The homeobox protein Barx2 is expressed in both smooth and skeletal muscle and is up-regulated during differentiation of skeletal myotubes. Here we use antisense-oligonucleotide inhibition of Barx2 expression in limb bud cell culture to show that Barx2 is required for myotube formation. Moreover, overexpression of Barx2 accelerates the fusion of MyoD-positive limb bud cells and C2C12 myoblasts. However, overexpression of Barx2 does not induce ectopic MyoD expression in either limb bud cultures or in multipotent C3H10T1/2 mesenchymal cells, and does not induce fusion of C3H10T1/2 cells. These results suggest that Barx2 acts downstream of MyoD. To test this hypothesis, we isolated the Barx2 gene promoter and identified DNA regulatory elements that might control Barx2 expression during myogenesis. The proximal promoter of the Barx2 gene contained binding sites for several factors involved in myoblast differentiation including MyoD, myogenin, serum response factor, and myocyte enhancer factor 2. Co-transfection experiments showed that binding sites for both MyoD and serum response factor are necessary for activation of the promoter by MyoD and myogenin. Taken together, these studies indicate that Barx2 is a key regulator of myogenic differentiation that acts downstream of muscle regulatory factors.

Most skeletal muscle develops from mesenchymal premuscle condensations that are determined early in development (1). Several types of transcription factors control the specification and differentiation of skeletal myoblasts within these condensations. Critical among these are four basic helix-loop-helix myogenic regulatory factors (MRFs): MyoD, Myf5, myogenin, and MRF4, each of which binds to E-box sequences in the promoters of skeletal muscle genes (see Ref. 2 for review). Each of the four MRFs can induce skeletal muscle differentiation when expressed in nonmuscle cells (3–7). However, during normal skeletal muscle development, the MRFs function in a hierarchical fashion: MyoD and Myf5 are involved in myoblast specification, whereas myogenin and MRF4 control the terminal differentiation of myoblasts into myotubes (8, 9). MyoD also promotes myoblast differentiation by inducing the expression of myogenin and MEF (myocyte enhancer factor) 2 family proteins. In addition, several homeobox genes, including Msx1, Pax3, and Pax7 influence the expression of myoblasts by regulating the expression of particular MRFs (10–12). However, few homeobox genes have been described that act downstream of MRFs or that influence myotube fusion directly without influencing the expression of MRFs.

One component of the morphogenetic program specified by homeobox genes is the control of cell adhesion, a process that is essential to condensation, migration, and fusion of cells in several developmental contexts (13, 14). Barx1 and Barx2 are two closely related homeobox proteins that were found in separate efforts to identify factors that control the expression of cell adhesion molecules (CAMs) (15, 16). In an earlier study, we discovered Barx2 and showed that it is expressed in precise patterns during mouse embryonic development in the nervous system, neural crest-derived craniofacial structures, lung buds, and limb mesenchyme (16). More recently, other researchers have shown that chicken and murine Barx2 variants are expressed in both skeletal and smooth muscle (17, 18). For example, the chicken Barx2 homologue (Barx2b) is expressed in the early myotome and persists after differentiation of muscle groups in the limb, neck, and cloaca (17). In addition, Herring et al. (18) showed that murine Barx2 is expressed in adult skeletal muscle and smooth muscle-containing tissues. Barx2 is also expressed in C2C12 myoblasts, and its expression increases dramatically when myoblasts fuse to form myotubes (18). Collectively, these observations suggest roles for Barx2 in both skeletal and smooth muscle development.

In this study we examined the role of Barx2 in muscle development. Inhibition of Barx2 expression in limb bud cell cultures inhibits myotube formation, whereas overexpression of Barx2 accelerates the appearance of myotubes. However, overexpression of Barx2 does not induce ectopic MyoD expression in limb bud cultures, nor is it sufficient to specify a myoblast fate in multipotent C3H10T1/2 mesenchymal cells that do not express MyoD. These results suggest that Barx2 acts downstream of MyoD to promote myogenic differentiation but cannot substitute for MyoD function. In support of this conclusion, we found that both MyoD and myogenin can bind to and activate the Barx2 promoter in C2C12 myoblasts. In C3H10T1/2 cells overexpression of Barx2 induces a smooth muscle-like appearance with increased smooth muscle-α-actin expression and formation of stress fibers. Overall our data suggest two roles for Barx2 in muscle development: in MyoD-positive skeletal myoblasts Barx2 controls myotube fusion, whereas in MyoD-negative mesenchymal cells Barx2 may promote differ-
EXPERIMENTAL PROCEDURES

Morpholino Antisense Oligonucleotide Inhibition of Barx2 Expression in Limb Bud Cultures—Morpholino oligonucleotides (ODNs) that are antisense to a region of the Barx2 mRNA near the initiation codon were designed and synthesized by Gene Tools LLC. Control sense ODNs were also prepared. Dissociated mesenchymal cells were prepared from E10.5 mouse limb buds and 2 x 10^6 cells were plated in 10 μl aliquots at a density of 2 x 10^5 cells/ml in CMRL-1066 (Invitrogen) containing 2% foetal bovine serum (FBS) in four-well culture dishes. After 24 h incubation to allow the micromass cultures to adhere, sense and antisense ODNs were introduced using LipofectAMINE 2000 (Invitrogen). The cells were cultured for an additional 24 h before fixing with 4% paraformaldehyde and staining with either anti-MyoD or anti-Barx2 antibodies as described below.

Retroviral Transduction of Barx2 in Limb Bud Cultures—Retroviral vectors were constructed that contain either full-length mouse Barx2 cDNA or a cDNA fragment that encodes the homeodomain, Barx basic region, and COOH-terminal activation domain (HD-BBR-C) (19). A control retroviral vector was constructed that contains the green fluorescent protein gene. The retroviral vector was based on the murine control retroviral vector was constructed that contains the green fluorescent protein gene. 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Nuclear envelopes were prepared as described previously (22). For qualitative gel shift experiments, equal amounts of C2C12 nuclear protein from C2C12 myoblasts or myotubes were used in each binding reaction.

Isolation of the Murine Barx2 Gene—The murine Barx2 gene was isolated from a 129/SvJ murine genomic BACmid library in the

2 The Blau Lab Homepage www.stanford.edu/group/blau/.
Fig. 1. Barx2 is required for the formation of myotubes in primary limb bud mesenchymal cultures. Limb bud cells from E10.5 embryos were cultured in micromass conditions and treated for 6 h with morpholino ODNs that were antisense to Barx2, or with control sense ODNs. Cells were then cultured for 24 h in low-serum conditions, fixed, and stained with polyclonal antibodies to either Barx2 or MyoD. Cultures treated with sense ODNs formed myotubes and expressed both Barx2 and MyoD at high levels (left panels). In contrast, cultures treated with Barx2 antisense ODNs did not form myotubes and did not express Barx2, however, MyoD expression is still apparent (right panels).

Relative binding of the protein to the E-box probes was determined by measuring the intensity of the probe-protein complexes formed in gel mobility-shift experiments using a PhosphorImager (Amerham Biosciences). The relative binding data shown in Fig. 6 were derived from four independent experiments using different C2C12 extract preparations.

RESULTS

Inhibition of Barx2 Expression Prevents Myotube Formation in Primary Limb Bud Cell Cultures—To determine whether Barx2 is involved in the differentiation of skeletal myoblasts, we inhibited Barx2 expression in primary myoblasts using morpholino antisense ODNs and then examined their ability to form myotubes. Morpholino ODNs are RNase H-independent, resistant to nucleases, and have a good targeting predictability (24, 25). For this study, limb bud mesenchymal cells from E10.5 mouse embryos were cultured in micromass conditions; these micromass cultures have been shown to contain cells that are committed to either a myoblast or chondrocyte fate (26). The limb buds were dissected, dissociated, and cultured in low serum under micromass conditions as described under “Experimental Procedures.” The cultures were treated immediately after plating with either Barx2 antisense ODNs or control sense ODNs and examined 24 h later for formation of myotubes and for expression of Barx2 and MyoD.

As shown in Fig. 1, cultures treated with ODNs antisense to Barx2 showed negligible expression of Barx2 (Fig. 1, top right panel) and formed few if any myotubes within the 24-h culture period. Many cells showed nuclear expression of MyoD, however, these cells were not fused and did not show the characteristic elongated nuclei of myotubes (Fig. 1, bottom right panel). In contrast, cultures that were treated with the sense ODNs contained many cells that expressed Barx2 and MyoD and that were fused into multinucleated myotubes (Fig. 1, left panels). These results indicate that Barx2 expression is required for myoblasts to differentiate into myotubes in culture. Immunohistochemical staining showed that Barx2 antisense-treated cultures do not express Barx2, but retain appreciable, albeit diffuse, expression of MyoD. The observation that MyoD staining in antisense-treated cultures is diffuse relative to the control cultures may be due in part to the condensed and elongated morphology of nuclei in myotubes.

Barx2 Overexpression Accelerates Myotube Formation in Primary Limb Bud Cells—To determine whether overexpression of Barx2 can promote myotube formation, a retroviral system was used to overexpress Barx2 proteins in limb bud cultures. Micromass cultures were infected with retroviral constructs that express either full-length Barx2 protein or a fragment of Barx2 containing the homeodomain, Barx basic region (BBR), and the carboxyl-terminal domain (Barx2/HDBBRC). The Barx2/HDBBRC fragment contains the DNA binding and transactivation domains of Barx2 and was previously shown to activate the promoter of the Barx2 target gene N-CAM, which is a cell adhesion molecule known to influence myotube fusion (19, 27). Control cultures were infected with a retroviral construct expressing green fluorescent protein. The cells were cultured in micromass conditions (28) for 24 h in media containing 10% serum to allow integration of the virus into dividing cells and the expression of Barx2 proteins. The cultures were then incubated for a further 24 h in media containing 2% serum to induce myotube formation.

As shown in Fig. 2, micromass cultures that were infected with retroviral constructs expressing either full-length Barx2 (not shown) or the Barx2/HDBBRC fragment (Fig. 2, top right panel) formed many myotubes within 24 h of culture. In contrast, cells infected with the control retrovirus produced very few myotubes within 24 h (Fig. 2, top left panel). Immunohistochemical staining with antibodies to MyoD revealed that only MyoD-positive cells fused to form myotubes. Thus Barx2 can-
not promote myobute fusion in the absence of MyoD (Fig. 2, middle panels). Moreover, when the number of MyoD-positive cells was counted in three separate fields, their numbers did not change after infection with the Barx2 construct (data not shown). Thus Barx2 does not induce ectopic expression of MyoD. Together these results indicate that Barx2 acts downstream of, but does not substitute for, MyoD activity. The myotubes that formed after infection of limb bud cells with the Barx2/HBBRC-expressing retrovirus also showed increased expression of SM-α-actin, an early marker of skeletal muscle differentiation (29, 30) (Fig. 2, bottom panels).

Barx2 Promotes Myobute Formation in C2C12 Myoblasts but Not in C3H10T1/2 Fibroblasts—The results of both Barx2 inhibition and overexpression experiments in primary limb bud cultures suggest that Barx2 acts downstream of MyoD to promote the differentiation of cells that are already committed to the myobute fate. To formally test whether Barx2 acts upstream or downstream of MyoD, we compared the effects of overexpressing Barx2 in MyoD-positive C2C12 myoblasts and in MyoD-negative C3H10T1/2 mesenchymal cells.

A population of C2C12 myoblasts that overexpress Barx2 was generated by stable transfection of a pcBarx2 expression plasmid, and a control population was established by stable transfection of the empty pcDNA3 vector. Overexpression of Barx2 protein in pcBarx2-transfected cells was confirmed by Western blotting with anti-Barx2 antibodies (see Fig. 3D). The effect of Barx2 overexpression on myobute formation was examined by plating pcBarx2-transfected and control cells at the same initial density and culturing in media containing 2% horse serum. Under these conditions, cultures of pcDNA3-transfected C2C12 cells differentiated relatively slowly, forming myobutes after 4 days (Fig. 3A, left panel). In contrast, C2C12 cells that were transfected with the pcBarx2 plasmid showed significantly accelerated differentiation, forming myobutes within 2 days of serum withdrawal (Fig. 3A, right panel). These results are similar to those obtained in primary limb bud cultures and provide further evidence that expression of Barx2 promotes the differentiation of MyoD-expressing myoblasts.

To test whether Barx2 is also involved in myobute determination, we next examined the effect of overexpressing Barx2 in the mesenchymal progenitor cell line C3H10T1/2 that does not express MyoD. Ectopic expression of MyoD was previously shown to convert C3H10T1/2 cells into skeletal myoblasts that fuse into myobutes when cultured in low serum conditions (31). If Barx2 were able to induce the expression of MRFs such as MyoD it may be expected to convert C3H10T1/2 cells into myoblasts. C3H10T1/2 cells were transiently transfected with either the pcBarx2 plasmid, a pcDNA expression plasmid containing the MyoD cDNA (pcMyoD), or the empty vector. To identify transfected cells, a plasmid encoding enhanced yellow fluorescent protein (EYFP) was co-transfected with the expression vectors. The cells were then incubated in media containing 2% horse serum and the formation of myobutes by the EYFP-labeled cells was examined over the next 4 days. Consistent with previous reports (31), ectopic MyoD expression induced myobute formation in nearly all of the transfected cells after 4 days of serum deprivation (Fig. 3B, right panel). In contrast, Barx2-transfected C3H10T1/2 cells did not form myobutes (Fig. 3B, middle panel). These results indicate that, unlike MyoD, Barx2 is not sufficient to specify a skeletal myobute fate when expressed in multipotent mesenchymal cells.

Interestingly, when pcBarx2-transfected and control pcDNA3-transfected C3H10T1/2 cells were grown in high serum conditions, pcBarx2-transfected cells showed morphological changes that are consistent with the differentiation of smooth muscle cells (SMCs) or myofibroblasts, including cell spreading and formation of stress fibers (Fig. 3C) (32, 33). C3H10T1/2 cells and other fibroblastic cell lines have been previously reported to differentiate into both SMCs and myofibroblasts in response to particular signals (34–36). Using immunohistochemistry, we found that Barx2-transfected
C3H10T1/2 cells contained increased amounts of SM-α-actin, which is also consistent with these cellular phenotypes (Fig. 3C). These results suggest that in the absence of MyoD expression, Barx2 influences the differentiation of mesenchymal progenitors into contractile cell types other than skeletal myoblasts.

In these studies, Barx2 overexpression increased the expression of SM-α-actin in all three cellular systems tested: primary myoblasts, C2C12 myoblasts, and C3H10T1/2 cells. To quantify this effect, we examined the expression of two cytoskeletal proteins that are regulated during myogenesis, SM-α-actin and desmin in Barx2- and pcDNA3-transfected C2C12 cells. Quantitative Western blotting was used to measure the amount of SM-α-actin, desmin, and β-actin protein in extracts of cells grown in high serum conditions (Fig. 3D). After normalization to β-actin, the amount of SM-α-actin was, on average, 1.7-fold higher in pcBarx2-transfected cells than in pcDNA3-transfected cells in three separate experiments. In contrast the expression of desmin did not change significantly (Fig. 3D). This indicates that Barx2 can activate particular genes that are associated with the differentiation of multiple contractile cell types. Collectively our studies in primary limb bud cultures and cell lines suggest two functions for Barx2 in muscle differentiation. In committed myoblasts that express MyoD, Barx2 promotes, and is required for, formation of myotubes, whereas in multipotent mesenchymal cells that lack MyoD, Barx2 may induce a smooth muscle or myofibroblast phenotype.

Identification of the Murine Barx2 Gene—The results of the studies described above suggest that Barx2 acts downstream of MyoD during myoblast differentiation. To determine whether MyoD or other MRFs may directly regulate the expression of Barx2, we isolated the murine Barx2 gene and characterized its promoter. The Barx2 gene was isolated by screening a BACmid murine genomic library using a probe from the 5’ end of the Barx2 cDNA sequence. Two clones of ~80 kb containing the Barx2 gene were analyzed by restriction endonuclease digestion and Southern blotting. Several overlapping restriction fragments containing the Barx2 gene were mapped and sequenced, revealing four exons spanning greater than 30 kb (Fig. 4). The size of the first intron was not determined precisely; however, Southern blots indicated that it spans at least 20 kb. In addition, a 3-kb segment upstream of the first exon was isolated and sequenced.

Comparison of the sequence of the murine Barx2 gene with the recently published human Barx2 gene (37) revealed considerable similarity over a 1-kb region upstream of the translation initiation codon. In particular, the sequence that corresponds to the transcription start site (TSS) in the human gene (37) is conserved, suggesting that transcription begins at the same site in the murine gene (Fig. 5A). DNA sequence analysis of the region upstream of the predicted TSS revealed potential binding sites for a number of transcription factors, including sequences that match the Sp1 consensus motif (G(A/G)GGC(A/G)GG(A/T)). A sequence containing a perfect match to the Sp1 consensus sequence (GGGCGGGGCT) is located ~340 nucleotides upstream of the TSS; an additional cluster of motifs that partially match the Sp1 consensus is located ~80 nucleotides upstream of the TSS. Because no TATA box elements are contained within the 3-kb region upstream of the first exon, this GC-rich region is likely to represent the core promoter. Several other conserved sequence elements were identified within a 400-bp segment upstream of the TSS. These include two E-box motifs, recognition sites for MEF and Ets family proteins, and a noncanonical CArG box/SRE (Fig. 5A).

The Murine Barx2 Promoter Contains Enhancer and Repressor Sequences That Function in Mesenchymal Cells—To identify positive and negative regulatory regions of the Barx2 promoter, six deletion constructs were generated, either by PCR amplification or by cleaving the promoter at natural restriction sites (Fig. 5B). These promoter fragments were cloned into the promoterless pGL3basic luciferase reporter vector and their activities were examined after transfection into C3H10T1/2 cells.

The shortest Barx2 promoter construct tested (construct 0.44), containing the region between nucleotides ~438 and ~80 relative to the predicted transcription start site, showed 7-fold greater activity than the pGL3basic plasmid suggesting that this region contains a core promoter (Fig. 5C). A fragment spanning nucleotides ~1012 to ~80, relative to the TSS (construct 1.0), showed ~2-fold greater activity than construct 0.44, indicating that the region between nucleotides ~438 and ~1012 contains an enhancer. Addition of the segment between nucleotides ~1012 and ~1214 (construct 1.2) led to a 2-fold decrease in promoter activity, relative to construct 1.0, indicating that this 200-bp region contains a repressor (Fig. 5C). Two additional constructs, 1.5 and 1.7, spanning nucleotides ~1446 to ~80 and ~1732 to ~80, respectively, showed levels of activity similar to that of construct 1.2. However, deletion of the segment between nucleotides ~1012 and ~1462 from construct 1.7 (construct 1.7Ksp1) increased activity ~8-fold. These results suggest that the region between ~1012 and ~1214 nucleotides contains a repressor that masks an enhancer activity located between ~1462 and ~1732 nucleotides upstream of the TSS (Fig. 5D).

MyoD and Myogenin Bind to an E-box Element within the Barx2 Promoter; Binding to this Element Is Increased during Differentiation of C2C12 Cells—The proximal region of the Barx2 promoter contains recognition motifs for several transcription factors that are associated with muscle development. Among these elements are two sequences corresponding to the E-box consensus (CANNTG) that may be recognized by myo-
genic basic helix-loop-helix factors such as MyoD or myogenin (see Fig. 5A). To determine whether these motifs can bind to MyoD or myogenin, we prepared two probes, designated E1 and E2, corresponding to each of the motifs, and tested their binding to in vitro translated MyoD and myogenin proteins in gel mobility shift experiments. MyoD and myogenin formed complexes with the E2 probe that were supershifted by antibodies to a Myc epitope tag located at the NH2 terminus of the proteins (Fig. 6A). In contrast, neither MyoD nor myogenin bound to the E1 probe (data not shown).

The E1 and E2 probes were also tested for binding to nuclear extracts of C2C12 cells in gel mobility shift experiments. MyoD and myogenin formed complexes with the E2 probe that were supershifted by antibodies to a Myc epitope tag located at the NH2 terminus of the proteins (Fig. 6A). In contrast, neither MyoD nor myogenin bound to the E1 probe (data not shown).

As shown in Fig. 6A, the complexes formed between the E2 probe and the myotube nuclear extract appeared more intense than those formed with the myoblast nuclear extract. To quantify the difference in binding, we measured the intensities of the probe-protein complexes that formed with the E2 probe using equivalent amounts of nuclear protein prepared from myoblast or myotube cultures in four separate gel mobility shift experiments (see “Experimental Procedures”). On average, the intensity of the E2 probe-protein complex formed with myotube nuclear extract was 1.9-fold greater than that formed with myoblast nuclear extract (Fig. 6A). These data indicate that the E2 element binds to proteins that are enriched during myogenic differentiation of C2C12 cells, and that these proteins are likely to include MyoD and myogenin.

Binding of Serum Response Factor (SRF) to the Barx2 Promoter—The proximal Barx2 promoter contains a noncanonical CArG box/SRE immediately adjacent to the MyoD/myogenin binding site. This element is a recognition motif for SRF, which has been shown in previous studies to interact with MyoD and myogenin at the promoter of particular muscle-specific genes (38, 39). To determine whether SRF binds to the Barx2 promoter, we prepared a probe corresponding to the Barx2 CArG box/SRE and tested its ability to bind to nuclear extracts from C2C12 cells. The CArG box/SRE probe formed complexes with nuclear extracts from both C2C12 myoblasts and myotubes; this binding was eliminated by mutation of the CArG box consensus motif (Fig. 6C). Because the sequence of the Barx2 CArG box/SRE (CCCAAAAAGG) diverges from the canonical recognition motif for SRF (CC(A/T)6GG), we examined whether...
antibodies to SRF could supershift or block the formation of complexes between the Barx2 CArG box probe and C2C12 nuclear extracts. The predominant probe-protein complex was supershifted by antibodies to SRF, suggesting that the complex contains SRF (see lane 5 in Fig. 6C).

In C2C12 cells, SRF regulates both mitogenic genes such as c-fos, as well as muscle-specific genes (40, 41). However, SRF has been reported to bind with higher affinity to the CArG box/SRE from the c-fos gene promoter than to the noncanonical CArg boxes/SREs present in many muscle-specific genes (40). To examine the relative strength of binding of SRF to the Barx2 CArg box/SRE, we compared binding of C2C12 nuclear extracts to the Barx2 CArg box/SRE probe as well as to a control probe corresponding to the c-fos CArg box/SRE (40). The Barx2 CArg box/SRE probe formed much less intense complexes than the c-fos CArg box/SRE probe when incubated with equivalent amounts of nuclear protein from C2C12 cells. Collectively these data suggest that both E-Box binding proteins and SRF may regulate expression of Barx2 in skeletal muscle cells.

**MyoD and Myogenin Activate the Proximal Barx2 Promoter in C2C12 Cells**—The results of the gel mobility shift analyses described above indicated that the proximal Barx2 promoter region is bound by factors involved in muscle-specific gene regulation. To examine the role of these elements in the regulation of the Barx2 gene, C2C12 myoblasts were transfected with various Barx2 promoter constructs, together with expression plasmids that encode MyoD and myogenin (Fig. 7). Constructs 1.0 and 0.44 showed lower levels of basal activity in C2C12 cells than in C3H10T1/2 cells (see Fig. 5). However, co-transfection of a MyoD expression plasmid activated these constructs approximately 13- and 11-fold, respectively (Fig. 7). Expression of myogenin activated these two constructs 6- and 5-fold, respectively (Fig. 7). Deletion of a 220-bp SmaI restriction fragment from construct 0.44 (0.44Sma) prevented its activation by both MyoD and myogenin. The 220-bp SmaI fragment alone (construct Sma) also functioned as a promoter in C2C12 myoblasts and was activated by MyoD and myogenin 7- and 4-fold, respectively (Fig. 7). These results indicate that the SmaI restriction fragment within the Barx2 proximal promoter, which contains Sp1, E-box, and CArg box/SRE motifs, is necessary and sufficient for promoter activation by MyoD, and to a lesser extent, by myogenin.

The E2 and CArg Box/SRE Motifs Are Required for Activation of the Barx2 Promoter by MyoD—To examine the role of the MyoD/myogenin binding site (E2) in activation of the Barx2 promoter by MyoD and myogenin, this element was mutated within the 0.44-kb Barx2 promoter construct. Because SRF has been shown to act cooperatively with MyoD (38), the SRF binding site (CArg box/SRE) was also mutated within the 0.44-kb construct. C2C12 cells were co-transfected with pGL3basic, the 0.44-bp SmaI restriction fragment within the Barx2 promoter that spans the E2 and CArg box/SRE elements was mutated. Deletion of a 220-bp SmaI restriction fragment from construct 0.44 (0.44Sma) prevented its activation by both MyoD and myogenin. The 220-bp SmaI fragment alone (construct Sma) also functioned as a promoter in C2C12 myoblasts and was activated by MyoD and myogenin 7- and 4-fold, respectively (Fig. 7). These results indicate that the SmaI restriction fragment within the Barx2 proximal promoter, which contains Sp1, E-box, and CArg box/SRE motifs, is necessary and sufficient for promoter activation by MyoD, and to a lesser extent, by myogenin.

To determine whether these two elements are sufficient for activation by MyoD, we generated a synthetic construct containing a segment of the Barx2 promoter that spans the E2 and CArg box/SRE motifs. This 60-bp segment (hereafter referred to as the myogenic regulatory region, or MRR) was synthesized (lanes 1 and 2, arrow at left). This complex was supershifted by antibodies to SRF (lanes 3–5, arrow at right). The Barx2 CArg probe formed a much less intense complex with the C2C12 extract than the c-fos CArg box/SRE, suggesting that the Barx2 CArg box/SRE has a lower affinity for SRF (compare lanes 3 and 6).
and inserted upstream of a minimal TATA box promoter driving the luciferase reporter gene (see Fig. 8B). When transfected into C2C12 cells, this synthetic promoter construct (MRR) showed ~7-fold greater activity than that of the minimal promoter alone (construct pLuc). Co-transfection with a MyoD expression plasmid activated the MRR construct ~6-fold, relative to transfection of the empty pcDNA3 plasmid (Fig. 8B). In contrast, co-transfection of a myogenin expression plasmid did not significantly activate the MRR construct (data not shown), suggesting that activation of the Barx2 promoter by myogenin (see Fig. 7) involves additional sequences outside of the MRR.

To examine whether the E2 or CArG box/SRE elements are required for activation of the MRR by MyoD, we prepared constructs in which either the E2 or CArG box/SRE motif was mutated, and tested their ability to be activated by MyoD in cellular transfection experiments. Mutation of either the E2 or CArG box/SRE motif reduced the activity of the MRR promoter construct to the level of the minimal promoter alone. Similar to the results obtained when the E2 and CArG box/SRE elements were mutated in the native promoter construct, mutation of either element with the MRR was sufficient to prevent activation by MyoD. These results indicate that activation of the Barx2 promoter by MyoD requires an accessory factor that binds to the CArG box/SRE element, most likely SRF.

**DISCUSSION**

The homebox protein Barx2 is expressed in several phases of mesenchymal tissue morphogenesis, including the formation of pre-chondrogenic and pre-muscle mesenchymal condensations and the differentiation of skeletal and smooth muscle (16–18). Barx2 is also expressed in adult skeletal and smooth muscle, and its expression in C2C12 myoblasts is increased during differentiation into myotubes (18). In this study we showed that expression of Barx2 is required for the differentiation of MyoD-positive limb bud cells into myotubes, and that overexpression of Barx2 accelerates myotube formation by both primary myoblasts and C2C12 cells. However, overexpression of Barx2 did not increase the number of MyoD-positive cells in limb bud cultures. This result, together with the observation that overexpression of Barx2 does not convert MyoD-negative C3H10T1/2 cells into skeletal myoblasts, strongly suggests that Barx2 does not activate MyoD but rather acts downstream of MyoD. Importantly, the latter observation also indicates that Barx2 cannot substitute for MyoD activity.

Taken together, our results suggest that the role of Barx2 in myoblast differentiation may be distinct from that of other homeobox genes previously shown to influence muscle development, such as Pax3, Pax7, Lbx1, and Msx1. Each of these factors has been shown to act upstream of MRFs in myoblast determination. For example, Pax3 is expressed in myogenic progenitor cells and proliferating myoblasts and is required for the activation of MyoD and thus myogenesis (10). Lbx1 also acts upstream of MRFs to specify myoblasts and both induces, and is induced by, Pax3 (11). Msx1 antagonizes the activity of Pax3 by direct interaction leading to repression of myogenesis (12, 42) and ectopic expression of Max1 in C2C12 myotubes reduces the expression of MRFs leading to de-differentiation of myotubes (43). In contrast to the activities of these factors, our study indicates that Barx2 promotes myotube formation without inducing the expression of MyoD; thus Barx2 is the first homeobox gene shown to have a significant role in myoblast differentiation downstream of MyoD expression.

The mechanism by which Barx2 promotes myotube formation is yet to be determined. However, because the portion of the Barx2 protein that contains the DNA binding and activation domains was sufficient to promote myotube formation, it is likely that Barx2 activates particular genes that are required for the differentiation of skeletal myoblasts. For example, in this study we found that Barx2 up-regulated SM-α-actin expression in both primary limb bud cultures as well as in the C2C12 myoblast cell line. Moreover, the observation that Barx2 increased SM-α-actin expression in proliferating myoblasts suggests that Barx2 induces cellular changes that prime skeletal myoblasts for differentiation. Other downstream targets of Barx2 that are likely to influence myoblast fusion include CAMs. Barx2 was previously shown to regulate the N-CAM promoter and to induce cadherin-6 expression (19, 44). Previous studies have shown that both N-CAM and particular cadherins, such as N-cadherin, can promote myoblast fusion (27, 45). In addition, two new Ig family CAMs, CDO and BOC, were shown recently to play an essential role in myoblast fusion via
their heterophilic interaction (46, 47). In future studies, it will be important to determine whether these and other CAMs are direct targets of Barx2 in myoblasts.

Our analysis of the Barx2 gene promoter provides a molecular mechanism for the control of Barx2 expression during myogenic differentiation. We identified a proximal region of the Barx2 promoter containing an E-box (E2) that binds to both MyoD and myogenin, and additional binding sites for SRF and MEF2 proteins. Binding of nuclear proteins to the E2 element was found to increase during differentiation of C2C12 myoblasts, suggesting that the binding of MRFs to this promoter element mediates the previously reported induction of Barx2 expression during myotube formation (18). Both the E2 element and an adjacent CArG box/SRE, which binds to the SRF, are required for activation of the Barx2 promoter by MyoD. This result is similar to previous observations that both E-box and CArG/SRE motifs are involved in activation of the SM-actin and dystrophin genes by MyoD (38, 48), and suggests that MyoD and SRF cooperatively activate Barx2 expression in C2C12 cells.

SRF also plays a central role in the differentiation of smooth muscle (41, 49, 50) and has been shown to interact with various homeodomain proteins including variants of both Barx1 and Barx2 (18, 51–54). In particular, Barx2 can stimulate the binding of SRF to its cognate CArG box/SRE motif (18), and interaction of chicken Barx1b with SRF regulates smooth muscle cell-specific expression of the β-tropomyosin gene (54). Our studies also suggest a role for Barx2 in SMC differentiation. Overexpression of Barx2 in C3H10T1/2 cells increased SM-actin expression and promoted cell spreading and formation of stress fibers. These morphological changes are consistent with differentiation of SMCs or myofibroblasts, cells that have contractile properties like smooth muscle, but retain characteristics of fibroblasts (33, 55, 56).

Overall, our studies suggest that Barx2 regulates multiple muscle differentiation pathways. In committed myoblasts Barx2 expression can be induced by MRFs such as MyoD and myogenin and increased expression of Barx2 then promotes myotube formation. In uncommitted mesenchymal cells that do not express MRFs, Barx2 may promote differentiation into other contractile cell types such as SMCs. The mechanisms by which Barx2 influences the differentiation of both skeletal and smooth muscle could be elucidated by chromatin immunoprecipitation or microarray analysis to identify targets of Barx2 that are specifically regulated in each of these contexts.

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