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Activation of *Fgf4* Gene Expression in the Myotomes Is Regulated by Myogenic bHLH Factors and by Sonic Hedgehog

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The *Fgf4* gene encodes an important signaling molecule which is expressed in specific developmental stages, including the inner cell mass of the blastocyst, the myotomes, and the limb bud apical ectodermal ridge (AER). Using a transgenic approach, we previously identified overlapping but distinct enhancer elements in the *Fgf4* 3′ untranslated region necessary and sufficient for myotome and AER expression. Here we have investigated the hypothesis that *Fgf4* is a target of myogenic bHLH factors. We show by mutational analysis that a conserved E box located in the *Fgf4* myotome enhancer is required for *Fgf4-lacZ* expression in the myotomes. A DNA probe containing the E box binds MYF5, MYOD, and bHLH-like activities from nuclear extracts of differentiating C2-7 myoblast cells, and both MYF5 and MYOD can activate gene expression of reporter plasmids containing the E-box element. Analyses of *Myf5* and *MyoD* knockout mice harboring *Fgf4-lacZ* transgenes show that *Myf5* is required for *Fgf4* expression in the myotomes, while *MyoD* is not, but *MyoD* can sustain *Fgf4* expression in the ventral myotomes in the absence of *Myf5*. Sonic hedgehog (*Shh*) signaling has been shown to have an essential inductive function in the expression of *Myf5* and *MyoD* in the epaxial myotomes, but not in the hypaxial myotomes. We show here that expression of an *Fgf4-lacZ* transgene in *Shh*−/− embryos is suppressed not only in the epaxial but also in the hypaxial myotomes, while it is maintained in the AER. This suggests that *Shh* mediates *Fgf4* activation in the myotomes through mechanisms independent of its role in the activation of myogenic factors. Thus, a cascade of events, involving *Shh* and bHLH factors, is responsible for activating *Fgf4* expression in the myotomes in a spatial- and temporal-specific manner.

INTRODUCTION

Embryonic development requires a complex program of events coordinated by extracellular signaling molecules that activate or repress transcription of specific gene subsets and thus influence the proliferative state or identity of the target cells. The expression of these signaling molecules has to be rigorously and specifically regulated, and the identification of the regulatory mechanisms that control expression of these molecules is fundamental to an understanding of the process of development.

Members of the fibroblast growth factor (FGF) family have been shown to influence a wide range of developmental events. Experiments using gene knockout and transgenic technology, as well as evidence from human genetics, have revealed that FGF signaling plays a major role in many aspects of development, including postimplantation mouse development, gastrulation, midbrain formation, myogenesis, growth and patterning of the limb, and bone morphogenesis. An important role in development has been demonstrated for FGF4. This growth factor is essential for postimplantation mouse development, since embryos homozygously null for the *Fgf4* gene undergo uterine implantation but do not develop substantially thereafter, and is also likely to be involved in limb growth and patterning (Basilico and Moscatelli, 1992; Goldfarb, 1996).

The pattern of expression of *Fgf4* is highly specific, being both spatially and temporally restricted to very discrete embryonic tissues (Niswander and Martin, 1992; Drucker

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and Goldfarb, 1993). *Fgf4* RNA is first detected in the inner cell mass (ICM) of the blastocyst at embryonic day 4.5 (E4.5) and then in the primitive streak (E7), the branchial arches and somitic myotomes (E9.5–13.5), and the apical ectodermal ridge (AER) of the limb bud (E10.5–11.5). Although the pattern of expression suggests a role for *Fgf4* during myogenesis, the early embryonic lethality of the *Fgf4* null embryos has precluded the study of its role in muscle development. *Fgf4* RNA is first detected in the myotomes at E9.5 just prior to or at approximately the same time as *Fgf6*. By E14.5, when *Fgf6* RNA is widely expressed in skeletal muscle, *Fgf4* RNA is no longer detected in any muscle cells (Niswander and Martin, 1992; Han and Martin, 1993). *Fgf5* is also known to be expressed in the myotome, with transcripts first becoming detectable at E10.5 (Haub and Goldfarb, 1991). The profile of *Fgf4* gene expression among skeletal muscle groups is overlapping, but distinct from, that of *Fgf5*, thereby revealing myoblast heterogenicity and suggesting distinct roles for multiple FGFs in muscle development (Drucker and Goldfarb, 1993).

Using a transgenic approach we previously defined distinct regulatory elements in the 3′ untranslated region (3′ UTR) of the *Fgf4* gene capable of driving *lacZ* gene expression in a manner which faithfully recapitulates the expression of the endogenous gene in the mouse blastocyst, myotomes, and developing limb (Fraidenraich *et al.*, 1998). While the *cis* and *trans*-acting elements governing *Fgf4* expression in the blastocyst have been previously well defined in our laboratory (Curatola and Basilico, 1990; Dailey *et al.*, 1994; Yuan *et al.*, 1995), the DNA elements controlling expression in the myotomes and AER appear to be overlapping but distinct. Within the myotomal/AER core enhancer element there are two E boxes (E-box 1 and E-box 2) and mutation of both E boxes abolished transgene expression in the myotomes, but not in the AER (Fraidenraich *et al.*, 1998).

E boxes (CANNTG) represent the consensus binding site for a large group of transcriptional regulators, the bHLH proteins, that includes the myogenic factors MYOD, MYF5, myogenin, and MRF4 (Molkentin and Olson, 1996; Cossu *et al.*, 1996b). Myogenic bHLH factors are expressed in overlapping but distinct patterns during muscle development, and a number of genetic experiments show that their activity is essential for establishing myoblast identity and muscle differentiation. Particularly *MyoD* and *Myf5* are thought to play a very early but partially redundant role in muscle development, since deletion of both of these genes results in complete suppression of muscle formation and in the absence of skeletal myoblasts, while the ablation of either *Myf5* or *MyoD* alone does not significantly affect muscle development (Rudnicki *et al.*, 1992, 1993; Braun *et al.*, 1992).

Although *MyoD* and *Myf5* proteins presumably regulate the expression of genes that collaborate to establish or maintain myogenic commitment, no direct target gene encoding for a growth factor in muscle progenitors has been yet identified. Grass *et al.* (1996) found that the expression of members of the FGF family was absent or delayed in the somites of *Myf5* mutant embryos. In this report we investigate the hypothesis that *Fgf4* is a target of myogenic bHLHs. We show by mutational analysis that the conserved E-box 2 but not E-box 1 is required for *Fgf4-lacZ* expression in the myotomes and cannot be compensated for by other E boxes situated in the 3′ UTR. Accordingly, an E-box 2 DNA probe binds efficiently MYF5, MYOD, and bHLH-like activities from nuclear extracts of differentiating C2-7 myoblast cells, and both MYF5 and MYOD can stimulate gene expression of reporter plasmids containing E-box 2 sequences. To ascertain which of the myogenic bHLH factors influences *Fgf4* expression in the myotomes we crossed *Myf5* and *MyoD* knockout mice with *Fgf4-lacZ* transgenic mouse lines. The results of these analyses show that *Myf5* is required for *Fgf4* expression in the myotomes, but that *MyoD* can also contribute to the activity of the *Fgf4* myotome enhancer in the absence of *Myf5*. Finally, we present evidence indicating that *Fgf4* gene expression in the myotomes is also dependent on Sonic hedgehog (*Shh*) signaling. Taken together, these data represent the first description of the molecular mechanisms that control the epaxial lineage expression of a myotome-specific gene.

MATERIAL AND METHODS

Plasmid DNAs

To make the constructs for the transient transgenesis experiments an *Fgf4-lacZ* plasmid containing the *Fgf4* murine promoter, the *Fgf4* splicing sequences, and bacterial β-galactosidase coding sequences, and the SV40 poly(A) site was used (Fraidenraich *et al.*, 1998). In the murine genomic sequences, nucleotide 1 corresponds to an *Eco*RI site located 3823 and 5257 nt upstream of the ATG initiation codon and TGA termination codon of the murine *Fgf4* gene, respectively. To make constructs 2 and 3, a 337-bp fragment (nt 6260–6597) from the *Fgf4* 3′ UTR was inserted 3′ of the SV40 poly(A) site. Construct 2 carries a mutation in E-box 1 and construct 3 in E-box 2 (G to A substitutions in nt 6267 and 6362, respectively). To make construct 4 a 1744-bp fragment (nt 5705–7449) from the *Fgf4* 3′ UTR that carries a mutation in E-box 2 was inserted. Mutations were performed by PCR (Fraidenraich *et al.*, 1998). To make the *Fgf4*-CAT constructs an *Fgf*-CAT plasmid derived from the pCAT3-Basic Vector (Promega) and containing the minimal *Fgf4* murine promoter (nt −64 to nt +150) was used (Ambrosetti *et al.*, 2000). To generate constructs 3a1 and 3a2, the 178- (nt 6251–6429) or 171-bp (nt 6429–6600) fragment, respectively, was inserted 5′ of the *Fgf4* promoter. To make constructs 8X(E-box 1) and 8X(E-box 2), oligonucleotides consisting of two repeats of 20 nt encompassing the E-box 1 and E-box 2 motifs (nt 6255–6274 and nt 6349–6368, respectively) were multimerized by self-ligation and inserted 5′ of the *Fgf4* promoter.

Transient Transgenesis Experiments

Fgf4-lacZ constructs were linearized using the appropriate restriction enzymes, agarose gel-purified, electroeluted, concentrated with Elutip-D minicolumns (S&S), and resuspended at a concentration of 100–300 μg/ml. Standard microinjection of DNA into

the pronucleus of fertilized eggs was achieved at the NYU Transgenic Mouse Facility. Swiss Webster mice were used.

Cell Culture, Transfections, and CAT Assay

HeLa, NIH-3T3, and C2-7 myoblast cells were grown in DMEM supplemented with 10% calf serum (CS). To induce C2-7 cell differentiation, the cells were grown in DMEM plus 1% horse serum (HS). $CaPO_4$ transfections were performed as described in Curatola and Basilico (1990).

Nuclear Extracts, Whole-Cell Extracts, and EMSA

For nuclear extracts (NE), pelleted C2-7 myoblast cells (grown in 10% CS or 1% HS) were resuspended in NE buffer (0.1 mM EDTA and EGTA, 1 mM DTT, protease inhibitors, pH 7.9) containing 10 mM KCl and 0.6% NP-40. Nuclei were pelleted and resuspended in NE buffer containing 400 mM NaCl. For whole-cell extracts (WCE) cells were harvested, resuspended in 20 mM Tris–HCl, pH 7.9, 450 mM KCl, 20% glycerol, 1 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 0.02% NP-40 and freeze–thawed. Oligonucleotides were labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. EMSAs were performed as in Dailey *et al.* (1994) using 6 μl of NE or 2 μl of WCE and, where indicated, 10-, 100-, or 200-fold molar excess of competitor oligonucleotide or 2 or 4 μl of a monoclonal antibody reactive with MYOD.

Interbreeding, Collection of Embryos, and X-gal Staining

Myf5+/− (Rudnicki *et al.*, 1992), *MyoD*−/− (Braun *et al.*, 1992), and *Shh*+/− (Chiang *et al.*, 1996) mice were first bred with two mouse lines carrying transgenes that encompass the myotomal enhancer element Hom3a (Fig. 3). The progeny were subsequently interbred to obtain *Myf5*−/−:*Fgf4-lacZ*, *MyoD*−/−:*Fgf4-lacZ*, and *Shh*−/−:*Fgf4-lacZ* embryos. Additionally, *Myf5*+/− and *MyoD*+/− transgenics were intercrossed to produce mice *Myf5*+/−:*MyoD*+/−:*Fgf4-lacZ*. After another round *Myf5*−/−:*MyoD*−/−, *Myf5*−/−:*MyoD*+/−, and *Myf5*+/−:*MyoD*−/− transgenics were produced. Embryos were collected between E10.5 and E13.5, fixed, and stained with X-gal as described previously (Fraidenraich *et al.*, 1998). Genomic DNA was isolated from mouse tails or from embryonic yolk sacs (Fraidenraich *et al.*, 1998), and the mice or embryos were genotyped by Southern blot analysis. At least three embryos of each genotype containing one allele of the *lacZ* transgene were derived and characterized. Whole embryos were photographed using a Leica stereomicroscope.

Immunohistochemistry

X-gal-stained embryos were fixed in 4% paraformaldehyde and sectioned transversely using a Zeiss cryostat or a Zeiss microtome. Immunostaining was performed using the biotin/avidin system (Vectastain), according to the manufacturer's instructions (Vector Laboratories, Inc.) using the primary antibodies mAb 5.8A against mouse MYOD (kindly provided by P. Loughton), mAb D33 against mouse desmin (DAKO, Denmark), antibody 5C-302 against Myf5 (Santa Cruz), mAb F5D against myogenin (developed by W. E. Wright, Developmental Studies Hybridoma Bank), and mAb MF20 against myosin heavy chain (MHC) (Bader *et al.*, 1982).

Whole-Mount in Situ Hybridization

Whole embryos were dissected at E11.5 and processed as described by Henrique *et al.* (1995). For detection of *Fgf4* mRNA, two digoxigenin-labeled antisense probes corresponding to the *Fgf4* first exon or homology region 2 of the *Fgf4* 3′ UTR were used together (Fraidenraich *et al.*, 1998). For detection of *MyoD* mRNA, a digoxigenin-labeled antisense probe was transcribed from a PCR-derived DNA template corresponding to the carboxyl terminus of the *MyoD* coding sequence (nt 761–1061) using T3 RNA polymerase. Whole embryos were photographed using a Leica stereomicroscope.

RESULTS

A Conserved E Box Situated in the 3′ UTR of the Fgf4 Gene Is Required for Fgf4-lacZ Expression in the Myotomes

We previously identified a 337-bp myotomal enhancer element in a conserved region within the 3′ UTR of the murine *Fgf4* gene (homology region 3a, Hom3a) by its ability to direct expression of a *Fgf4-lacZ* transgene to the trunk and tail myotomes (Fraidenraich *et al.*, 1998). The *Fgf4* promoter sequences have no activity in isolation. The AER enhancer elements overlap with the myotomal enhancer sequences but are distinct, since mutation of two E boxes present in the Hom3a region abolishes transgene expression in the myotomes but not in the AER (Fraidenraich *et al.*, 1998). These observations prompted us to investigate if both E boxes are required for transgene activity, or only one E box is important, and whether these elements are redundant.

In order to answer these questions, we created three new constructs and produced transgenic embryos, which were harvested at E11.5 and subjected to X-gal staining (Fig. 1). Mutation of E-box 1 (construct 2) did not alter the pattern of expression of the *Fgf4* transgene (Fig. 1B), compared to the pattern of the transgene driven by the intact Hom3a (data not shown and Fraidenraich *et al.*, 1998). Mutation in E-box 2 (construct 3), however, led to a complete loss of transgene expression in the myotomes (Fig. 1C). The expression of a construct bearing a mutation in E-box 2 in the context of most of the 3′ UTR sequences that contain six additional E boxes (including E-box 1) was also examined (construct 4). There was no detectable expression of this transgene in the myotomes (Fig. 1D), indicating that the absence of E-box 2 function cannot be compensated for by other E boxes of the 3′ UTR. Ectopic areas of expression were observed, such as in the dorsal root ganglia (Fig. 1D). We sectioned these embryos and confirmed the absence of staining in the myotomes. Expression in the AER was weak but detectable when constructs 2 and 3 were used and stronger in the case of construct 4 (data not shown).

To confirm that the pattern observed in the myotomes with the construct 1 or 2 transgenics is similar to that of the endogenous *Fgf4* transcripts, we performed whole-mount *in situ* hybridization analysis on E11.5 embryos with DIG

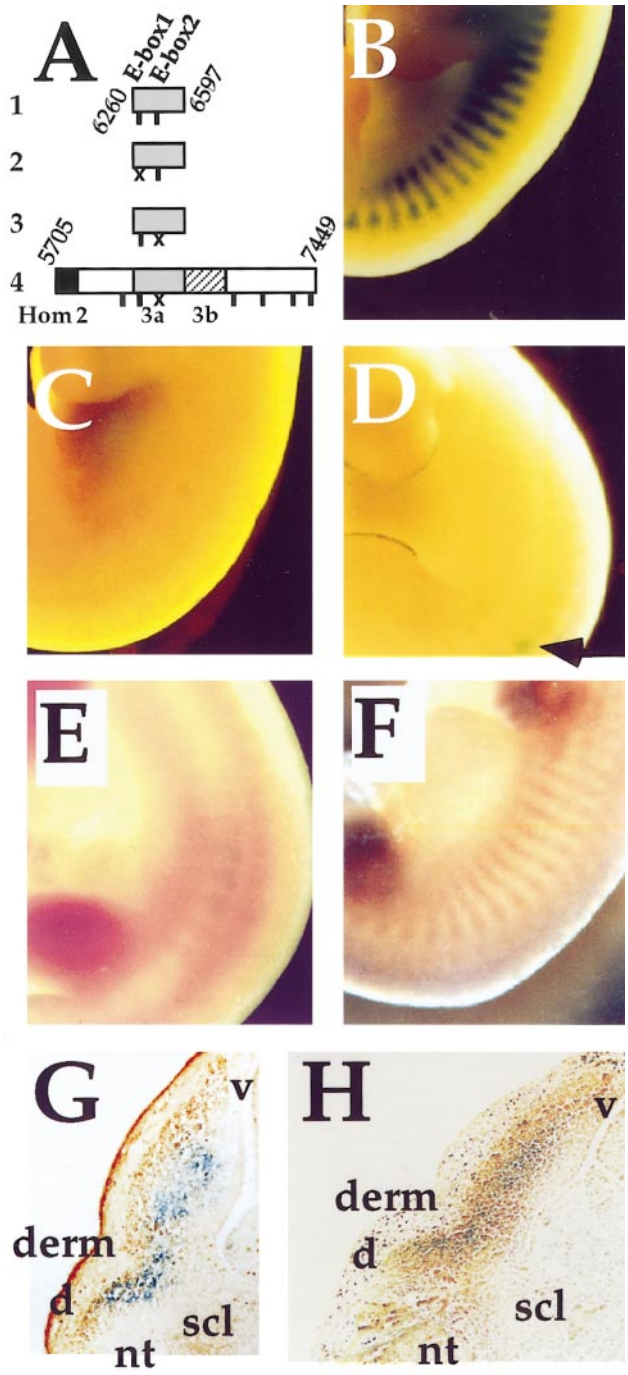


FIG. 1. E-box 2 but not E-box 1 is required for *Fgf4-lacZ* expression in the myotomes. (A) Schematic representation of the enhancer elements used to create *Fgf4-lacZ* transgenic embryos (see Material and Methods for details). Fragment 2 is identical to fragment 1 (Hom3a; Fraidenraich *et al.*, 1998) but carries a mutant E-box 1; fragment 3 carries a mutant E-box 2; fragment 4 contains most of the 3′ UTR sequences including Hom2, intervening sequences, Hom3a carrying a mutant E-box 2, Hom3b, and flanking sequences. For coordinates see Material and Methods. At least five independent transgenic embryos of each group were examined in transient assays with consistent results: Vertical bars, intact E boxes; x, mutant E box. Hom 2, 3a, and 3b, 3′ UTR homology regions 2 (black rectangle), 3a (gray rectangles), and 3b (hatched rectangles). (B) X-gal staining of an E11.5 embryo carrying construct 2. (C) X-gal staining of an E11.5 embryo carrying construct 3. (D) X-gal staining of an E11.5 embryo carrying construct 4. Ectopic staining is detected in the dorsal root ganglia (arrow). (E and F) Whole-mount *in situ* hybridization of wild-type E11.5 embryos using antisense DIG probes for *Fgf4* (E) and *MyoD* (F) mRNAs as described under Material and Methods. (G and H) An E11.5 *Fgf4-lacZ* transgenic embryo was X-gal stained, sectioned at the interlimb level, and immunostained with anti-MYOD (G) or anti-MYF5 (H) antibodies as described under Material and Methods. d, dorsal; v, ventral; scl, sclerotome; derm, dermatome; nt, neural tube.

probes for *Fgf4* and *MyoD*. The pattern of expression of *Fgf4* mRNA was very similar to the transgene pattern (Fig. 1E, compare to Fig. 1B). Interestingly, both *Fgf4-lacZ* and *Fgf4* mRNA were observed in a subset of the myotomes. *MyoD* is known to be expressed throughout the myotome at E10.5 (Cossu *et al.*, 1996a). The expression of *MyoD* mRNA was dorsoventrally more extended than that observed for *Fgf4* (Fig. 1F, compare to Figs. 1E and 1B). This is more clearly seen in Fig. 1G, which shows a section of a transgenic embryo (E11.5) stained with X-gal and immunostained with anti-MYOD antibodies. While MYOD-positive cells demarcate the entire myotome, including the dorsal and ventral domains, β-gal-positive cells are observed only in a central area with respect to the dorsoventral axis, predominantly in the epaxial domain. The β-gal-positive domain contacts the sclerotomal layer, but not the dermatome. We also determined the expression of *Myf5* in the myotomes and compared it with that of *Fgf4-lacZ* (Fig. 1H). Also in this case, *Myf5* expression was more extended than that of *lacZ*, which was confined to the central area of *Myf5* expression. Our observation that *Fgf4* (both *Fgf4-lacZ* and *Fgf4* mRNA) is expressed in a subset of the myotomes is in agreement with previous observations by Drucker and Goldfarb (1993), who also reported that *Fgf4* mRNA detection in the myotomes is confined to a centrally located area.

The E-box 2 Element but Not E-box 1 Binds MYF5 and MYOD

The myogenic bHLH transcription factor family can be divided into two functional groups. MYF5 and MYOD appear to be required for the determination of skeletal myoblasts. Myogenin and MRF4 act later in the program, and their role is likely to be that of differentiation factors (Rudnicki *et al.*, 1993; Braun *et al.*, 1994; Rawls *et al.*, 1995; Megeney and Rudnicki, 1995). The temporal pattern of expression of *Fgf4* in the myotomes and its apparent requirement for the E-box 2 element suggested that either MYF5 or MYOD might be involved in activation of *Fgf4*

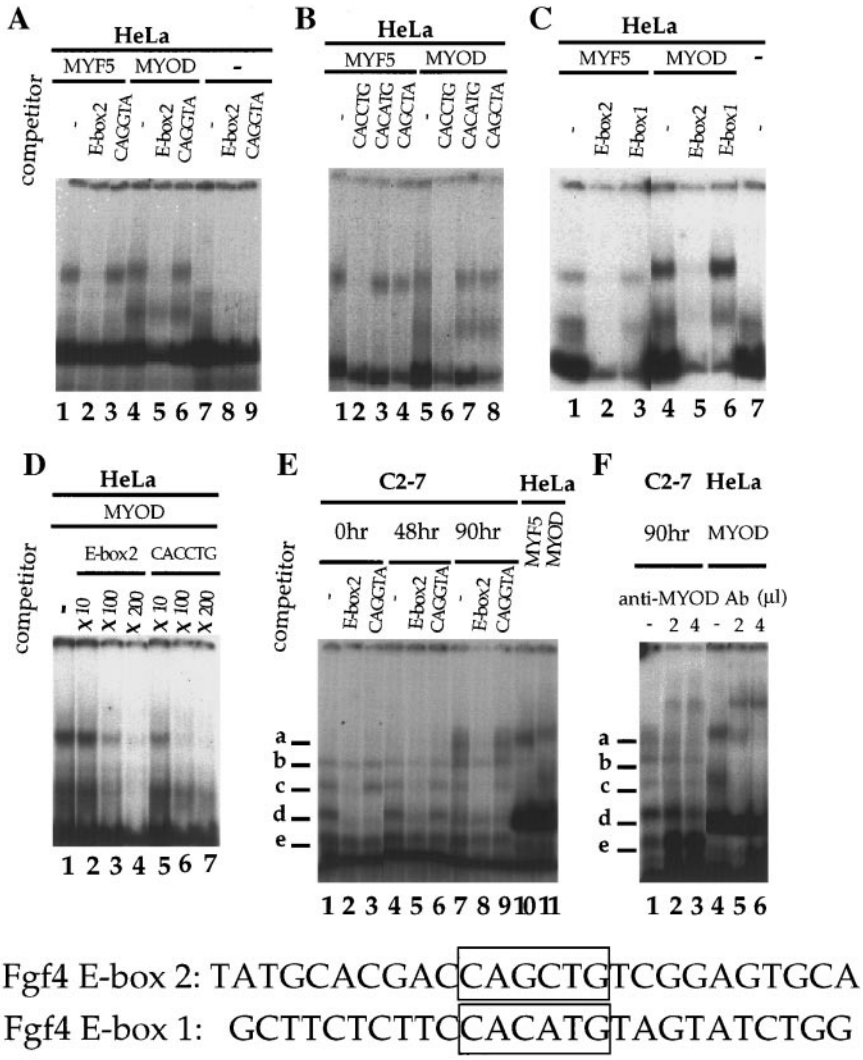


FIG. 2. The *Fgf4* E-box 2 site binds MYF5, MYOD, and nuclear cell extracts derived from differentiating C27-myoblasts. (A through D) Electrophoretic mobility shift of an *Fgf4* E-box 2 probe (see sequence at bottom) in a HeLa whole-cell extract transfected with *Myf5* (A, lanes 1–3; B, lanes 1–4; C, lanes 1–3; see also E, lane 10), *MyoD* (A, lanes 4–6; B, lanes 5–8; C, lanes 4–6; D, lanes 1–7; see also E, lane 11) expression plasmids or untransfected (A, lanes 7–9; C, lane 7). 26-mer DNAs were used to comp ete the binding of the probe. E-box 2, *Fgf4* E-box 2 (A, lanes 2, 5, and 8; C, lanes 2 and 5; and D, lanes 2–4); CAGGTA, *Fgf4* E-box 2 with the second half of the E box mutated (A, lanes 3, 6, and 9); CACCTG, DNA from the MCK enhancer (B, lanes 2 and 6, and D, lanes 5–7); CACATG, E-box 1 element plus E-box 2 flanking sequences (B, lanes 3 and 7); CAGCTA, *Fgf4* E-box 2 with a G to A substitution (B, lanes 4 and 8); E-box 1, *Fgf4* E-box 1 (see sequence at bottom). (E and F) Electrophoretic mobility shift of *Fgf4* E-box 2 probe in a nuclear cell extract derived from C2-7 myoblasts grown in 1% HS for 0 (E, lanes 1–3), 48 (E, lanes 4–6), and 90 h (E, lanes 7–9, and F, lanes 1–3). (E) DNAs used for competition: E-box 2 (lanes 2, 5, and 8) and CAGGTA (lanes 3, 6, and 9). The formation of complexes a, b, c, d, and e is detected. (F) Anti-MYOD antibodies were incubated with the extracts derived from differentiating C2-7 myoblasts (lanes 1–3) or from *MyoD*-transfected HeLa cells (lanes 4–6) as described under Material and Methods.

gene expression in the myotomes. To test whether E-box 2 binds MYF5 and MYOD and to study the basis of the functional specificity of the two E boxes, we performed EMSAs using whole-cell extracts derived from HeLa cells transfected with *Myf5* or *MyoD* expression plasmids.

Probe E-box 2, consisting of E-box 2 plus flanking sequences (*Fgf4* E-box 2, Fig. 2) was incubated with extracts derived from HeLa cells transfected with *Myf5* and *MyoD* expression plasmids. Two bands representing the formation of complexes between E-box 2 and MYOD and one band representing the formation of a complex between E-box 2 and MYF5 are detected in transfected but not in untrans-

fected cells extracts. Binding was competed when the incubation was performed in the presence of excess of cold E-box 2, but not in the presence of excess of mutant E-box 2 in which we substituted the second half of the E-box 2 element (CAGGTA) (Fig. 2A). Probe E-box 1, consisting of E-box 1 and flanking sequences (*Fgf4* E-box 1, Fig. 2), did not produce any specific complexes using these extracts (data not shown).

The binding of E-box 2 to MYF5 and MYOD was further characterized using four additional competitors. We used a sequence from the muscle creatine kinase enhancer containing a functional E box (CACCTG; Jaynes *et al.*, 1988) and another DNA corresponding to E-box 2 with the two central nucleotides substituted (CACATG). This E box is identical to the E-box 1 element but the flanking sequences correspond to E-box 2. The third DNA oligomer was another mutant E-box 2, in which we substituted the sixth nucleotide of E-box 2, disrupting again the E box (CAGCTA). The G to A substitution had been used to create mutant E boxes in the transgenic analyses of Fig. 1 and previously (Fraidenraich *et al.*, 1998). The fourth DNA oligomer was E-box 1 and flanking sequences. The binding of MYOD and MYF5 to E-box 2 was competed with CACCTG and E-box 2 with similar affinities, but not with CACATG, CAGCTA, or E-box 1 (Figs. 2B–2D and data not shown). Thus the E-box 2 element is capable of binding myogenic bHLH factors, while the E-box 1 is not, in agreement with the hypothesis that the requirement for E-box 2 shown *in vivo* reflects the binding and activation by myogenic factors.

Expression of Fgf4-lacZ in the Myotomes of Myf5- but Not of MyoD-Deficient Embryos Is Severely Impaired

The requirement for E-box 2 and its binding to MYF5 and MYOD led us to investigate whether these factors play a role in controlling *Fgf4* gene expression. To this end, we crossed two *Fgf4-lacZ* transgenic mouse lines with mice deficient for *Myf5* or *MyoD* to obtain transgenic, *Myf5* or *MyoD* null embryos. Both transgenes express *lacZ* under the control of most of the sequences of the 3′ UTR (Fraidenraich *et al.*, 1998), which include the intact Hom3a myotomal enhancer element (Fig. 3). We tested therefore the ability of *Fgf4* Hom3a to drive *lacZ* expression in the myotomes in the context of *MyoD* or *Myf5* null embryos.

Embryos were harvested between E10.5 and E13.5 and subjected to X-gal staining. An identical pattern of staining was observed in the trunk and tail myotomes and derivative muscles including the intercostal and deep back muscles with both *Fgf4-lacZ* transgenic lines analyzed in wild-type embryos. The absence of *MyoD* did not affect the expression of the *Fgf4* transgene at any of the developmental stages examined (Figs. 3C, 3F, 3I, and 3L, compare with wild-type embryos in 3A, 3D, 3G, and 3J). In contrast, the expression of the *Fgf4* transgene was severely compromised in the *Myf5* null embryos (Figs. 3B, 3E, 3H, and 3K). At E10.5–E11.0, X-gal staining of the myotomes was absent in the *Myf5* KO embryos, although *lacZ* expression was activated in the limb-bud AERs and was indistinguishable from that of the wild-type embryos (Fig. 3B vs Fig. 3A). At E11.5 and E12.5 in the *Myf5* KO embryos, only weak positive areas were detected in the myotomes (Figs. 3E and 3H) and they were localized to the ventral portion of the myotomes of the interlimb somites and to small centers in the dorsal myotomes (see arrows). At E13.5 the muscles derived from the trunk and tail myotomes presented little or no X-gal staining, with only a few areas in the intercostal and abdominal musculature being weakly β-gal positive. However, X-gal staining in muscles derived from the rostral myotomes appeared to be less affected by the lack of *Myf5*. Accordingly, weak *lacZ* expression was detected in the forebut not in the hindlimbs of the *Myf5* KO embryos.

E11.5 embryos subjected to X-gal staining were coronally sectioned at the interlimb level and counterstained with hematoxylin. Figures 3M and 3N show the restricted expression of *lacZ* in the myotomes of *Myf5*−/− embryos, which is confined to the ventral myotomes, compared to the wild type. On the other hand, *lacZ* expression in the *MyoD*−/− embryos was essentially identical to that of the wild type (data not shown). Together these results indicate that Myf5 is required for full expression of the *Fgf4* gene in the myotomes, while *MyoD* is dispensable. In *Myf5*−/− embryos a delay in the formation of myotomes until *MyoD* is turned on (E10.5–11) has been observed. Thus the absence of *Fgf4* transgene expression in the *Myf5*−/− embryos could be due to delay in the formation of the myotomes. However, the expression of our *Fgf4* transgenes is also strongly inhibited at all subsequent stages of muscle development (from E10.5 to E13.5) and thus this latter explanation seems unlikely.

In the Absence of Myf5, MyoD Is Required for Fgf4-lacZ Expression in the Ventral Myotomes

The *in vivo* experiments suggest that *Myf5* is required for *Fgf4* expression in the myotomes. However, EMSAs of Fig. 2 showed that not only MYF5, but also MYOD, can bind E-box 2 and thus could also be involved in activating *Fgf4* gene expression. Thus, we asked whether the residual domain of *lacZ* expression in the ventral myotome of the *Myf5*-deficient embryos was *MyoD* dependent.

Fgf4-lacZ transgenic mice carrying a single copy of each of the genes *MyoD* and *Myf5* were crossed to produce transgenic embryos deficient for both *MyoD* and *Myf5* and analyzed for *lacZ* expression (Fig. 4). Embryos deficient for both genes do not develop muscle (Rudnicki *et al.*, 1993) and did not show any *lacZ*-positive cells (Fig. 4D, compare to Figs. 4A and 4B).

We then produced *Myf5*−/−:*MyoD*+/− *Fgf4-lacZ* E11.5 embryos which were stained with X-gal (Fig. 4C) and subsequently sectioned at the interlimb level. Mice lacking *Myf5* and one copy of *MyoD* display no obvious morphological abnormalities in size and form muscles (Rudnicki *et*

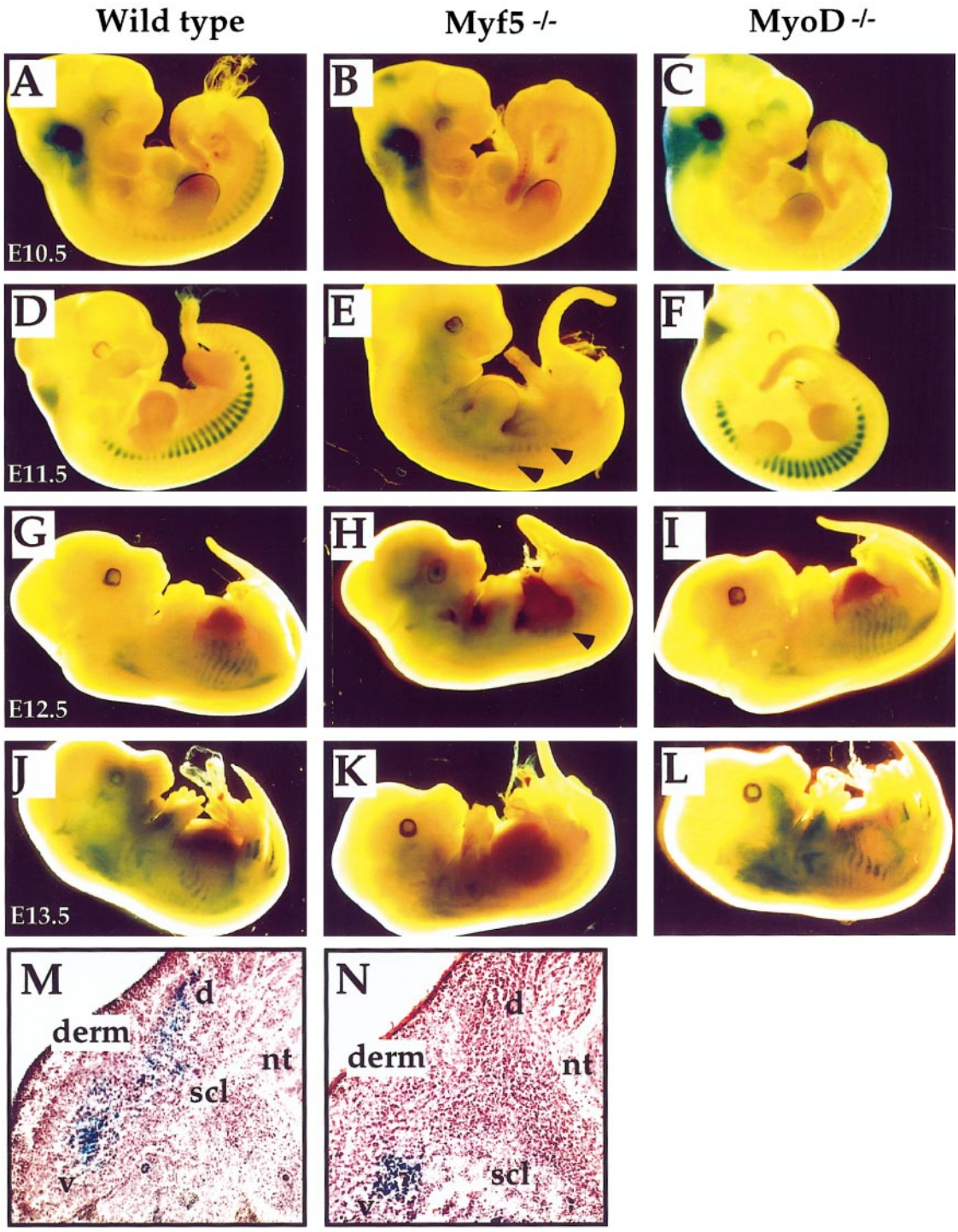
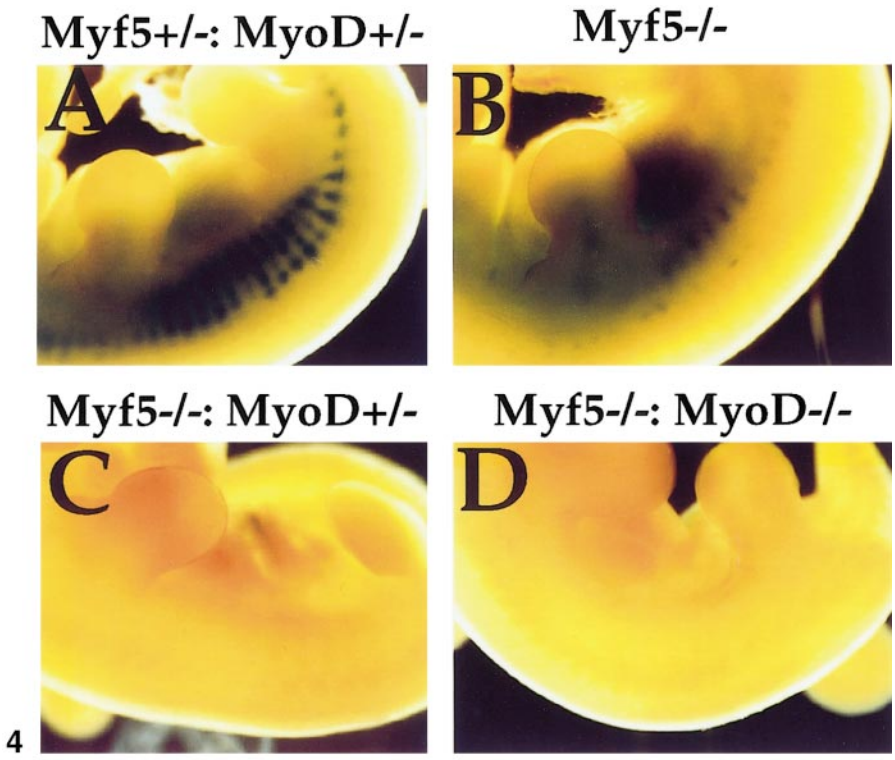


FIG. 3. *Fgf4-lacZ* expression in the myotomes of *Myf5*- but not of *MyoD*-deficient embryos is severely impaired. Wild-type (A, D, G, and J), *Myf5* null (B, E, H, and K), and *MyoD* null (C, F, I, and L) embryos transgenic for *Fgf4-lacZ* (see Material and Methods) were harvested at E10.5–11.0 (A, B, and C), E11.5 (D, E, and F), E12.5 (G, H, and I), and E13.5 (J, K, and L) and subjected to X-gal staining. The two constructs used to create transgenic mouse lines were described elsewhere (constructs 2 and 5 in Fraidenraich *et al.*, 1998). Construct 2 bears a fragment of the *Fgf4* 3′ UTR consisting of Hom3a, Hom3b, and 5′ and 3′ flanking sequences. Construct 5 3′ UTR fragment includes Hom2, intervening sequences, Hom3a, Hom3b carrying a 20-bp deletion corresponding to the SOX/OCT binding sites, and flanking sequences. The deletion in the SOX/OCT binding sites has been shown not to affect the pattern of expression of the transgene in the myotomes (Fraidenraich *et al.*, 1998). The ectopic staining in the brain at E10.5–11.0 (A, B, and C) results from a position effect (Fraidenraich *et al.*, 1998). Residual areas of stain are detected in the *Myf5*-deficient embryos at E11.5 and E12.5 (arrowheads). (M and N) Wild-type and *Myf5*−/− embryos from D and E were sectioned at the interlimb level and counterstained with hematoxylin. d, dorsal; v, ventral; derm, dermatome; scl, sclerotome; nt, neural tube.



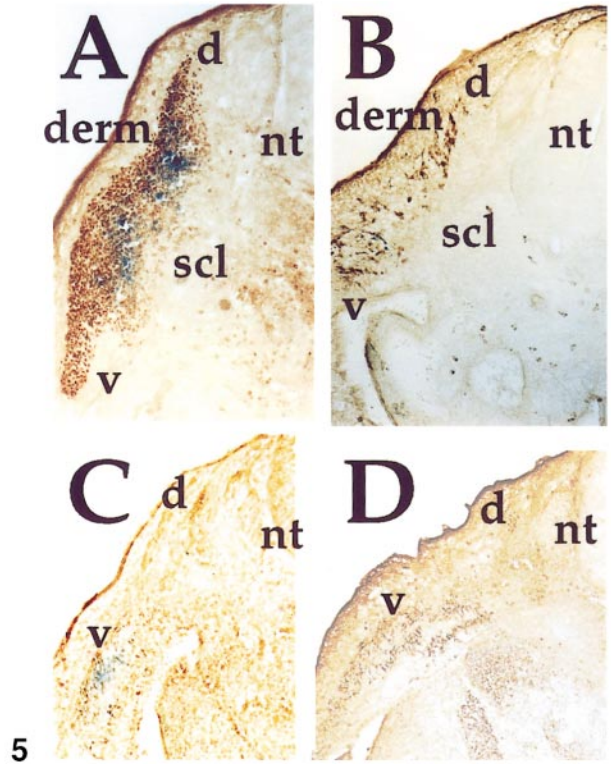


FIG. 4. In the absence of *Myf5*, *MyoD* is required for *Fgf4-lacZ* expression in the ventral myotomes. (A through D) *Myf5*+/−:*MyoD*+/− (A), *Myf5*−/− (B), *Myf5*−/−:*MyoD*+/− (C), and *Myf5*−/−:*MyoD*−/− (D) embryos transgenic for *Fgf4-lacZ* were harvested at E11.5 and subjected to X-gal staining.

FIG. 5. The myotomes of *Myf5*−/−:*MyoD*+/− embryos express desmin. E.11.5 wild-type (A) and *Myf5*−/−:*MyoD*+/− (B) *Fgf4-lacZ* transgenic embryos X-gal stained were sectioned at the interlimb level and immunostained with anti-MYOD antibodies. E12.5 *Myf5*−/− (C) and *Myf5*−/−:*MyoD*+/− (D) *Fgf4-lacZ* transgenic embryos X-gal stained were sectioned at the interlimb level and immunostained with anti-desmin antibodies. d, dorsal; v, ventral; scl, sclerotome; derm, dermatome; nt, neural tube.

al., 1993). Although one copy of *MyoD* appears capable of rescuing skeletal myogenesis in mice lacking *Myf5* (Rudnicki *et al.*, 1993), essentially no *lacZ*-positive cells were observed (Fig. 4C). The sections were immunostained with anti-MYOD or anti-desmin antibodies (Fig. 5). At E11.5 a few MYOD-positive cells were observed that, however, did not express *lacZ* (Fig. 5B). At E12.5 again LacZ staining was absent, but expression of desmin, a marker of muscle differentiation, was clearly detectable in the myotomes of *Myf5*−/−:*MyoD*+/− embryos (Fig. 5D), in line with the previous observation (Rudnicki *et al.*, 1993) that these mice develop normal muscles. The observation that the residual activity of the FGF4 myotome enhancer observed in *Myf5* null embryos is abolished in a *MyoD*+/− background supports the hypothesis that this residual activity is MYOD dependent. Furthermore, since *MyoD*+/−:*Myf5*−/− embryos form muscles, these results suggest that FGF4 may not be critical for skeletal myogenesis.

An Fgf4-CAT Reporter Plasmid under the Control of E-box 2 but Not of E-box 1 Is Transactivated by MYF5 and MYOD

In the previous sections we showed that E-box 2 but not E-box 1 can bind MYF5 and MYOD, is required for transgene expression in the myotomes, and cannot be substituted by any other E box of the 3′ UTR. Furthermore, *Myf5* appeared to be essential for activity of the *Fgf4* enhancer in the myotome, although *MyoD* could partially compensate for the absence of *Myf5*. We therefore performed a series of transfection experiments to determine whether the introduction of myogenic bHLHs in cell lines devoid of these factors activated the expression of *Fgf4*-CAT reporter plasmids controlled by E-box 2.

Figure 6A shows the constructs used in these experiments. Construct 3a1 contains the 5′ half of Hom3a, including E-box 1 and E-box 2, while construct 3a2 contains the 3′ half of Hom3a. Construct 8X(E-box 1) or construct 8X(E-box 2) is a multimer of a 20-mer DNA unit containing E-box 1 or E-box 2.

We transfected constructs 3a1 and 3a2 in C2-7 myoblasts and NIH 3T3 fibroblasts. The basal activities for both constructs were similarly low. However, when the cells were cotransfected with expression plasmids for *Myf5* or *MyoD*, approximately 10-fold induction of the activity of 3a1 and no induction of 3a2 occurred (Figs. 6B and 6C). We also performed the same analysis with HeLa cells and obtained similar results (data not shown). We next tested the constructs 8X(E-box 1) and 8X(E-box 2) in HeLa cells. A robust transactivation of 8X(E-box 2) but not of 8X(E-box 1) CAT activity occurred when either *Myf5* or *MyoD* expression plasmid was cotransfected (Fig. 6D).

Taken together these results indicate that the DNA elements comprising E-box 2 can serve as a target for transactivation by MYF5 and MYOD, while the E-box 1 elements do not respond to *Myf5* and *MyoD* expression. In addition, they confirm the hypothesis that the first half of the Hom3a region is essential for myotome expression.

While MYOD or MYF5 or both are present in proliferating myoblasts and differentiated myotubes, myogenin and MRF4 are activated upon differentiation. However, the inhibitory HLH protein Id, which lacks the basic DNA-binding domain, has been shown to inhibit MYOD/E complexes from binding DNA in proliferating myoblasts (Benezra *et al.*, 1990). Upon differentiation, when Id protein levels fall, these hetero-oligomeric complexes now bind with high affinity to response elements in muscle-specific genes. We tested the ability of the E-box 2 element to bind myogenic bHLH-like activities and to activate an *Fgf4*-CAT plasmid in C2-7 myoblasts undergoing differentiation. At different time points during differentiation nuclear extracts were prepared and incubated with the E-box 2 probe (Fig. 2E). At 90 h after serum starvation cells became positive for MHC. Concomitantly, EMSA analysis showed the appearance of a broad gel-shift band (denoted complex a). Complex a presented a mobility similar to those of MYF5- and MYOD-containing complexes and was competed with E-box 2 but not with CAGGTA. Other bands of faster mobility (complexes b through e) were also detected, but appeared to be nonspecific (i.e., were not efficiently competed) or did not vary upon differentiation. We then transfected 8X(E-box 1) or 8X(E-box 2) into myoblasts and allowed the cells to differentiate for 90 h. A robust activation of 8X(E-box 2) but not of 8X(E-box 1) construct was observed (Fig. 6E).

In an attempt to identify some of the components of the complexes appearing in the band-shift experiments, we performed a supershift experiment with extracts from differentiating C2-7 cells, probe E-box 2, and the anti-MYOD Ab. Only complex a was significantly supershifted and the supershifted complex presented a mobility similar to that observed for MYOD:anti-MYOD Ab control (Fig. 2F). This indicates that MYOD is a major component of complex a. A residual binding activity, however, was observed in the faster mobility area of the broad complex a, which was less affected by the addition of the anti-MYOD Ab, suggesting that nuclear factors other than MYOD may be contained in complex a. Interestingly, the MYF5 control band (Fig. 2E, lane 10) runs slightly faster than the MYOD control band (Fig. 2E, lane 11) and with a mobility similar to that observed for the residual binding activity of complex a. All the other bands observed in the nuclear extracts from C2-7 cells were not supershifted (Fig. 2F).

Taken together these results indicate that *in vitro*, the *Fgf4* myotome enhancer can be activated both by MYOD and by MYF5 and does not show any preference for MYF5 over MYOD, contrary to what is observed *in vivo*. As discussed in detail later, we tend to interpret these results as indicating that both MYOD and MYF5 can equally activate the enhancer, but that *in vivo*, the pattern and timing of gene expression of these factors make MYF5 the most relevant element.

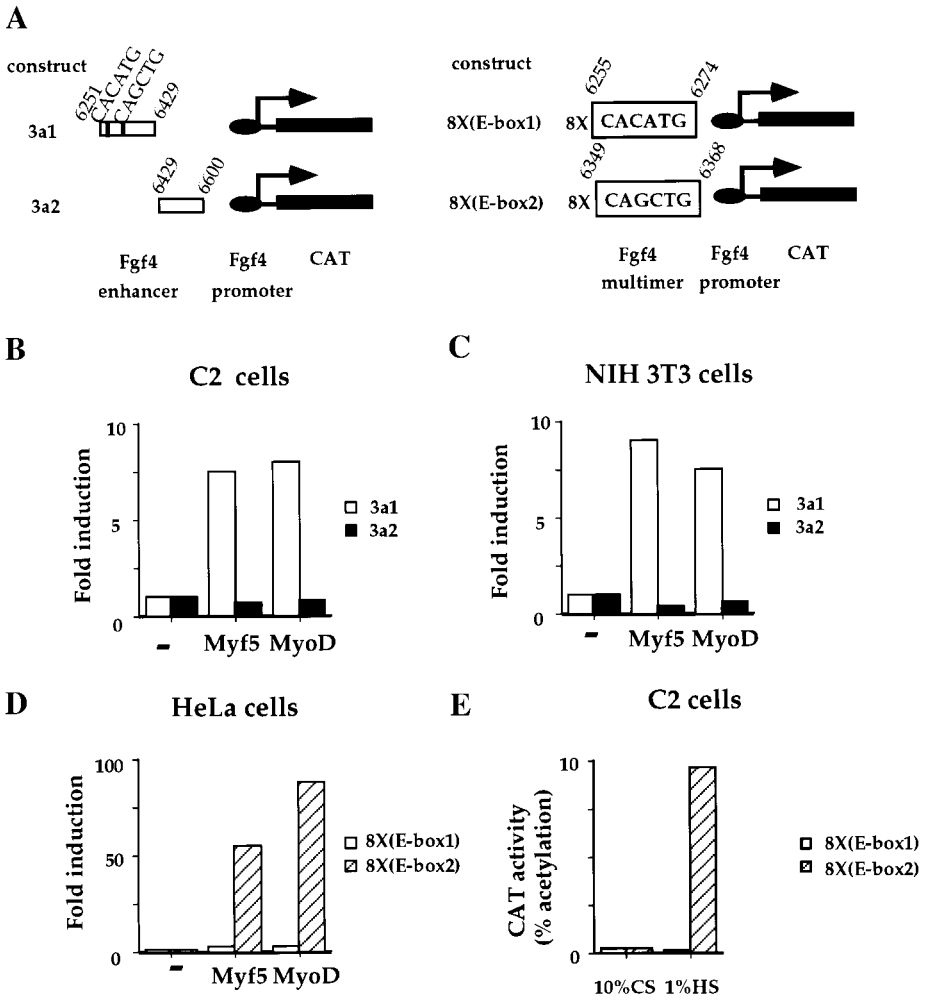


FIG. 6. An *Fgf4*-CAT plasmid under the control of E-box 2 but not of E-box 1 is transactivated by MYF5 and MYOD. (A) Schematic representation of the constructs used to transfect various cell lines. 3a1 contains E-boxes 1 and 2. 3a2 contains no E box. 8X(E-box 1) or 8X(E-box-2) contains eight repeats of a 20-bp unit encompassing E-box 1 or E-box 2. For coordinates see Material and Methods. (B and C) Construct 3a1 or 3a2 was transfected in C2-7 myoblasts. (B) or NIH 3T3 cells (C) in the presence or absence of CMV-*Myf5* or CMV-*MyoD* expression plasmids as indicated below the histograms. (D) Construct 8X(E-box1) or 8X(E-box 2) was transfected in HeLa cells in the presence or absence of CMV-*Myf5* or CMV-*MyoD* expression plasmids as indicated below the histogram. CAT activity generated by each transfected reporter construct alone was given the value of 1 in B, C, and D. (E) C2-7 cells transfected with 8X(E-box1) or 8X(E-box 2) were allowed to proliferate (10% CS) or induced to differentiate (1% HS). The values represent the averages of at least three experiments.

Fgf4-lacZ Is Not Activated in the Myotomes of Shh Null Embryos

Shh signals from the notochord and the neural plate have been shown to play a key role in influencing the expression of myogenic bHLH factors (Munsterberg *et al.*, 1995). While *Shh* has an essential inductive function in the early activation of *Myf5* and *MyoD* in the dorsal myotome, *Shh* signaling is not required for expression of *Myf5* and *MyoD* in the ventral myotome (Borycki *et al.*, 1999).

We tested the activation of the *Fgf4-lacZ* transgene in the myotomes of the *Shh*−/− embryos. X-gal staining was undetectable or extremely weak in the myotomes and derivative muscles of E10.5, E11.5, E12.5, and E13.5. *Shh*−/− embryos, including both dorsal and ventral domains (Figs. 7A and 7B and data not shown). In only a few cases could we detect very weak β-gal expression in the hypaxial myotomes, but not in epaxial myotomes (Fig. 7B). Immunohistochemistry performed on transverse sections of E11.5 *Shh*−/− *Fgf4-lacZ* transgenic embryos at the interlimb or tail level revealed remarkable features. Anti-MYOD or anti-myogenin antibodies did not detect any MYOD- or myogenin-positive cells in the dorsal domain. The ventral domain was, however, positive for MYOD and myogenin and was considerably expanded toward the scle-

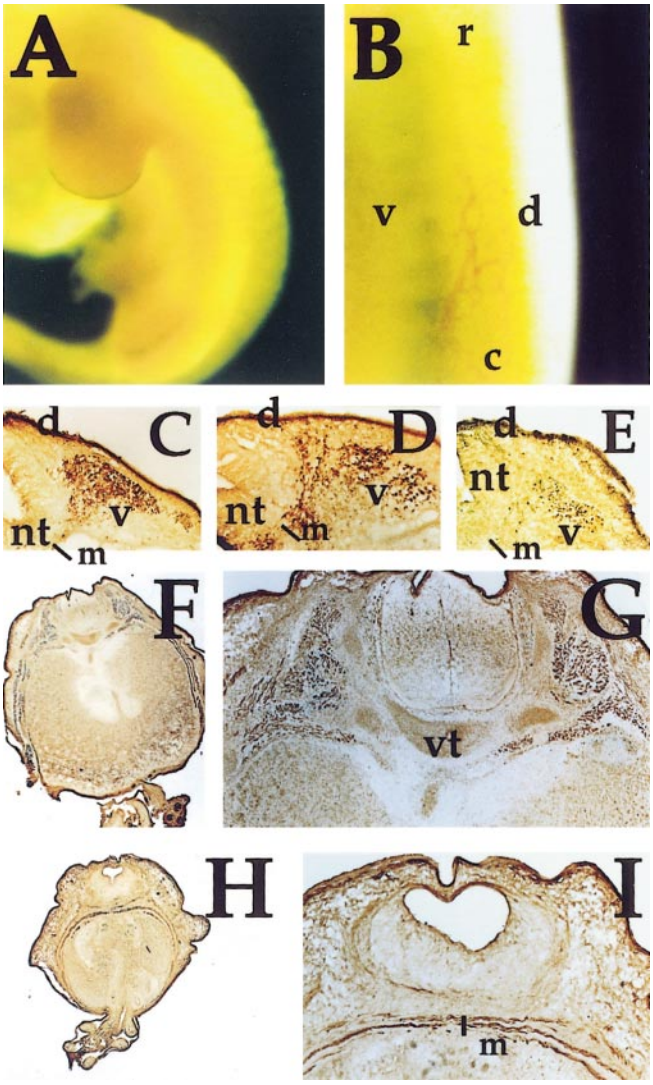


FIG. 7. *Fgf4-lacZ* expression is severely impaired in the myotomes of the *Shh* null embryos. (A and B) *Shh*−/−:*Fgf4-lacZ* transgenic embryos were harvested at E10.5 (A) or E11.5 (B) and X-gal stained. (C–E) An E11.5 *Shh*−/−:*Fgf4-lacZ* embryo was X-gal stained, sectioned at interlimb (C, E) and tail (D) levels, and immunostained with anti-MYOD (C, D) or anti-myogenin (E) antibodies. (F–I) Wild-type (F and G) and *Shh*−/− (H and I) transgenic embryos for *Fgf4-lacZ* were harvested at E13.5, X-gal stained, sectioned at the interlimb level, and immunostained with anti-MHC antibodies. d, dorsal; v, ventral; r, rostral; c, caudal; m, midline; nt, neural tube; vt, vertebrate.

rotome (Figs. 7C–7E), particularly at the tail level. The absence of *Shh* allowed MYOD-positive cells to occupy adjacent ventral regions in close proximity to the notochord and the neural plate. MYOD-positive cells from both left and right myotomes joined at the midline in the proximity of the notochord and no discernible separation between the two myotomes was observed (Fig. 7D). No *lacZ*-positive cells were observed. These observations indicate that the presence of myogenic bHLH factors in the ventral myotome of the *Shh* null embryos is insufficient for *Fgf4* gene expression in the myotomes and suggest that *Fgf4* expression is also dependent on *Shh* signaling.

Immunohistochemistry performed on transverse sections of E13.5 *Shh*−/− embryos *Fgf4-lacZ* transgenics using anti-MHC Ab revealed a complete absence of the dorsal musculature and of a *lacZ*-positive domain (Figs. 7H and 7I, compare to a section of a wild-type embryo in Figs. 7F and 7G). In the hypaxial musculature a wall of ectopic MHC-positive cells continues without interruption through the midline (Fig. 7I, compare to Fig. 7G), and this could be a consequence of the expansion of the ventral domain observed at E11.5 (Fig. 7C).

It has been proposed (Laufer *et al.*, 1994; Niswander *et al.*, 1994) that *Fgf4* expression in the limb-bud AER requires *Shh* signaling originating from the zone of polarizing activity (ZPA). Surprisingly, however, the limb-bud AERs of E10.5 and E11.5 *Shh* null embryos were clearly *lacZ* positive (Fig. 7A and data not shown).

DISCUSSION

The experiments presented in this report were aimed at identifying the mechanisms underlying the expression of *Fgf4* in the somitic myotomes of the developing embryo. We showed earlier that the *Fgf4* gene contains cassette-like DNA elements, located in the 3′ UTR of the gene, that direct gene expression to specific developmental stages and structures. The best characterized of these elements is the *Fgf4* EC cell enhancer, which corresponds to the Hom3b conserved region of the *Fgf4* 3′ UTR and is responsible for *Fgf4* expression in the ICM of the blastocyst as well as in EC and ES cells. This enhancer is synergistically activated by two transcriptional regulators, SOX2 and OCT-3, that bind to adjacent sites on the *Fgf4* enhancer DNA (Curatola and Basilico, 1990; Dailey *et al.*, 1994; Yuan *et al.*, 1995; Fraidenraich *et al.*, 1998).

Previous results, obtained through a transgenic analysis of the *Fgf4* elements required to drive β-gal expression in specific structures of the developing mouse embryo, had indicated that another conserved *Fgf4* DNA fragment termed Hom3a, also located in the 3′ UTR of the gene, contained *cis* elements necessary and sufficient for both myotome and AER expression. The myotomes and AER DNA elements overlapped, but appeared to be distinct since mutations of the two E boxes contained in Hom3a abolished myotome, but not AER, expression (Fraidenraich *et al.*, 1998). Here we show that only the second of these two E boxes is essential for the activity of the *Fgf4* myotome enhancer and that the myogenic factor MYF5 plays a major role in the activation of this element. MYOD, which can also bind and stimulate this element *in vitro*, can only partially compensate for the absence of MYF5. We also show that *Shh* is required for *Fgf4* expression in the myo-

tomes and that the role of *Shh* may not result solely from its ability to activate the myogenic factors, suggesting that other transcriptional regulatory factors are involved.

A Conserved E box Located in the Hom3a Region Controls Fgf4 Expression in the Myotomes

As alluded to above, the myotome enhancer element we have identified contains two E boxes, which are known binding sites for the family of bHLH transcription factors, including the myogenic factors MYOD and MYF5. The sequences of these two E boxes differ in the two central nucleotides (CACATG, box 1; CAGCTG, box 2), which are known to influence the specificity of binding of bHLH factors. By analyzing transgenic embryos in which *lacZ* expression was controlled by the Hom3a *Fgf4* region, we found that only E-box 2 was essential, while E-box 1 appeared to play no role. Accordingly, we found that DNA probes corresponding to E-box 2 and flanking sequences bound MYOD and MYF5 in gel-shift assays, while E-box 1 did not show appreciable activity. Furthermore, another *lacZ* construct containing a mutated E-box 2 within most of the 3′ UTR sequences failed to activate β-gal expression in the myotomes. This transgene contains six intact E boxes in addition to the mutated E box. This strongly suggests that E-box 2 cannot be substituted by any other E box present in the 3′ UTR. It is worth mentioning that none of the additional E boxes has the exact sequence (CAGCTG) of E-box 2. The requirement for E-box 2 appeared to be specific for expression in the myotomes but not for the limb-bud AER where *lacZ* expression was not perturbed by mutating E-box 2.

It is interesting to note that the Hom3a element is overall highly conserved in the human, mouse (Curatola and Basilico, 1990), chicken (unpublished), and zebrafish (B. Draper, in preparation) *Fgf4* genes, but only E-box 2 is perfectly conserved in these four species. Previous studies on regulation of expression of myogenic factors through E-box elements showed that mutation of one E box in the myogenin promoter in the context of a minimal sequence (133 bp) abrogated *lacZ* expression in the myotomes (Yee and Rigby, 1993). However, when this mutation was analyzed in the context of longer fragments containing other E boxes, no abrogation was observed (Yee and Rigby, 1993; Cheng *et al.*, 1993). A core motif in the *MyoD* distal enhancer contains four E boxes. However, mutations of these E boxes did not alter the pattern of *lacZ* transgene expression in the myotomes (Goldhamer *et al.*, 1995). Recent studies performed on the *MyoD* core enhancer identified a 30-bp segment which responds to *Myf5*. Interestingly, an E box with the sequence CAGCTGTT lies in the center of this element (Kucharczuk *et al.*, 1999). The sequence CTGTC/T—present also in E-box 2—has been also shown to act as a preferred half site for MYOD binding (Blackwell and Weintraub, 1990).

Myf5 Appears to Be the Main Myogenic Factor Involved in Fgf4 Expression in the Myotomes

The results described above prompted us to examine the role of *Myf5* and *MyoD* on *Fgf4* gene expression in the myotomes. *Myf5* is the first of the myogenic bHLH genes to be expressed during mouse embryogenesis, with transcripts observed initially in the dermamyotome and later in the myotome between E8.0 and E14.5 (Ott *et al.*, 1991), while *MyoD* is expressed in the myotome beginning at E10 and throughout development and adulthood (Sassoon *et al.*, 1989). *Myf5*-null mice do not form the distal part of the ribs but exhibit normal skeletal muscle development at birth because of the expression of *MyoD* (Rudnicki *et al.*, 1993). They show, however, a delay in myotome maturation and it has been suggested that this delay of early myotome maturation is responsible for the rib cage defect (Braun *et al.*, 1992). More recent studies have led to the suggestion that in the *Myf5* KO embryos there is a delay in the development of the epaxial (paraspinal and intercostal) musculature, but the hypaxial (limbs and abdominal wall) musculature develops on schedule (Kablar *et al.*, 1997). Previous studies have described that the expression of members of the FGF family was absent or delayed in the somites (E9.5–10.5) of *Myf5* null embryos (Grass *et al.*, 1996), but did not investigate their expression in the myotomes at later stages.

By crossing our transgenic lines with *Myf5* or *MyoD* knockout mice we have observed that *lacZ* expression was unchanged in the myotomes of *MyoD*−/− embryos, but was almost completely suppressed in a *Myf5*−/− background. However, some residual X-gal staining was observed in the ventral portion of *Myf5* null myotomes. By examining *Myf5*−/−:*MyoD*+/− or *MyoD*−/− embryos we could show that the residual transgene expression in the absence of *Myf5* requires the expression of *MyoD*. Thus, *MyoD* could compensate, at least partially, for the absence of *Myf5* in the ventral myotomes.

These observations have to be interpreted also in the context of our *in vitro* experiments, which show that both MYF5 and MYOD bind equally to the E-box 2 sequence in the *Fgf4* myotome enhancer and can stimulate gene expression from a CAT reporter plasmid under the control of myotome enhancer sequences. We believe that the best interpretation of these data is that MYF5 is the main myogenic factor required to drive *Fgf4* gene expression in the myotomes and that it can be replaced by MYOD in the ventral myotomes, but that this requirement does not reflect an intrinsic ability of MYF5 and not MYOD to activate the myotome enhancer. Rather we believe that in principle both MYF5 and MYOD can transactivate the *Fgf4* myotome enhancer, but that *in vivo* only *Myf5* is expressed in the specific temporal and spatial pattern required for *Fgf4* enhancer activation. This hypothesis finds support in a recent finding obtained by P. Soriano (manuscript in preparation). Knock-in mice in which the *Myf5* gene is replaced by *MyoD* and in which therefore *MyoD* is expressed under the regulatory elements of *Myf5* do not display the rib

defects of *Myf5* KO mice. Thus in this case, *MyoD* can substitute for *Myf5* function.

It is likely that another transcription factor(s) must cooperate with MYF5 to promote *Fgf4* expression in the myotomes. The hypothesis is not only supported by the general consensus that specific regulation of gene expression during development results from the combinatorial action of multiple transcription factors, but also from the following observations: (i) Both *Myf5* and *MyoD* expressions in the myotomes of E11.5 embryos are much more widespread than that of *Fgf4*, which is restricted to a central portion of the myotomes; (ii) many tissues and cell lines (e.g., differentiated myoblasts) express *MyoD* and *Myf5* but not *Fgf4;* and (iii) our preliminary results indicate that transgenic constructs containing only the 5′ 160 nucleotides of the Hom3a region (i.e., the myotomal enhancer), which include E-box 2, are not capable of driving *lacZ* expression in the myotomes, indicating that other DNA elements located in the 3′ half of the myotome enhancer are required. In this view, *Myf5* could be expressed in cells of the epaxial lineage which coexpress another factor instrumental for *Fgf4* activation, and *MyoD* could be mostly expressed in cells of the hypaxial lineage, which lack the hypothetical coactivator. The 3′ half of Hom3a contains several AT-rich regions, including a consensus for myocyte enhancer factor 2 (MEF2) binding site—YTA(A/T)$_4$TAR (Black and Olson, 1998). The combinatorial action of proteins from the myogenic bHLH and the MEF2 families appears to represent a transcriptional code specific for skeletal muscle gene activation. However, expression of *Mef2* is broadly detected in myogenic and nonmyogenic lineages during mouse embryogenesis (Black and Olson, 1998) and thus may not explain the restricted pattern of expression of *Fgf4*.

Shh Is Required for Fgf4 Gene Expression in the Myotomes

The establishment of the myogenic program is specified by a precise number of instructions emanated from surrounding tissues. Thus for example, *Shh* signals from the notochord and floor plate, in combination with *Wnt* family members which are expressed in dorsal regions of the neural tube, induce myogenic bHLH gene expression in the somite (Munsterberg *et al.*, 1995). Another key regulator of somitic myogenesis is the transcription factor PAX3. *Wnt* and *Shh* signals can induce somitic expression of *Pax3* concomitantly with the expression of *Myf5* and prior to *MyoD*. Indeed, infection of embryonic tissues *in vitro* with a retrovirus encoding PAX3 is sufficient to induce expression of *MyoD, Myf5,* and myogenin in paraxial and lateral plate mesoderm in the absence of inducing tissues (Maroto *et al.*, 1997). Furthermore, *Pax3* (*Splotch*):*Myf5* double-mutant embryos were found to be devoid of *MyoD*, suggesting that *MyoD* acts genetically downstream of these genes for myogenesis (Tajbakhsh *et al.*, 1997). Targeted gene disruption of *Shh* showed defects in tissues that are beyond the normal sites of *Shh* transcription, confirming the proposed role of SHH as an extracellular signal required for the tissue-organizing properties of several vertebrate patterning centers (Chiang *et al.*, 1996). In particular during myogenesis, *Shh* has an essential inductive function in the activation of *Myf5* and *MyoD* in the epaxial somite cells but is not required for the expression of *Myf5, MyoD,* and *Pax3* in the hypaxial dermamyotomal cells (Borycki *et al.*, 1999). We analyzed the pattern of expression of *Fgf4-lacZ* in the myotomes of *Shh* mutant embryos and found no detectable expression. While the absence of β-gal expression in the presumptive dorsal myotome was expected, since no myogenic factors are present, the fact that *Fgf4* is not activated in the ventral myotomes suggests that *Fgf4* expression in the myotomes is dependent on *Shh* signaling and that the myogenic bHLH factors are not sufficient for *Fgf4* expression in the absence of *Shh*. We interpret this finding as indicating that *Shh* induces the expression of other transcription factors which cooperate with MYF5 in the activation of the *Fgf4* myotome enhancer and that, in their absence, no *Fgf4* expression is detected. Since PAX3 is expressed in the hypaxial myotomes of *Shh*−/− embryos (Borycki *et al.*, 1999), however, PAX3 is unlikely to be such a factor. We cannot, however, exclude the possibility that in the absence of *Shh,* the integrity of the hypaxial myotome is compromised and the expression levels of the myogenic factors are reduced. This hypothesis is supported by the observation that the ventral somites of *Shh* null embryos undergo considerable apoptosis (Borycki *et al.*, 1999; our unpublished observations) that could lead to extremely reduced levels of bHLH factors. We are currently undertaking mutational analysis of the *Fgf4* myotome enhancer to identify MYF5 coactivators and to address if they are *Shh* dependent.

It has been proposed that *Fgf4* expression in the limb-bud AER requires *Shh* signaling from the limb mesenchyme ZPA and that *Shh* and *Fgf4* were under a complex form of reciprocal regulation (Niswander *et al.*, 1994; Laufer *et al.*, 1994). However, we found that *Fgf4-lacZ* was expressed in the limb-bud AER of *Shh* null embryos at E10.5–E11.5. This is in agreement with recent findings showing that expression of the BMP antagonist gremlin is sufficient to induce *Fgf4* in the AER and establish the SHH/FGF4 feedback loop, although only until E10.25 and in a restricted portion of the AER (Zuniga *et al.*, 1999). FGF4 activation was independent of *Shh* signaling. BMPs and FGFs may antagonize one another during limb-bud development (Niswander and Martin, 1993). Therefore, inhibition of BMP activity in the limb-bud compartments by BMP antagonists might override BMP-mediated *Fgf4* repression (Pizette and Niswander, 1999). We have identified a *cis*-acting negative regulatory element controlling *Fgf4* expression in the AER (Fraidenraich *et al.*, 1998) and this element could be a target of transcriptional repression by *Bmp* signaling.

As in the case of the AER, somitic myogenesis is also under negative regulation (Reshef *et al.*, 1998). *Bmp* signaling serves to inhibit the activation of *MyoD* and *Myf5* in *Pax3*-expressing cells, and the BMP antagonist noggin,

ectopically expressed lateral to the somite, expands *MyoD* expression and induces formation of a lateral myotome (Reshef *et al.*, 1998). The somites of noggin null embryos show a severe reduction of *MyoD* expression (McMahon *et al.*, 1998). The fact that *Fgf4* is not expressed in the dorsal nor in the ventral myotomes of *Shh* null embryos, however, suggests that *Fgf4* cannot be initially activated through a *Shh*-independent mechanism. Thus it is unlikely that *Fgf4* expression in the myotomes is initiated by antagonists of BMP as has been proposed for *Fgf4* expression in the limb-bud AER.

Developmental Role of Fgf4 Expression in the Myotomes

Although our experiments were focused on the identification of the mechanisms regulating *Fgf4* expression during embryogenesis, and not directly aimed at understanding the role of *Fgf4* signaling in development, some of the data presented here have a bearing on this subject. Several *Fgf*s are expressed in the myotomes, most notably *Fgf4*, *5*, and *6*, but the phenotypes of *Fgf5* and *Fgf6* KO mice do not suggest a specific, unique role in muscle development, while the early lethality of *Fgf4* null embryos has precluded the assessment of its role in the myotomes (Herber *et al.*, 1994; Floss *et al.*, 1997; Feldman *et al.*, 1995). Our data suggest that FGF4 is not essential for skeletal myogenesis. (I) *Fgf4-lacZ* gene expression is greatly reduced and restricted to the ventral portion of the myotomes in *Myf5* null mice, which, however, form a normal skeletal musculature. (II) We have observed a total absence of *Fgf4* transgene expression in *Myf5* null, *MyoD*+/− mice, which although not viable, can develop skeletal muscles. Thus either *Fgf* signaling is not essential for muscle development or the role of FGF4 in this process is redundant with that of FGF5 or FGF6. The creation of compound null mice, and experiments aimed at specifically abolishing *Fgf4* expression in the myotome, which are currently in progress in our laboratory, should eventually answer these questions.

In conclusion, the experiments presented in this report show that *Fgf4* expression in the myotomes, studied through a sensitive system of transgenic expression, depends on a specific and conserved enhancer element located in the 3′ UTR and that this element must interact with the myogenic factor MYF5 to direct sustained *Fgf4* expression. *MyoD* can partially compensate for the absence of *Myf5* in the hypaxial lineage. Furthermore, other factors, some of which are likely to be controlled by *Shh* signaling, must cooperate with MYF5 in promoting expression of *Fgf4* in the myotomes.

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