Identification of Barx2B, a Serum Response Factor-associated Homeodomain Protein*

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CC(A/T)6GG or serum response elements represent a common regulatory motif important for regulating the expression of many smooth muscle-specific genes. They are multifunctional elements that bind serum response factor (SRF) and are important for serum induction of genes, expression of muscle-specific genes, and differentiation of vascular smooth muscle cells. In the current study, a yeast two-hybrid screen was used to identify proteins from mouse intestine that interact with SRF. A novel homeodomain-containing transcription factor, called Barx2b, was identified that specifically interacts with SRF and promotes the DNA binding activity of SRF. Northern blotting, RNase protection analysis, and Western blotting revealed that Barx2b mRNA and protein are expressed in several smooth muscle-containing tissues, as well as in skeletal muscle and brain. In vitro binding studies using bacterial fusion proteins revealed that the DNA-binding domain of SRF interacts with a region of Barx2b located amino-terminal of the homeobox domain. The results of these studies support the hypothesis that interaction of SRF with different homeodomain-containing proteins may play a critical role in determining the cell-specific functions of SRF.

Analysis of several smooth muscle-specific genes has, thus far, failed to identify any smooth muscle-restricted transcription factors that control their expression (reviewed in Ref. 1). Data have suggested that expression of a single smooth muscle-specific protein may be regulated by distinct response elements and distinct transcription factors in different smooth muscle tissues. For example, the telokin promoter was found to require an estrogen response element for high levels of expression in uterine smooth muscle, but this element is not required for expression in intestinal smooth muscle (2). In addition, several fragments of the SM22α promoter that have been shown to be sufficient to mediate expression in arteriolar smooth muscle do not result in detectable expression in visceral smooth muscle (3, 4). CC(A/T)6GG (CARG) 1 or serum response elements (SREs) have been shown to be critical for the activity of most smooth muscle-specific promoters characterized (4–11); thus, it appears likely that CARG elements represent a common regulatory motif important for regulating the expression of smooth muscle-specific genes. CARG or SRE elements bind serum response factor (SRF), a protein that plays an important role in both the expression of muscle-specific genes and serum induction of genes (12). Consistent with its role in regulating expression of muscle-specific genes, although SRF is widely expressed, it is present at the highest levels in skeletal, cardiac, and smooth muscle-containing tissues (13). In addition to regulating the activity of smooth muscle-specific promoters, SRF has also been directly shown to be required for the differentiation of proepicardial cells into coronary vascular smooth muscle cells (14).

It is likely that SRF bound to CARG elements in smooth muscle-specific genes may be interacting with other tissue-restricted factors to mediate tissue-specific gene expression. Given its broad pattern of expression, it seems likely that the interaction of SRF with other factors determines whether SRF functions to activate muscle-specific genes or to confer serum inducibility to a gene. For example the smooth muscle α-actin gene contains several CARG elements that are required for transcriptional activity; however, the endogenous gene or a reporter gene containing a 1063-bp fragment of the promoter is not induced by serum. In contrast, a truncated 191-bp α-actin promoter fragment is induced by serum (15), suggesting that within this single gene, the function of SRF bound to CARG elements is dependent on interactions with factors that bind to other elements within the gene. Similarly, although SRF has been shown to be required for telokin promoter activity, endogenous telokin is not induced by serum. 2 In the current study, we have used a yeast two-hybrid screen to identify proteins from mouse intestine that interact with SRF. These studies show that a novel homeodomain-containing transcription factor, Barx2b, specifically interacts with SRF and promotes the DNA binding activity of SRF.

MATERIALS AND METHODS

Yeast Two-hybrid Screen—To identify proteins that interact with SRF, we performed a yeast two-hybrid screen of a mouse intestine cDNA library using the yeast strain Y190 (Matchmaker Two-hybrid system; CLONTECH). This yeast strain contains LacZ and His3 reporter genes downstream of a minimal ADH promoter and three DNA binding sites for GAL4. The human cDNA for SRF (a generous gift from Dr. R. Prywes, Columbia University, New York, NY) was fused to the GAL4 DNA-binding domain in plasmid pG5BF9 (CLONTECH). A cDNA library was constructed in pGAD10, from mouse intestinal mRNA, using a two-hybrid cDNA construction kit according to the manufacturer’s directions (CLONTECH). The resultant cDNAs are fused to the carboxyl terminus of the GAL4 transcription activation domain. In the presence of 45 mM 3-amino-1,2,4-triazole, the SRF-GAL4BD fusion protein did not result in colony growth or β-galactosidase activity. The identity of positive clones was determined by direct DNA sequencing.

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1 The abbreviations used are: CARG, CC(A/T)6GG; SRE, serum response element; SRF, serum response factor; bp, base pair(s); SSPE, saline/sodium phosphate/EDTA; GST, glutathione S-transferase; TK, thymidine kinase.

2 B. P. Herring, unpublished observation.

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To verify that SRF interacted with the sequenced clones, these clones were reintroduced into yeast together with SRF-GAL4BD plasmid. True positive clones were identified by their ability to activate the LacZ reporter gene.

**Lambda Library Construction and Screening**—The remaining cDNA fragments obtained from construction of the mouse intestine pGAD10 cDNA library were ligated to EcoRI-digested and dephosphorylated agar11 arms. The lambda library was packaged, amplified, and screened by standard procedures (16). Nick translation filters were hybridized at 65 °C overnight with a 32P probe corresponding to the entire Barx2 clone obtained from the yeast library screen and to nucleotides 364–692 of the previouly described mouse Barx2 cDNA. Filters were washed in 2× SSPE (1× SSPE = 180 mM NaCl, 10 mM NaH2PO4, 1 mM EDTA, pH 7.4) + 1.0% SDS at room temperature for 10 min, 2× SSPE + 1.0% SDS at 65 °C for 10 min, and 0.2× SSPE + 0.1% SDS at 65 °C for 10 min. Lambda DNA was isolated using Lamdasorb (Promega, Madison, WI) and digested with NcoI, and the resulting fragments were subcloned into pGEM Zf and sequenced by automated sequencing.

**Northern Blotting**—Total RNA was isolated from adult tissues using a single-step guanidinium isothiocyanate procedure (16). 15 μg were separated on a 1.2% formaldehyde agarose gel and transferred to a nylon membrane under vacuum. Hybridization was carried out at 65 °C overnight with the same Barx2b probe used for lambda library screening. Final wash conditions were 2× SSPE + 1.0% SDS for 10 min at 65 °C.

**RNase Protection Assays**—A 538-bp fragment of the Barx2b cDNA (corresponding to nucleotides 594–1122) was subcloned into pGEM T7 (Promega). The plasmid was linearized with SmaI and a 32P-labeled antisense riboprobe generated using SP6 polymerase and a Maxi Script in vitro Transcription kit (Ambion, Austin, TX) according to the manufacturer's directions. The Barx2b riboprobe was gel-purified on a 6% polyacrylamide/8 M urea gel and eluted overnight at 37 °C. Ribonuclease protection assays were then performed according to the manufacturer's directions (Standard RPA II kit; Ambion). Briefly, 1× 105 cpm of gel-purified Barx2b riboprobe was coprecipitated with 20 μg of RNA and hybridized overnight at 42 °C. Samples were digested with RNase AT1 at a 1:150 dilution for 30 min at 37 °C and then inactivated and precipitated. Samples were solubilized in 0.5% SDS, 10 mM Tris-Cl, pH 8, 1 mM EDTA and 10% glycerol, and 1 μl dithiothreitol. All binding reactions were incubated for 15 min at room temperature followed by 1 h at 5 °C, except where indicated. For antibody supershift experiments, antibody was added after the 15-min room temperature incubation. For experiments in which the time of incubation was varied, proteins were allowed to incubate in reaction buffer for 10 min at room temperature before the addition of probe. Incubations were then performed for the specified times, and the reactions were immediately loaded onto a 4% polyacrylamide gel (containing 6.75 mM Tris, pH 7.9, 3.3 mM sodium acetate, pH 7.9, 1 mM EDTA, and 2.5% glycerol). Unlabeled competitor double-stranded oligonucleotides at 200-fold excess were included in some reactions as indicated in the figure legends. The sequences of the sense strand of each probe were as follows: (a) CARG probe, ACCCTATCCC-CTATGGGAGCTGAAAGGGA; (b) SRE probe, CAGGATGTCCATATTCTAGGAGCAG; and (c) Barx2b probe, (two binding sites) CTACCCCTTATGGGACCTGAAAGGGA. Underlined nucleotides are not part of the natural sequences but were added for labeling purposes.

**Mammalian Expression and Reporter Gene Assays**—For expression in mammalian cells, a fragment of the Barx2b cDNA encoding the coding region was amplified by polymerase chain reaction and cloned into pGL3 Basic luciferase reporter (Promega). The mammalian expression vector pCMV5 provides a cytomegalovirus promoter reporters, which drives expression of the reporter gene in a variety of cell types. To verify that SRF interacted with the sequenced clones, these clones were cloned into the pGL3 Basic luciferase reporter vector and then transfected into mammalian cells. In vitro binding experiments, GST-SRF fusion proteins were expressed in bacteria. Wild type Barx2b and six deletion mutants were expressed as glutathione S-transferase (GST) fusion proteins. Deletion fragments were generated by polymerase chain reaction and subcloned into pGEX4T (Amersham Pharmacia Biotech); all constructs were verified by DNA sequencing.

**Gel Mobility Shift Assay**—A mobility shift assay was performed in a final volume of 15 μl. Binding mixtures contained 0.2 ng (1.5 × 105 cpm) of end-labeled double-stranded DNA probe, 200 ng of salmon sperm DNA, 4.5 μg of bovine serum albumin, and various amounts of purified recombinant protein as indicated in the figure legend. The binding buffer containing 12 mM HEPES, pH 7.9, 60 mM KCl, 4 mM MgCl2, 10% glycerol, and 1 mM dithiothreitol. All binding reactions were incubated for 15 min at room temperature followed by 1 h at 5 °C, except where indicated. For antibody supershift experiments, antibody was added after the 15-min room temperature incubation. For experiments in which the time of incubation was varied, proteins were allowed to incubate in reaction buffer for 10 min at room temperature before the addition of probe. Incubations were then performed for the specified times, and the reactions were immediately loaded onto a 4% polyacrylamide gel (containing 6.75 mM Tris, pH 7.9, 3.3 mM sodium acetate, pH 7.9, 1 mM EDTA, and 2.5% glycerol). Unlabeled competitor double-stranded oligonucleotides at 200-fold excess were included in some reactions as indicated in the figure legends. The sequences of the sense strand of each probe were as follows: (a) CARG probe, ACCCTATCCC-CTATGGGAGCTGAAAGGGA; (b) SRE probe, CAGGATGTCCATATTCTAGGAGCAG; and (c) Barx2b probe, (two binding sites) CTACCCCTTATGGGACCTGAAAGGGA. Underlined nucleotides are not part of the natural sequences but were added for labeling purposes. Polyclonal antibodies to its amino-terminal and C-terminal were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

**Mammalian Expression and Reporter Gene Assays**—For expression in mammalian cells, a fragment of the Barx2b cDNA encoding the coding region was amplified by polymerase chain reaction and cloned into pGL3 Basic luciferase reporter (Promega). The rabbit telokin promoter-luciferase reporter gene used includes nucleotides −256 to +147 of the telokin gene, as described previously (18). The minimal TK promoter used comprised nucleotides −113 to −29 of the thymidine kinase gene; CArG-TK comprised two copies of the telokin CArG element upstream of the minimal TK promoter. Plasmids were transfected into rat A10 smooth muscle cells, REF52 fibroblasts, or COS cells using Fugene (Roche).

A10 cells were grown in high-glucose Dulbecco's modified Eagle's medium containing 50 units/ml penicillin, 50 μg/ml streptomycin, and 20% fetal bovine serum. REF52 and COS cells were grown in media supplemented with 10% fetal bovine serum. Cells were transfected in serum-free media supplemented with 10% fetal bovine serum. Cells were transfected by lipofectin (1.4 × 107 cells/dish in 35-mm dishes. At 16–18 h after transfection, cells were harvested and washed once with phosphate-buffered saline, pH 7.4, replaced with 2 ml of complete media, and incubated with a total of 2 μg of plasmid DNA (1 μg of promoter-luciferase, 0.5 μg of Barx2b expressing plasmid, and 0.5 μg of GLUTA3 expressing plasmid) and 3 μl of Fugene in 0.1 ml of Dulbecco's modified Eagle's medium (Life Technologies, Inc.). Twenty-four h later, 10-μl extracts of (400 μl/dish) were prepared for dual luciferase assays. Assays were performed using a dual luciferase reporter assay system according to the manufacturer's directions (Promega). Reporter gene luciferase activities were normalized to the luciferase activity of the internal control. For analysis of
Barx2b protein expression, COS cell lysates were generated 36 h after transfection using radioimmune precipitation buffer and analyzed by Western blotting as described above.

RESULTS

Identification of Barx2b as a SRF-binding Protein in Mouse Intestine—To identify proteins that interact with SRF, we performed a yeast two-hybrid screen of a mouse intestine cDNA library in the yeast strain Y190 (Matchmaker Two-hybrid system; CLONTECH). In a screen of \(10^7\) cDNAs, 2 of the galactosidase-positive colonies were identified as encoding a novel form of the homeodomain-containing protein Barx2 (19–22). These clones were found to be dependent on SRF for activation of the reporter genes upon retransformation of plasmids into Y190 yeast cells.

Barx2 Expressed in Intestine Is Distinct from Barx2 Previously Cloned from Mouse Day 11.5 Embryos—The nucleotide sequence of two cDNA clones isolated from the yeast two-hybrid screen of a mouse intestinal cDNA library spanned nucleotides 364–800 of the previously described mouse Barx2 cDNA (20). Both of the mouse intestinal cDNA clones have an insert of 84 nucleotides just 3' of the homeobox domain (nucleotides 952–1041 in the intestinal clone, Fig. 1A) that is not present in the previously described Barx2 cDNA. The Barx2 clone isolated was used as a probe to screen a mouse intestine \(lgt11\) cDNA library to isolate a full-length cDNA. Five cDNA clones were isolated from this screen, the longest of which was 1852 bp (Fig. 1A). All of the cDNA clones isolated contained the same nucleotide insert as that found in the original clones identified by the two-hybrid screen. The longest clone extends the published Barx2 cDNA sequence an additional 240 nucleotides at the 5' end and has a short poly(A) tail at the 3' end. The 5' region also contains an additional in-frame translation start site (Fig. 1B). Because of the similarity between our clone and the previously published chicken Barx2b cDNA (19) (73% amino acid identity; Fig. 2) and the expression of both proteins in skeletal muscle (Fig. 3), it is likely that our cDNA represents the mouse homologue of chicken Barx2b, hence we have referred to the protein encoded by our cDNA as mouse Barx2b. Human Barx2 also contains the inserted region present in mouse Barx2b and is homologous to the mouse protein (87% amino acid identity; Fig. 2) (21, 22).

Although an open reading frame extends to the 5' end of our clone, three lines of evidence support the designation of nucleotides 297–299 as the translation start site. First, there is a very high degree of sequence homology between the mouse and human Barx2 cDNAs 3' of the predicted translation start site, but 5' of this region, the sequences diverge (21, 22). Second, the size of the Barx2 mRNA estimated from Northern blots is 1.8 kb, similar to the size of the cDNA (Fig. 3A). Third, the recombinant protein expression without an epitope tag has the same apparent molecular mass as the protein present in tissues (Fig. 3C).

Barx2b Expression Is Tissue-restricted—The expression pattern of Barx2b was examined by Northern blotting and by RNase protection analysis. Together, these data demonstrated that in the adult mouse, Barx2b mRNA is expressed at high levels in several smooth muscle-containing tissues including the ileum, stomach, uterus, aorta, and lung. It is also expressed at significant levels in skeletal muscle and brain, and no expression is detected in the spleen, kidney, liver or heart. During mouse embryonic development, there is a large increase in Barx2b expression between embryonic day 10 and embryonic...
day 15 (Fig. 3). Results from Western blotting experiments are generally consistent with the Northern blots and confirm that Barx2 protein is expressed in a tissue-restricted manner in adult mice (Fig. 3C). However, it was noted that the levels of Barx2 protein and mRNA do not correlate perfectly, particularly in the lung and aorta, suggesting that Barx2 protein levels may also be regulated by posttranscriptional mechanisms.

Barx2b Binds Directly to SRF in Vitro—To confirm results obtained from the yeast two-hybrid analysis, GST pull-down assays were used to determine whether SRF interacts directly with Barx2b. Fig. 4 shows that GST-SRF binds directly to Barx2b. This interaction was specific because no Barx2b bound to GST alone. To map the interaction between SRF and Barx2b, several deletion constructs were generated, expressed in bacteria, and used in GST pull-down assays (Figs. 4 and 5). Dele-
Fig. 4. SRF interacts directly with Barx2b. A and B, full-length human SRF was expressed as a GST-fusion protein. GST-SRF (+) and GST alone (−) were immobilized on glutathione-Sepharose beads and incubated with bacterial lysates containing either wild type or various deletion mutants of Barx2b, as indicated. After extensive washing of the beads, bound protein complexes were dissolved in SDS sample buffer and analyzed by immunoblotting with anti-T7 epitope antibodies (top panels). The presence of GST-SRF in each of the + samples was confirmed by immunoblotting with anti-SRF antiserum (middle panels). The presence of GST in each of the − samples was confirmed by immunoblotting with anti-GST antibodies (data not shown). C, full-length wild type Barx2b and the Δ6 fragment of Barx2b expressed as GST-fusion proteins and GST alone were immobilized on glutathione-agarose beads and incubated with bacterial lysates containing full-length human SRF expressed as a T7-tagged protein. SRF bound to the Barx2b fusion proteins was then visualized by Western blotting with an anti-SRF antibody (top panel). The presence of SRF in each of the binding assays was confirmed by Western blotting of the supernatant fractions from the assays (bottom panel). The presence of GST-Δ6 and GST was confirmed by Ponceau stain of the Western blot (data not shown). The schematic structures of the various Barx2b deletion mutants, together with a summary of their ability to bind to SRF, are shown at the bottom of the figure. The homeobox domain is shaded, and the insert present in Barx2b but not Barx2 is stippled.

Barx2b Increases the Affinity of SRF for a CArG Box—To directly assess the effects of Barx2b on the ability of SRF to bind to DNA, gel mobility shift assays were performed. Identical mobility-shifted complexes were observed on the telokin CArG probe. When a consensus Barx2 binding site was used as a probe, Barx2b but not SRF resulted in a fast-migrating mobility-shifted complex (Fig. 6). The mobility-shifted complex could be supershifted with antibodies to SRF but not by antibodies to either Barx2 or ets (Fig. 6). In the absence of SRF, Barx2b produced a fast-migrating mobility-shifted complex on the cFos SRE probe but not on the telokin CArG probe. When a consensus Barx2 binding site was used as a probe, Barx2b but not SRF resulted in a fast-migrating mobility-shifted complex. A similarly migrating complex was formed in the presence of both Barx2b and SRF, and this complex could be supershifted with antibodies to Barx2b but not SRF. Barx2b Increases the Affinity of SRF for a CArG Box—To directly assess the effects of Barx2b on the ability of SRF to bind to DNA, gel mobility shift assays were performed. Identical mobility-shifted complexes were observed on the telokin CArG probe. When a consensus Barx2 binding site was used as a probe, Barx2b but not SRF resulted in a fast-migrating mobility-shifted complex (Fig. 6). The mobility-shifted complex could be supershifted with antibodies to SRF but not by antibodies to either Barx2 or ets (Fig. 6). In the absence of SRF, Barx2b produced a fast-migrating mobility-shifted complex on the cFos SRE probe but not on the telokin CArG probe. When a consensus Barx2 binding site was used as a probe, Barx2b but not SRF resulted in a fast-migrating mobility-shifted complex. A similarly migrating complex was formed in the presence of both Barx2b and SRF, and this complex could be supershifted with antibodies to Barx2b but not SRF.
The dimerization domain of SRF is shaded (indicates that no binding was detected). The MADS DNA binding and summary of their ability to bind to Barx2b (Fig. 7). DNA such that the SRF-DNA complex formed more quickly in the presence of Barx2b (Fig. 7). Because we were unable to obtain any evidence for a SRF-Barx2b complex in gel mobility shift assays, we investigated the ability of Barx2b to alter the kinetics of SRF binding to DNA. Under conditions of limiting SRF protein, Barx2b increased the affinity of SRF for DNA such that the SRF-DNA complex formed more quickly in the presence of Barx2b (Fig. 7).

**DISCUSSION**

Results show that Barx2b is a novel homeodomain-containing protein that interacts with SRF and increases the affinity of SRF for DNA. This function of Barx2b is analogous to that reported previously for Phox or Mhox (23). The enhancement of DNA binding by Phox1/Mhox was found to be primarily kinetic and additional cellular proteins such as TFII-I are required in vivo to stabilize the SRF-homeodomain complexes (24). Similarly, we were unable to demonstrate the presence of a tertiary Barx2b/SRF/DNA complex in vitro, suggesting that the enhancement of SRF DNA binding mediated by Barx2b is also largely kinetic and perhaps requires other endogenous proteins to stabilize the complex in vitro. Together, these data suggest that members of several families of homeobox-containing genes can interact with SRF and modulate its ability to bind DNA. The interaction of different classes of homeodomain proteins with SRF may thus represent a general mechanism by which the DNA binding activity of SRF is modulated in different tissues. In further support of this proposal, Nkx3-1, a member of the NK family of homeodomain-containing proteins, has recently been shown to promote the interaction of SRF with a CArG element in the smooth muscle $\gamma$-actin promoter and to potentiate the activity of this smooth muscle-specific promoter (25). In contrast to the effects of Nkx3-1 on the activity of the smooth muscle $\gamma$-actin promoter, Barx2b, in either the absence or presence of exogenous SRF, did not alter the activity of the telokin promoter in smooth muscle cells (data not shown). This result implies that either additional accessory factors are required for Barx2b to activate the promoter or that the effects of Barx2b may be promoter-specific and that the telokin promoter may not represent a physiological target. Additional studies will be required to identify physiological targets of Barx2b in muscle tissues.

Unlike Phox/Mhox, the homeodomain alone of Barx2b was not sufficient to mediate interaction with SRF (Ref. 23 and Fig. 4). A 10-amino acid region amino-terminal of the Barx2b homeodomain was found to be necessary but not sufficient to bind SRF. The ability of two nonoverlapping fragments of Barx2b (amino acids 1–123 and 124–283) to bind to SRF suggests that there are two distinct SRF binding sites; however, the 1–123 fragment appears to have a lower affinity than the 124–283 fragment (Fig. 4). A similar bipartite binding site has been reported on Nkx2.5 in which two distinct regions of the Nkx2.5 homeodomain were shown to mediate SRF binding (26). The 10-amino acid region of Barx2b that is critical for SRF binding contains several potential phosphorylation sites for casein kinase II (Ser^{123}, Ser^{125}, and Thr^{127}) and one potential site (Thr^{131}) of phosphorylation by proline-directed kinases. This observation raises the possibility that in vivo the interaction of Barx2b with SRF may be regulated by signaling cascades.

Barx2b isolated from adult mouse intestine is identical to the previously described mouse Barx2 except for the presence of an additional 25 amino acids at the amino terminus and the presence of a 30-amino acid insert close to the carboxyl terminus of the molecule. The sequence identity between these molecules suggests that they likely represent alternatively spliced versions of the same gene. During early mouse embryonic development (embryonic days 9.5–12.5), Barx2 has been shown to be expressed at high levels in neural and craniofacial structures and to control the expression of L1 neural adhesion molecule (20). The probes used for these studies, however, would not distinguish between Barx2 and Barx2b. In chicken, cBarx2b has also been shown to be expressed in myogenic cells in the myotome and in the skeletal muscles of the limb as well as in neural tissues (19). In the current study, we have shown that in addition to being expressed in adult brain and skeletal muscle, mouse Barx2b is also expressed at high levels in several adult smooth muscle tissues including the gut, uterus, and aorta. We also found that Barx2b is the predominant isoform present during the later stages of mouse development (embryonic day 15; Fig. 3); we were unable to detect the previously described mouse Barx2 in adult tissues by RNase protection analysis. These data suggest that Barx2/Barx2b may play roles in regulating gene expression in several different tissue lineages. The insert present in the carboxyl-terminal region of Barx2b is rich in acidic, glutamine, and proline residues, suggesting that this region may be involved in transcription activation and that Barx2b may thus possess functions distinct from those of Barx2.

The mechanisms by which Barx2 and Barx2b regulate gene expression are likely to be complex, involving both activation
and repression resulting from Barx2b binding to homeodomain binding sites as well as indirect regulation of gene expression through its interaction with SRF. Similar to Nkx2.5, Barx2b not only interacts with SRF, but it also binds directly to some, but not all, CArG elements. For example, Barx2b bound directly to the serum response element from the cFos gene, but no binding was detected to the CArG element from the telokin gene (Fig. 6). The ability of Barx2b to interact with SRF is likely to be shared by Barx2 because the region of Barx2b shown to interact with SRF is completely conserved in Barx2. Barx2 and Barx2b may regulate the activity of SRF by two different mechanisms, enhancement of the ability of SRF to bind to DNA (Fig. 7) and regulation of the interaction of SRF with other accessory factors (27). Recent findings that suggest that Barx2 can also interact with other transcription factors of the cAMP-response element-binding protein family further demonstrate the complexities of gene regulation that can be mediated by Barx2 (28).

The interaction of SRF with tissue-restricted transcription factors is likely to be important in determining the function of SRF on a given promoter in specific cell types. The ability of SRF to activate muscle-specific gene expression in addition to mediating growth factor responsiveness of genes likely results from complex protein-protein and protein-DNA interactions that are both promoter- and cell type-dependent. To confer growth factor responsiveness to genes, SRF interacts with members of the ets family of transcription factors, such as p62TCF, and these proteins interact with SRF together with DNA sequences that flank the SRF binding site (29). In contrast, in muscle tissues, which express high levels of Phox/Mhox, the expression of Phox/Mhox prevents the interaction of SRF with SAP-1, a member of the ets family of ternary complex factors (27). In skeletal muscle, SRF bound to a CArG element of the cardiac muscle α-actin gene has been shown to cooperate with muscle-specific myogenic factors bound to an adjacent E box, and this interaction is required for transcriptional activation (30). Similarly, in cardiac muscle, SRF bound to CArG elements interacts with the homeodomain-containing protein Nkx2.5 to activate transcription of the cardiac α-actin gene (26). In addition, Nkx3-1 but not Nkx2-5 has been shown to activate the smooth muscle γ-actin promoter (25). Together, these data suggest that the interaction of SRF with tissue-restricted homeodomain proteins, such as Barx2b and members of the Nkx family, may be one means by which SRF mediates its cell-specific functions.

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REFERENCES
1. Owens, G. K. (1998) Acta Physiol. Scand. 164, 623–635
2. Smith, A. F., Bigsby, R. M., Word, R. A., and Herring, B. P. (1998) Am. J. Physiol. 274, C1188—C1195
3. Li, L., Minjo, J. M., Mercer, B., and Olson, E. N. (1996) J. Cell Biol. 132, 849–859
4. Kim, S., Ip, H. S., Lu, M. M., Clendenin, C., and Parmacek, M. S. (1997) Mol. Cell. Biol. 17, 2266–2278
5. Herring, B. P., and Smith, A. F. (1997) Am. J. Physiol. 272, C1394—C1404
6. Browning, C. L., Culberson, D. E., Aragon, I. V., Fillmore, R. A., Croissant, J. D., Schwartz, R. J., and Zimmer, W. E. (1998) Dev. Biol. 194, 18–37
7. Hautmann, M. B., Madsen, C. S., Mack, C. P., and Owens, G. K. (1998) J. Biol. Chem. 273, 8398–8406
8. Mack, C. P., and Owens, G. K. (1999) Circ. Res. 84, 852–861
9. Madsen, C. S., Hershey, J. C., Hautmann, M. B., White, S. L., and Owens, G. K. (1997) J. Biol. Chem. 272, 6332–6340
10. Zilberman, A., Dave, V., Miano, J., Olson, E. N., and Periasamy, M. (1998) Circ. Res. 82, 566–575
11. Miano, J. M., Carlson, M. J., Spencer, J. A., and Misra, R. P. (2000) J. Biol. Chem. 275, 9814–9822
12. Norman, C., Runswick, M., Pollock, R., and Treisman, R. (1988) Cell 55, 989–1003
13. Belaguli, N. S., Schildmeyer, L. A., and Schwartz, R. J. (1997) J. Biol. Chem. 272, 18222–18231
14. Landerholm, T. E., Dong, X. R., Lu, J., Belaguli, N. S., Schwartz, R. J., and Majesky, M. W. (1999) Development 126, 2053–2062
15. Stoflet, E. S., Schmidt, L. J., Elder, P. K., Korf, G. M., Foster, D. N., Strauch, A. R., and Getz, M. J. (1992) Mol. Biol. Cell 3, 1073–1083
16. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. D., Smith, J. A., and Struhl, K. (eds) (1994) Current Protocols in Molecular Biology, Greene Publishing Associates and John Wiley and Sons, New York
17. Gallagher, P. J., Herring, B. P., Griffin, S. A., and Stull, J. T. (1991) J. Biol. Chem. 266, 23936–23944
18. Herring, B. P., and Smith, A. F. (1996) Am. J. Physiol. 270, C1656–C1665
19. Smith, D. M., and Tabin, C. J. (1999) Mech. Dev. 80, 203–206
20. Jones, F. S., Kioussi, C., Copertino, D. W., Kallunki, P., Holst, B. D., and Edelman, G. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2632–2637
21. Hjalt, T. A., and Murray, J. C. (1999) Genomics 62, 456–459
22. Kramer, A., Wallace, L., Thiagalingam, A., Jones, C., Lengauer, C., Minahan, L., Ma, Y., Kalikin, L., Feinberg, A. P., Jabs, E. W., Tunacliffle, A., Baylin, S. B., Ball, D. W., and Nelkin, B. D. (2000) Gene (Amst.) 250, 171–180
23. Gruneberg, D. A., Natesan, S., Alexandre, C., and Gilman, M. Z. (1992) Science 257, 1089–1095
24. Gruneberg, D. A., Henry, R. W., Brauer, A., Novina, C. D., Cheryiyath, V., Roy, A. L., and Gilman, M. (1997) Genes Dev. 11, 2482–2493
25. Carson, J. A., Fillmore, R. A., Schwartz, R. J., and Zimmer, W. E. (2000) J. Biol. Chem. 275, 39061–39072
26. Chen, C. Y., and Schwartz, R. J. (1996) Mol. Cell. Biol. 16, 6372–6384
27. Ma, J. T., and Ng, S. Y. (1984) Biochem. Biophys. Res. Commun. 120, 1742–1747
28. Edelman, D. B., Meech, R., and Jones, F. S. (2000) J. Biol. Chem. 275, 21737–21745
29. Price, M. A., Rogers, A. E., and Treisman, R. (1995) EMBO J. 14, 2589–2601
30. Moss, J. B., McQuinn, T. C., and Schwartz, R. J. (1994) J. Biol. Chem. 269, 12731–12740