fig. 5. Mutation of the Six binding site in the 145-bp element within the larger *Myf5* −58/−57 sequence compromises transgene expression. X-gal staining of transgenic embryos at E11.5 carrying a *nlacZ* reporter, regulated by −3 kb adjacent to the *Myf5* gene, containing the promoter and the branchial arch sequence as a positive control, proceeded by a −58/−57 fragment containing the WT 145-bp element, −58/−57*baMyf5nlacZ* (*A*) or with the 145-bp MEF3 site mutated, −58/−57SixMut*baMyf5nlacZ* (*B* and *C*). ba, branchial arch; fl, forelimb; hl, hindlimb.

previously shown that the 145-bp element directs transgene expression in limb buds and mature somites (26). To have maximally robust expression, we had mutated the Pax3 binding site in this element in the context of a larger −58/−57 fragment placed in front of a proximal 3-kb *Myf5* promoter sequence, including the branchial arch regulatory element (28), as a positive control. This proximal region also directs some expression in the neural tube. We adopted the same strategy with a WT or mutated MEF3 site and monitored expression of the *nlacZ* reporter in transient transgenic experiments. Seven WT transgenic embryos all showed strong expression of *nlacZ* in both forelimbs and hindlimbs, as well as in the somites and branchial arches at E11.5 (Fig. 5*A*). Strong somitic expression is seen in the hypaxial and epaxial lips of the residual dermomyotome epithelium at this stage (23). Mutation of the MEF3 site impaired the transcriptional activity of the transgene in all *Myf5*-expressing territories except the branchial arch (Fig. 5 *B* and *C* and Table 1). Three out of 12 embryos showed no transgene expression in the limb buds (SI Fig. 8 *A–C*), and eight had incomplete expression mainly in the proximal part (SI Fig. 8 *D–K*). One embryo with a stronger expression in the limb buds also had extensive ectopic expression, indicating an integration site effect (SI Fig. 8*L*). Thus, in 11 of 12 embryos limb expression of the transgene was compromised, and we therefore conclude that the MEF3 site in the 145-bp element plays a role in its activity *in vivo*.

Discussion

We had shown previously that Six homeoproteins are implicated in the regulation of the myogenic regulatory genes *MyoD*, *Mrf4*, and *Myogenin*. Their somitic expression is affected in $Six1^{-/-}Six4^{-/-}$ double mutants (22). In the case of *Myogenin*, direct regulation of a MEF3 site by Six proteins has been demonstrated (21). In the context of hypaxial myogenesis, loss of myogenic progenitor

Table 1. Summary of the expression of the −58/−57*baMyf5nlacZ* and the −58/−57SixMut*baMyf5nlacZ* transgenes in limb buds and mature somites at E11.5

		−58/−57SixMut	
Territory	−58/−57	Negative	Partial
Limb buds	7/7	3/12	9/12
Mature somites	6 (1*)/7	6/12	6/12

Two examples of partial expression in the limb buds are illustrated in Fig. 5 *B* and *C*, and an example of partial expression in the somites is illustrated in Fig. 5C. Pictures of all of the embryos obtained are shown in SI Fig. 8.
*Incomplete expression.

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cells in the hypaxial dermomyotome of the *Six1*$^{-/-}$*Six4*$^{-/-}$ double mutant, probably partly due to negative effects on *Pax3* expression, precludes analysis of the effects of Six on skeletal muscles, such as those in the limbs, that derive from this part of the somite. However, analysis of hindlimb myogenesis in the *Six1*$^{-/-}$ mutant now reveals that *Myf5* expression, both transcript and protein, is severely reduced in Pax3^{+} progenitor cells in which *MyoD* activation still occurs. In the forelimb, which is more severely affected, *Myf5* transcripts are not detectable in the residual Pax3^{+} cells at E11.5, whereas a low level of *Myf5* expression can be detected at E12.5. This may reflect the sensitivity of detection or a delay in *Myf5* activation. We had previously reported higher Myf5 expression in *Six1*$^{-/-}$ forelimbs (20), and we can only suppose that after more extensive breeding it reflects a change in genetic background or that Myf5 antibodies used in the previous study detected a protein unrelated to Myf5. Further specific reduction of *Myf5* expression in the hindlimbs of *Six1*$^{-/-}$*Six4*$^{-/+}$ suggests that Six4, also expressed in myogenic progenitor cells, is responsible for the remaining *Myf5* transcription in the *Six1*$^{-/-}$ mutant. Although *MyoD* expression is observed, it is delayed by 1 or 2 days in hindlimb or forelimb buds, respectively (ref. 20 and unpublished data). This may reflect a direct effect of reduced Six1/4 levels on the initiation of *MyoD* transcription or an indirect effect due to the absence or reduction of *Myf5* expression, because a delay in *MyoD* activation was observed in the limb buds of *Myf5*$^{-/-}$ (*Mrf4*$^{-/-}$) mutant embryos (7). In the trunk, in the absence of Six1, Six4, and Myf5, *MyoD* is not expressed (J.D., M.B., and P.M., unpublished data), whereas its activation is delayed in the trunk in the absence of Myf5 (30). This would suggest that Six1/4 may also have a role in regulating *MyoD* during limb myogenesis.

We demonstrate that Six1 and Six4 act directly on *Myf5* activation through a MEF3 site present in the 145-bp regulatory element that directs *Myf5* expression in the limb buds. Another regulatory element contributes to partial limb expression of *Myf5* mainly in the hindlimbs, and this element may ensure some of the remaining expression of *Myf5* observed in the hindlimb buds of *Six1*$^{-/-}$. Mutation of the MEF3 site in the 145-bp element, which we show binds Six1/4 *in vitro* and *in vivo*, does not totally abolish its activity in all transgenic embryos. There is no apparent difference in the partial expression seen in E11.5 transgenic embryos between forelimbs and hindlimbs. The reduced expression of Myf5 seen in the *Six1*$^{-/-}$ mutant may reflect differences in Six4 levels, which fail to reach a threshold level for *Myf5* expression in the forelimb. We had previously shown that the activity of the 145-bp element is totally dependent on a site to which Pax3 binds *in vivo*. Here we show that Pax3 binds efficiently to its site when the MEF3 site is mutated; activation by Pax3 may partially override the requirement for Six in some cells of the limbs of *Six* mutant embryos. Meox2 is another factor that affects *Myf5* transcription, as shown by the phenotype of *Meox2* mutant embryos in which *Myf5* activation in the limb buds is delayed (31). It is not yet clear whether Meox2 also intervenes directly in the regulation of the 145-bp sequence. *Myf5* is not expressed in WT myogenic progenitor cells before they reach the limb buds (3) despite the fact that they express both Pax3 and Six1/4. Wnt signaling may be important in regulating the activation of *Myf5* in the limb bud, and Wnt6 is a candidate in this context (32). It remains to be seen whether Wnt signaling also directly impacts the 145-bp element.

The regulation of the 145-bp *Myf5* element by Pax3 and Six1/4 provides an example of the participation of both types of transcription factor in the regulation of myogenic progenitor cells. This is seen at the level of their entry into the myogenic program, as shown here. Unlike Pax3, which is down-regulated after initial expression of the myogenic determination genes (see ref. 33), Six1/4 continue to be expressed in Myogenin-positive cells in muscle fibers, and indeed Six factors play an important role in the activation of fiber type-specific genes such as *MCK* and *aldolase A* (19, 34). Pax3 and Six1/4 play an important upstream role in myogenic progenitor cells in the somite, regulating their behavior in the dermomyotome and their migration from it (see refs. 16, 18, 22, and 29). Furthermore, there are complex interactions between Pax3 and Six1/4, such that Six1/4 regulate *Pax3* expression in the hypaxial somite (22). The key roles of Six1/4 in many myogenic processes are summarized in SI Fig. 9.

Materials and Methods

Transfection Experiments. Primary myogenic cells were obtained from E10 hindlimbs of chick embryos and were grown in MEM/E199 medium (Invitrogen, Cergy Pontoise, France) complemented with 10% FCS. Four hours after plating, cells were transfected with Lipofectamine 2000 (Invitrogen). The WT or MEF3 mutant 145-bp *Myf5* limb element (26) was cloned upstream of the −35 to +45 minimal promoter of the *aldolase A* gene, linked to the firefly luciferase transgene (Promega, Charbonnières, France). Transfection efficiency was normalized by measuring the activity of the *TK-Renilla* transgene. Thirty-six hours after transfection, both luciferase and *Renilla* activities were determined on a Lumat LB 907 luminometer (Berthold, Thoiry, France).

ChIP. ChIP with embryonic extracts was performed as described previously (18). ChIP from C2 cells was performed with cells after 2 days in differentiation medium. For PCR, 10 ng of genomic DNA was amplified as a positive control. The histone H4 promoter, devoid of MEF3 binding sites, was amplified as a ChIP negative control. Primers used for amplification of the 145-bp element, histone *H4* promoter, and for the sequence at −200 kb 5′ to the 145-bp sequence are described in *SI Text*. For quantitative PCR experiments, immunoprecipitated DNA and input DNA were analyzed by real-time PCR using a Light Cycler Faststart DNA Master SYBR Green I Mix and a Light Cycler (Roche Applied Science, Meylan, France).

We thank Milan Esner and Catherine Baudin for their assistance. J.G. and L.B. were supported by a fellowship from the Ministère de la Recherche et de l'Education Nationale and from the Association Française Contre les Myopathies. Financial support for P.M.'s laboratory has been provided by the Institut National pour la Santé et la Recherche Médicale, the Association Française Contre les Myopathies, the Centre National de la Recherche Scientifique, Action Concertée Incitative 0220514, the Agence Nationale pour la Recherche, the Association pour la Recherche sur le Cancer, and the FP6 MYORES European Muscle Development Network of Excellence. The contribution of the Région Ile de France to the Institut Cochin animal care facility is also acknowledged. Work on skeletal myogenesis in M.B.'s laboratory is supported by the Pasteur Institute, the Centre National de la Recherche Scientifique, the Association Française Contre les Myopathies, and two European Union Networks of Excellence (Cells into Organs and MYORES).

1. Gros J, Scaal M, Marcelle C (2004) *Dev Cell* 6:875–882.
2. Buckingham M (2006) *Curr Opin Genet Dev* 16:525–532.
3. Tajbakhsh S, Bober E, Babinet C, Pournin S, Arnold H, Buckingham M (1996) *Dev Dyn* 206:291–300.
4. Tajbakhsh S, Buckingham ME (1994) *Proc Natl Acad Sci USA* 91:747–751.
5. Bober E, Franz T, Arnold HH, Gruss P, Tremblay P (1994) *Development (Cambridge, UK)* 120:603–612.
6. Delfini M, Hirsinger E, Pourquie O, Duprez D (2000) *Development (Cambridge, UK)* 127:5213–5224.
7. Kablar B, Krastel K, Ying C, Asakura A, Tapscott SJ, Rudnicki MA (1997) *Development (Cambridge, UK)* 124:4729–4738.
8. Kawakami K, Sato S, Ozaki H, Ikeda K (2000) *BioEssays* 22:616–626.
9. Xu PX, Zheng W, Huang L, Maire P, Laclef C, Silvius D (2003) *Development (Cambridge, UK)* 130:3085–3094.

10. Laclef C, Souil E, Demignon J, Maire P (2003) *Mech Dev* 120:669–679.
11. Zheng W, Huang L, Wei ZB, Silvius D, Tang B, Xu PX (2003) *Development (Cambridge, UK)* 130:3989–4000.
12. Ozaki H, Nakamura K, Funahashi J, Ikeda K, Yamada G, Tokano H, Okamura HO, Kitamura K, Muto S, Kotaki H, *et al.* (2004) *Development (Cambridge, UK)* 131:551–562.
13. Li X, Oghi KA, Zhang J, Krones A, Bush KT, Glass CK, Nigam SK, Aggarwal AK, Maas R, Rose DW, *et al.* (2003) *Nature* 426:247–254.
14. Oliver G, Wehr R, Jenkins NA, Copeland NG, Cheyette BN, Hartenstein V, Zipursky SL, Gruss P (1995) *Development (Cambridge, UK)* 121:693–705.
15. Xu PX, Woo I, Her H, Beier DR, Maas RL (1997) *Development (Cambridge, UK)* 124:219–231.
16. Heanue TA, Reshef R, Davis RJ, Mardon G, Oliver G, Tomarev S, Lassar AB, Tabin CJ (1999) *Genes Dev* 13:3231–3243.
17. Ohto H, Kamada S, Tago K, Tominaga SI, Ozaki H, Sato S, Kawakami K (1999) *Mol Cell Biol* 19:6815–6824.
18. Grifone R, Demignon J, Giordani J, Niro C, Souil E, Bertin F, Laclef C, Xu PX, Maire P (2007) *Dev Biol* 302:602–616.
19. Grifone R, Laclef C, Spitz F, Lopez S, Demignon J, Guidotti JE, Kawakami K, Xu PX, Kelly R, Petrof BJ, *et al.* (2004) *Mol Cell Biol* 24:6253–6267.
20. Laclef C, Hamard G, Demignon J, Souil E, Houbron C, Maire P (2003) *Development (Cambridge, UK)* 130:2239–2252.
21. Spitz F, Demignon J, Porteu A, Kahn A, Concordet JP, Daegelen D, Maire P (1998) *Proc Natl Acad Sci USA* 95:14220–14225.
22. Grifone R, Demignon J, Houbron C, Souil E, Niro C, Seller M, Hamard G, Maire P (2005) *Development (Cambridge, UK)* 132:2235–2249.
23. Hadchouel J, Carvajal JJ, Daubas P, Bajard L, Chang T, Rocancourt D, Cox D, Summerbell D, Tajbakhsh S, Rigby PW, *et al.* (2003) *Development (Cambridge, UK)* 130:3415–3426.
24. Hadchouel J, Tajbakhsh S, Primig M, Chang TH, Daubas P, Rocancourt D, Buckingham M (2000) *Development (Cambridge, UK)* 127:4455–4467.
25. Buchberger A, Nomokonova N, Arnold HH (2003) *Development (Cambridge, UK)* 130:3297–3307.
26. Bajard L, Relaix F, Lagha M, Rocancourt D, Daubas P, Buckingham M (2006) *Genes Dev* 20:2450–2464.
27. Pauli T, Seimiya M, Blanco J, Gehring W (2005) *Development (Cambridge, UK)* 132:2771–2782.
28. Summerbell D, Ashby PR, Coutelle O, Cox D, Yee S, Rigby PW (2000) *Development (Cambridge, UK)* 127:3745–3757.
29. Relaix F, Polimeni M, Rocancourt D, Ponzetto C, Schafer BW, Buckingham M (2003) *Genes Dev* 17:2950–2965.
30. Tajbakhsh S, Rocancourt D, Cossu G, Buckingham M (1997) *Cell* 89:127–138.
31. Mankoo BS, Collins NS, Ashby P, Grigorieva E, Pevny LH, Candia A, Wright CV, Rigby PW, Pachnis V (1999) *Nature* 400:69–73.
32. Geetha-Loganathan P, Nimmagadda S, Huang R, Scaal M, Christ B (2006) *Anat Embryol (Berlin)* 211:183–188.
33. Relaix F, Rocancourt D, Mansouri A, Buckingham M (2005) *Nature* 435:948–953.
34. Himeda CL, Ranish JA, Angello JC, Maire P, Aebersold R, Hauschka SD (2004) *Mol Cell Biol* 24:2132–2143.