The Smooth Muscle \(\gamma\)-Actin Gene Promoter Is a Molecular Target for the Mouse bagpipe Homologue, mNkx3-1, and Serum Response Factor*  

An evolutionarily conserved vertebrate homologue of the Drosophila NK-3 homeodomain gene bagpipe, Nkx3-1, is expressed in vascular and visceral mesoderm-derived muscle tissues and may influence smooth muscle cell differentiation. Nkx3-1 was evaluated for mediating smooth muscle \(\gamma\)-actin (SMGA) gene activity, a specific marker of smooth muscle differentiation. Expression of mNkx3-1 in heterologous CV-1 fibroblasts was unable to elicit SMGA promoter activity but required the coexpression of serum response factor (SRF) to activate robust SMGA transcription. A novel complex element containing a juxtaposed Nkx-binding site (NKE) and an SRF-binding element (SRE) in the proximal promoter region was found to be necessary for the Nkx3-1/ SRF coactivation of SMGA transcription. Furthermore, Nkx3-1 and SRF associate through protein-protein interactions and the homeodomain region of Nkx3-1 facilitated SRF binding to the complex NKE/SRE. Mutagenesis of Nkx3-1 revealed an inhibitory domain within its C-terminal segment. In addition, mNkx3-1/ SRF cooperative activity required an intact Nkx3-1 homeodomain along with the MADS box of SRF, which contains DNA binding and dimerization structural domains, and the contiguous C-terminal SRF activation domain. Thus, SMGA is a novel target for Nkx3-1, and the activity of Nkx3-1 on the SMGA promoter is dependent upon SRF.

Homeobox proteins are a class of developmentally regulated transcription factors that are important for embryonic patterning and differentiation (reviewed in Ref. 1). Although first identified in Drosophila, these regulators have been found in all metazoan species examined to date from fungi to humans. They constitute a family of transcription factors that are recognized by a conserved segment of 60 amino acids, referred to as the homeodomain, that usually recognizes a TTAATT-degenerate DNA consensus binding sequence found within the control elements of target genes (1–3). Drosophila and vertebrate homeobox genes are clustered on the genome, exhibit a high degree of structural conservation across species, and are expressed in a temporal and spatial sequence that is also conserved (1). Genome and expression analyses have now firmly established that there are a variety of homeodomain-containing proteins in vertebrates including a class of unique genes that are dispersed throughout the genome (1, 4, 5). Among the dispersed class of homeodomain proteins is the NK family, which was first defined by four genes (nk-1 through nk-4) identified in Drosophila. This family exhibits specific homology within the homeodomain and shares other regions of conserved sequence outside the homeodomain (4–7). In Drosophila, two nk genes, tinman and bagpipe, have been found to be closely linked and are expressed and participate in the specialization of mesoderm-derived heart and visceral organs (8, 9). Mutations in tinman block dorsal vessel (Drosophila heart equivalent) and visceral musculature formation; however, bagpipe mutations lead to only mid gut visceral musculature abnormalities. Thus, bagpipe may be a downstream target of tinman (8).

nkx3-1 and nkx3-2, two murine homologues of the bagpipe gene, have recently been identified (10–13). These genes demonstrate overlapping expression patterns in somites of early embryos (~8.5–9 embryonic days); however, only the nkx3-2 gene was expressed in the lateral and splanchic mesoderm (10, 12). In later stage embryos and in adults nkx3 genes are differentially expressed. Nkx3-1 is predominantly expressed in brain, kidney, blood vessels, and the male reproductive system (10, 11, 14), whereas Nkx3-2 is found in the lateral plate mesoderm surrounding the mid- hindgut and within cartilaginous condensations (12, 13). In adult tissues, nkx3 genes retain expression within the mesoderm-derived structures surrounding vascular (Nkx3-1 in the blood vessels) and visceral (Nkx3-2 in the mid- hindgut mesoderm) organs and may influence smooth muscle cell differentiation. At present, potential target genes regulated by the Nkx3 family have yet to be identified.

Smooth muscle cells are integral cellular components of most organs through their role in controlling vascular tone, gastrointestinal motility, fluids movement, and airway resistance. Differentiated smooth muscle cells are characterized by their expression of a unique subset of contractile protein isoforms including smooth muscle \(\alpha\)-actin (15–18), smooth muscle \(\gamma\)-actin (17–21), smooth muscle myosin heavy and light chains (22–24), calponin (25), SM22\(\alpha\) (26–28), and telokin (29–31). Furthermore, these cells possess the capability to express appropriate levels of these characteristic smooth muscle proteins even though they are derived from diverse embryonic origins (32). In contrast to skeletal and cardiac muscle cells, smooth muscle cells retain their capacity to modulate reversibly their phenotype during postnatal development (33, 34). This phenotypic modulation includes a reentry into the cell cycle and an

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altered expression of the characteristic proteins of the differentiated cell, effects that have been implicated in the pathogenesis of certain cardiovascular (35) and gastrointestinal (36) disease states. Thus, a knowledge of the molecular mechanisms that control smooth muscle development and cell-specific gene expression will provide insights into cellular differentiation and the physiological responses of these cells to injury and disease states.

We have examined the expression of the smooth muscle γ-actin (SMGA) gene as a model to understand the molecular mechanisms that regulate genes during smooth muscle differentiation. In aves (20, 21, 37) as in mammals (17–19), SMGA expression is restricted to smooth muscle tissues with the exception of the post-meiotic spermatocyte (38); thus SMGA provides an excellent marker for the smooth muscle phenotype. In addition, tissue-restricted expression of SMGA arises during early embryonic development of the vasculature and gastrointestinal tract (17, 18, 20, 21, 39), suggesting that the activation of SMGA transcription may require factors unique to the differentiating smooth muscle cell. Transcriptional regulation of the SMGA gene appears to require the interplay of positive- and negative-acting cis elements within the promoter. Two regions displaying positive acting transcriptional activity were mapped on the SMGA promoter, referred to as the specifier and modulator domains (21). A key cis element for smooth muscle-specific SMGA transcription found in both of these domains is the CARg/SRE (CC(A/T)6GG) motif. The positive acting transcriptional activity of the specifier and modulator domains is derived from the binding of SRF to the SRE sites within these domains, and we have demonstrated that SRF-containing complexes play a prominent role in the developmental activation of the SMGA gene (40). Based upon our studies and the many examples from SRF-dependent regulation of cardiac (6, 14, 41–43) and skeletal (44–48) muscle genes, we predicted that SRF requires association with other factors to regulate the SMGA gene.

Here we demonstrate the cooperative interaction of Nkx-3.1 with SRF-activating SMGA transcription. The conservation of structure and activity of actin gene transcriptional machinery permits examining these components utilizing a heterologous cotransfection assay. Transcriptional synergy is supported by a complex of regulatory elements that is composed of immediately adjacent NKE-SRE cis elements within the SMGA proximal promoter. Our studies demonstrate that the SMGA gene promoter serves as a target for the Nkx3 family of transcription factors, specifically the Nkx3-1 factor, and supports the hypothesis that appropriate transcriptional regulation of smooth muscle specific genes requires the combinatorial interactions of SRF with coaccessory trans-acting factors expressed within the smooth muscle cell.

MATERIALS AND METHODS

Recombinant Plasmids—Approximately 2300 bp of the avian smooth muscle γ-actin promoter and 5′ deletions have been cloned into pG3-L3 basic plasmid driving the expression of the reporter gene luciferase (21, 37, 40). The −1224, −176, and −108 5′ deletions of the smooth muscle γ-actin promoter were used in transfection experiments in the current study. Mutations were made to γ-actin promoter elements SRE1, SRE2, and NKE1 using multiple primer polymerase chain reaction (PCR) and verified by DNA sequencing. The SMGA SRE1 was disrupted by changing the wild type element from 5′-CCTATTAGG-3′ to 5′-CACCCCCCT-3′. The NKE1/SRE1 double mutant contained the mutations to both sites. PCR was used to isolate the SRE2-bp Nkx3-1 cDNA from 6-day embryoid bodies with forward primer 5′-GCTTCAGAAAGTCTACGAGG-3′ and reverse primer 5′-TTGGATCCTAGGAGAAGCAC-3′. The isolated cDNA was cloned into pCR (Invitrogen, TA Cloning Kit) and verified by sequencing. The Nkx3-1 fragment was excised from pCR with XbaI and BamHI and cloned into identical sites of the pCGN vector downstream of the cytomegalovirus promoter and HA epitope tag. Mutations of the Nkx3-1 sequence were made using PCR-based techniques (Stratagene, Excite PCR Mutagenesis Kit). The cDNAs were used as the template for these experiments, and all mutants were cloned into the same vector maintaining an HA epitope tag, resulting in the expression of the mutant proteins. All mutants were confirmed by DNA sequencing and Western analyses of lysates derived from transfected cells.

The −330-bp cardiac α-actin promoter cloned in front of a luciferase reporter gene has been previously described. The construction of the consensus NKE reporter construct A20 has also been described previously (41). Briefly, the 3× (NKE)-tata-luciferase construct was constructed by inserting a linker containing three copies of an intermediate strength NKE in front of the α-cardiac actin minimal TATA box driving the expression of luciferase. Human SRF expression vectors (pCGN-SRF, pCGN-SRFAC, and pCGN-SRFpm1) driven by the cytomegalovirus promoter have been previously described (41) and generously supplied by Ron Prywes. The construction and use of the pCGN-Nkx3-5 expression vector has been previously described (41–43).

Expression and Purification of Bacterially Expressed His-Nkx3-1 and GST-SRF—The Nkx3-1 homeodomain cDNA was isolated by PCR on the full-length cDNA using forward primer 5′-TCACCAAGCCAGCCACGAGAGAACGG-3′ and reverse primer 5′-TCGCTTTCGCTTGCTTATAGG-3′ (359–379 bp) and reverse primer 5′-TCGCTTTCGCTTGCTTATAGG-3′ (529–552 bp). The Nkx3-1 homeodomain protein consisted of 193 bp, including 13 bp 5′ to the homeodomain. To express Nkx3-1 in bacteria, the cDNAs for the full-length and homeodomains of Nkx3-1 were isolated by PCR, verified by DNA sequencing, and cloned into pRSET B (Invitrogen) vector. The full-length and homeodomain-only Nkx3-1 cDNA PCR products were cloned into pCRH (Invitrogen, TA Cloning Kit). The cDNA for the Nkx3-1 homeodomain region was excised from pCRH with HindIII and XhoI sites internal to the PCR primers, and ligated into the HindIII/XhoI sites of pRSET B, downstream of a 6× histidine tag. These constructs were used to transform BL21 (DE3) cells (Novagen). Freshly transformed cells were grown in 500 ml of LB broth containing 100 μg/ml ampicillin at 37 °C to an OD600 of 0.5. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and growth was continued for another 2 h. Cells were harvested and suspended in 20 ml of column buffer (20 mM Tris, pH 7.4, 200 mM NaCl, 1 mM EDTA), sonicated, and cellular debris removed by centrifugation. The fusion protein was purified by binding to a metal affinity column (CLONTECH), washed extensively, and then eluted with fractions of column buffer containing 10–200 mM imidazole. The concentration of the protein was determined by Bradford protein assay, and purity was determined by Coomassie staining after SDS-polyacrylamide gel electrophoresis. Full-length human SRF was expressed and purified as a glutathione S-transferase (GST) fusion protein as described previously (41–43). The concentration of the protein was determined by Bradford protein assay and purity determined by Coomassie staining after SDS-polyacrylamide gel electrophoresis.

Transfection Assays in CV-1 Fibroblast Cell Cultures—Monkey CV-1 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were plated at an approximate density of 5 × 10^4 cells per 6-cm plate. Cells were transfected 24 h post-plating using LipofectAMINE (Life Technologies, Inc.) as described previously (49). Briefly, each transfection reaction contained 1 μg of luciferase reporter plasmid (γ-actin, A20, or α-Cα) and various amounts of transactivator plasmids (pCGN-Nkx3.1 and/or pCGN-SRF). All transfections were balanced to 2 μg of DNA with empty vector in order to keep the level of DNA and CMV promoter constant in all transfection reactions. Cells were transfected 16–18 h after which the transfection media were removed and replaced with Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum and 10 μg/ml insulin for an additional 48 h. Cells were then harvested by washing with PBS and then scraped in 400 μl of 1x Reporter Lysis Buffer (Promega). Cellular debris was removed by centrifugation, and 30 μl of supernatant was analyzed for luciferase activity by mixing with 100 μl of luciferase substrate (20 mM Tris-HCl, pH 8.0; 4 mM MgSO4, 0.1 mM EDTA, 30 mM dithiothreitol, 0.5 mM ATP, 0.5 mM N-luciferin, 0.25 mM coenzyme A). Emitted luminescence was measured for 10 s. Protein concentrations were measured by the Bradford assay (Bio-Rad) and the physiological responses of these cells to injury and disease states.
used to normalize luciferase activity. In vivo Immunoprecipitation of the Nkx3-1/SRF Complex—Interactions between Nkx3-1 and SRF were examined by in vivo immunoprecipitation using previously described methodology (42). Briefly, 3T3 cells at late-logarithmic dialysis were treated with 2 μg of either pCGN-Nkx3.1 or pCGN-SRF plasmid DNA using Lipofectamine (Life Technologies, Inc.). Forty eight hours post-transfection the cells were washed twice in ice-cold PBS, harvested in 1 ml of ice-cold PBS, and collected by centrifugation at 4 °C. Cells were resuspended in EBC buffer (50 mM Tris, pH 8.0; 120 mM NaCl; 0.5% Nonidet P-40; 2 μg/ml leupeptin; 2 μg/ml pepstatin; and 1 mM phenylmethylsulfonyl fluoride), rocked at 4 °C for 15 min, and centrifuged at 4 °C. The supernatant was then transferred to a new tube and protein concentration determined (Bio-Rad), and 500 μg of protein extracts containing SRF and Nkx3-1 were incubated for 2 h at 4 °C with 4 μg of anti-SRF antibody (Santa Cruz Biotechnology) in a total of 500 μl of NETN buffer (20 mM Tris, pH 8.0; 100 mM NaCl; 1 mM EDTA; 5 mM MgCl2; 1 mM dithiothreitol; 0.05% Nonidet P-40; 1 mM phenylmethylsulfonyl fluoride). Non-transfected 3T3 cell extracts served as controls. Thirty micrograms of purified bacterially expressed proteins were incubated 10 min containing 1 μg of either poly(dI-dC) or poly(dG-dC) binding buffer (10 mM Tris, pH 8.0; 1 mM dithiothreitol; 1 mM sodium phosphate; 5% glycerol; 50 mM sodium chloride) and nanogram quantities of purified bacterially expressed proteins were incubated 10 min at room temperature, and then the probe was added (20,000 cpm) in 50 μl of gel shift assay buffer described above. The gel was prerun for 20 min, followed by sample electrophoreses for 2 h at 180 V. Binding complexes were visualized by autoradiography.

DNA Binding Mobility Shift and DNase I Footprinting Assays—Double-stranded oligonucleotides corresponding to the smooth muscle γ-actin NKE1/SRE1 elements were constructed consisting of 100 to 74 bp of the promoter (5′-CATCATAACGCCTATTTAGGGTCTT-3′). The oligonucleotide was end-labeled using the poly(dI-dC) kinase reassociation, and band shift assays were performed as described previously (40–43). Briefly, 20 μl containing 1 μg of either poly(dI-dC) or poly(dG-dC) binding buffer (10 mM Tris, pH 8.0; 1 mM dithiothreitol; 1 mM sodium phosphate; 5% glycerol; 50 mM sodium chloride) and nanogram quantities of purified bacterially expressed proteins were incubated 10 min at room temperature, and then the probe was added (20,000 cpm/reaction) and incubated for 15 min at room temperature. Binding complexes were then run on a 5% polyacrylamide gel in 0.5× TBE buffer. The gel was prerun for 20 min, followed by sample electrophoreses for 2 h at 180 V. Binding complexes were visualized by autoradiography.

RESULTS

The ability of SRF to activate transcription from this promoter. The −176 construct contains two CArG/SRE motifs located at −85 (CArG/SRE1) and −120 (CArG/SRE2), both of which were bound by smooth muscle nuclear complexes (37, 40); however, of the two CArG, SRE2 appeared to bind SRF with greater avidity.

We then asked if a vertebrate homologue of bagpipe, the homeodomain factor Nkx3-1, was capable of activating the SMGA promoter. Whereas Nkx3-1 alone was not able to activate appreciably the SMGA promoter/reporter constructs above vector controls, there was a robust increase in promoter activity when Nkx3-1 was expressed with SRF (Fig. 1, B and C). The activation of reporter gene activity obtained in cells expressing both factors was approximately 20–25-fold above vector controls. These values were significantly enhanced compared with the expression obtained from cells transfected singularly with SRF or with Nkx3-1 indicating a cooperative and/or synergistic interaction of these trans-factors upon SMGA promoter activity. Moreover, this synergistic response was maintained using constructs containing the first 176 base pairs of 5′-flanking sequence of the SMGA promoter (Fig. 1C).

Deletion of the distal CArG/SRE, SRE2, from the SMGA −176 promoter construct (Fig. 1D) eliminated Nkx3-1 and SRF-stimulated transcription, indicating the positive acting nature of this cis-acting element in the Nkx3-1/SRF-dependent transcriptional activation. Western blotting analyses of cellular lysates confirmed that both SRF and Nkx3-1 were expressed efficiently in the transfected cells (Fig. 1B).

Previously, we showed that Nkx2-5 cooperates with SRF to transactivate the cardiac α-actin promoter (41–43, 50). We asked if the tioman homologue, Nkx2-5, could substitute for Nkx3-1 in cotransfection experiments with the SMGA promoter. Unlike Nkx3-1, Nkx2-5 was not able to augment the basal transcriptional response of the −176 bp SMGA promoter obtained with SRF (Fig. 2B). In addition, we did not observe Nkx3-1/SRF-dependent transcriptional activation from the NKE target promoter A20 (41; data not shown). However, the A20 promoter, derived from preferred Nkx2-5-binding sequences and a minimal portion of sequences of the α-cardiac actin promoter, was activated with Nkx2-5 and SRF under the same conditions. Taken together, these data show that Nkx3-1 exhibits a synergism with SRF to activate transcription from the SMGA promoter. This activation is specific for the SMGA promoter and is directed through the initial 176 bases flanking the SMGA gene.

Multiple DNA Elements Are Required for Nkx3-1/SRF-dependent Activation of SMGA Transcription—There is a high level of sequence homology (70%) that extends over the initial 400 bp flanking the 5′ region of the SMGA gene across species (19, 21, 51). Furthermore, the structure and spacing of CArG motifs appear to be strictly maintained across species (21). We focused upon the NKE and CArG sites of the SMGA −176 promoter fragment as potential cis regulatory elements responsible for Nkx3-1/SRF transcriptional activation. To assess fully Nkx3-1 and SRF factor function, we mutated the two CArG/SRE motifs and a potential NKE site. As illustrated in Fig. 2, a mutation of CArG/SRE2 (−120) that abolished binding with purified SRF (40) also completely inhibited transcriptional activation of the SMGA−176 promoter by SRF. However, the mutant CArG/SRE2-containing SMGA promoter did not block SRF and Nkx3-1-dependent activity. Similarly, mutagenesis of the −85 CArG/SRE1 motif (CCTATTTAGG → CCTATCCGG) also abolished SRF-dependent activation. However, in contrast to mutated SRE2, mutated SRE1 blunted coactivation by SRF and Nkx3-1 via the mutated −176 promoter (Fig. 2A). The −90 bp element (CATCATTAG) resembled the consensus
binding sequence for TTF-1/Nkx2–1 and Nkx2-5 factors (41–43, 52). Mutagenesis of this potential NKE site reduced promoter SRF and Nkx3-1-dependent activity by approximately 40% (Fig. 2). Therefore, only CArG/SRE 1 appears to be required for the Nkx3-1/SRF response. However, the inability of SMGA promoter constructs containing just the NKE1/SRE1 motif to be activated by Nkx3-1 and SRF (–108, Fig. 1D) demonstrates that although this motif is necessary it is not

Fig. 1. A short portion (176 bp) of the SMGA promoter was sufficient to support synergistic transactivation by Nkx3-1 and SRF. A, a diagram of deletion mutants of the avian SMGA promoter used in this study illustrating the positions of an NKE (closed box) and six SRE (open box) motifs is shown. The negative numbers refer to the 5′ end position of the various DNA fragments with respect to the SMGA gene. B, CV-1 cells were transfected with the −1224 SMGA promoter-luciferase reporter gene (1 μg of DNA) and CMV promoter-directed expression vectors as described under “Materials and Methods.” Expression vectors for SRF and Nkx3-1 were assayed singularly (0.2 μg of DNA) and in combination where SRF was maintained at a constant level (0.2 μg), and Nkx3-1 was provided at increasing content (0.2–0.8 μg). All transactivation reactions were balanced to 1 μg of DNA content by the addition of empty CMV promoter vector. These data were generated from a minimum of four experiments, performed in duplicate. The fold induction represents the luciferase activity measured in lysates receiving various transactivation plasmids compared with the activity in lysates derived from cells that received the −1224 construct and vector control (vector lane). C, CV-1 cells were transfected as in B, except the cells received the SMGA −176 reporter construct. In addition to Nkx3-1, the −176 SMGA construct was assayed with an Nkx2-5 expression vector (41–43), both singularly (0.2 μg) and in combination with SRF (0.2 μg of SRF with 0.8 μg of Nkx2-5). Fold induction was derived from the data as above. D, CV-1 cells were transfected as described with the SMGA −108 promoter-luciferase reporter gene as the target. Fold induction was calculated from luciferase activities in lysates of cells transfected with SRF (0.2 μg) or Nkx3-1 (0.2 μg) alone or in combination (SRF at 0.2 μg; Nkx3-1 at 0.8 μg) compared with the activity measured in cells receiving empty vector. E, 50 μg of CV-1 cellular protein extracted from cells transfected with empty vector (control), pCGN Nkx3-1, and/or pCGN SRF as described above was sized by 10% SDS-PAGE, and the proteins were transferred to a nylon membrane. The membrane was probed with an anti-HA epitope monoclonal antibody, and the HA-tagged fusion proteins visualized by chemiluminescence. The numbers to the right of the blot show the migration of protein standards, and the positions of HA-tagged SRF and Nkx3-1 are indicated by the arrows.
totally sufficient to support the synergistic transcriptional response. Alignment of sequences near the proximal avian (21), human (19), and mouse (51) SMGA promoters emphasize the conservation of structure and spacing of the NKE1 and SRE motifs across diverse species (Fig. 2). Taken together, these data are consistent with the hypothesis that multiple DNA-binding elements are required for SRF and Nkx3-1/SRF-dependent transcriptional activation.

**Nkx3-1 Homeodomain and SRF Bound the NKE1/CArG1 Region of the SMGA Promoter**—We reasoned that in order for SRF and Nkx3-1 to function together, they might bind in the immediate vicinity of these promoter elements. To test this hypothesis, we performed DNA binding experiments using purified bacterially expressed proteins with a DNA segment containing their potential binding elements. A DNA probe containing sequences −2100 to −74 of the SMGA promoter (NKE/CArG probe) was incubated with purified Nkx3-1 homeodomain (Fig. 3A) or SRF (Fig. 3D) proteins, and the mixtures were separated on polyacrylamide gels to assay for protein binding. As shown in Fig. 3A, the homeodomain of the Nkx3-1 protein formed two shifted complexes with the NKE/CArG probe fragment. Both of the Nkx3-1 homeodomain complexes increased in intensity with added protein; however, the slower migrating complex demonstrated a more intense signal, indicative of stronger binding. DNA binding specificity was demonstrated by specific competition using a 50-fold excess of unlabeled NKE/CArG fragment but not with a DNA oligonucleotide duplex containing a sequence for GATA factor binding (53, 54). An excess of three synthetic NKE sites in A20, previously shown to bind Nkx2-5 with great avidity (41), competed away the more slowly migrating complex (Fig. 3B). This experiment shows that the Nkx3-1 homeodomain can bind a DNA containing an Nk homeodomain consensus core-binding site ((C/T)AAG)) (6, 41) as had been previously suggested (41). Furthermore, the slower migrating complex formed with the NKE/CArG element represents the interaction of the Nkx3-1 homeodomain at the NKE

**Fig. 2.** Evolutionarily conserved SMGA promoter proximal SRE1 and NKX-binding sites are required for Nkx3-1/SRF synergistic transcriptional activation. A, the transcriptional activity of the −176 SMGA promoter (wild type) and selected point mutants of the promoter by Nkx3-1 and SRF was assayed by cotransfection analysis in CV-1 cells. The specific base changes that inactivate SRE 2 (SRE2mut), SRE 1 (SRE1mut), and NKE 1 (NKE1mut) are shown below the diagram of the −176 SMGA promoter fragment. Activity of wild type and mutant promoter constructs was determined in cells overexpressing Nkx3-1 and SRF singularly or in combination. The fold activation was calculated from comparison of activities in lysates of cells expressing transactivator proteins to that of cells treated with empty vector. Each plot represents a minimum of four experiments assayed in duplicate. B, an alignment of the proximal promoter sequences elucidated from the avian (chick) (21), human (19), and mouse (51) SMGA genes is shown. The positions of SRE (CArG/SRE 2, CArG/SRE 1) and the NKE (NKE1) motifs are marked above the sequence.
mNkx3-1 and SRF Coactivate γ-SM Actin Promoter
sequence. Shifts with the NKE/CARG SRE1-mutated probe eliminated the migration of the more rapidly migrating species but still allowed for the appearance of a single Nkx2-5 and/or Nkx3-1 DNA-binding complex that specifically competed with A20. Another shifted species, presumably nonspecific, was also observed with Nkx3-1 and the SRE1 mutant probe that could not be competed with multimerized NKEs.

To map precisely Nkx3-1 binding, we performed footprinting experiments using an end-labeled fragment derived from the SMGA promoter (~108 to +15). The Nkx3-1 homeodomain protected a segment of sequence corresponding to the NKE1 element (~95 to ~89) under conditions of a low protein load (0.25 μg, Fig. 3C). With increasing protein, the Nkx3-1 homeodomain narrowly protected the 5’ boundary of CARG/SRE1 (~88 to ~79) of the SMGA promoter. Moreover, there is an appearance of new DNase I cleavage sites 3’ to the NKE1 sequence appearing with Nkx3-1-dependent DNase I protection (indicated by the arrowheads, Fig. 3D), suggesting an altered DNA conformation induced by homeodomain binding to DNA. Taken together, these results show that the homeodomain segment of the Nkx3-1 factor (amino acids 124–184 of the protein) is capable of binding to sequences within the proximal promoter of the SMGA gene, one site over a consensus NKE and a second weaker binding over a CARG/SRE.

We showed that a DNA fragment containing CARG/SRE sequences 1 and 2 was capable of binding bacterially synthesized, purified SRF (40). When the probe containing the NKE1/SRE1 sequence of the SMGA promoter was incubated with purified SRF a singular, prominent protein-DNA-binding complex was formed (Fig. 3D). This specific binding complex was efficiently competed with unlabeled NKE/CARG probe but not with a fragment bearing GATA-binding sites. SRF bound to the CARG/SRE1 segment (~88 to ~79) of the SMGA promoter as indicated by a DNase I footprinting experiment (Fig. 3D). At higher protein loads, the purified SRF broadens its DNase I protection of surrounding DNA sequences. Approximately 10-fold more protein was required to obtain measurable SRF binding upon SRE1 than the binding observed upon SRE2 in previous studies (40), suggesting a weak SRE1 binding relative to other SRF-binding sites within the SMGA promoter. In support of this observation, minimal DNase I protection was detected over the proximal SRE1 using DNA fragments containing both SRE1 and SRE2 motifs under conditions of limiting amounts of SRF (data not shown). These experiments demonstrated that SRF and Nkx3-1 factors bound to contiguous sequences within the SMGA proximal promoter and was buttressed by the co-transfection experiments with wild type and mutant SMGA promoter fragments (Figs. 1 and 2), which indicated that the NKE/CARG motif within the proximal promoter is the operative cis-acting element for Nkx3-1/SRF-mediated SMGA promoter activation.

**Nkx3-1 Homeodomain Was Sufficient to Facilitate SRF Binding to the SMGA NKE/CARG Motif**—In studies of the α-cardiac actin gene where SRF was found to cooperate with Nkx2-5/Csx to activate transcription, the factors appeared to bind equivalent sequences, specifically SRE motifs (41–43). Here we have mapped the binding sites for Nkx3-1 and SRF to adjacent sequences. The Nkx3-1 homeodomain was found to also exhibit a weak interaction with the SRE1 motif of the SMGA promoter. Furthermore, we have shown that although SRE1 of the SMGA promoter conforms to consensus SRE motifs (55), it is not an avid SRF-binding site. To determine whether the binding of one factor at the NKE/CARG element influences the binding of the other, we performed DNA binding and DNase I footprinting experiments (Fig. 4). As shown in Fig. 4A, 0.25 μg of Nkx3-1 homeodomain protein produced two complexes with the SMGA NKE/CARG probe, and 0.1 μg of purified SRF produced a single major protein-DNA complex. Incubating less SRF along with the probe (0.005 μg, lane 4, Fig. 4A) resulted in reduced intensity of the SRF-containing complex. In comparison, this amount of SRF was capable of avid binding to other SRE motifs of the SMGA gene (40) and of other genes (28, 41, 44, 43, 55).

The addition of Nkx3-1 homeodomain to the reaction increased the binding avidity of SRF to the Nke/CARG probe (lanes 5–7, Fig. 4A). We did not observe the formation of ternary higher order complexes regardless of the amount of Nkx3-1 protein added to the reaction mix. The same result was obtained in DNase I footprinting analyses (Fig. 4B). Low levels of SRF did not produce a noticeable footprint upon the −108/+15 SMGA gene fragment (lane 2, Fig. 4B). However, with the addition of the Nkx3-1 homeodomain, the region of the DNA probe corresponding to the Nke/CARG1 element was protected from digestion. Significant binding was observed even at the lowest amount of Nkx3-1 added into the reaction (0.25 μg, lane 3, Fig. 4B). Also as the Nkx3-1 homeodomain content was increased, the added Nkx3-1 homeodomain and SRF completely protected the Nke/CARG portion of the probe (lane 5). Additionally, there was the appearance of new DNase I cleavage sites with added Nkx3-1 as that observed when the homeodomain was assayed singularly (Fig. 3C). These experiments demonstrate that when included in the same reaction Nkx3-1 and SRF bind adjacent elements within the SMGA promoter, namely the Nke/CARG1 motif, and there is enhanced SRF binding when Nkx3-1 is added to the reaction. Evaluation of the reciprocal interaction, adding increasing amounts of SRF with a constant amount of Nkx3-1 homeodomain, did not enhance

**Fig. 3. Nkx3-1 and SRF independently bind to closely juxtaposed NKE and SRE1 elements.** Fusion proteins containing Nkx3-1 (HisNkx3-1) homeodomain and GST (GSH-SRF) (electrophoretic mobility shift assay) and DNase I footprinting analyses to localize their binding sites upon the SMGA promoter NKE/CARG element. A–C represent experiments utilizing His-Nkx3-1 homeodomain fusion protein. A, Nkx3-1 homeodomain protein binding was analyzed by electrophoretic mobility shift assay. Two complexes were formed with increasing quantities of the Nkx3-1 fusion protein (100, 200, and 400 ng; lanes 2–4, respectively) on the NKE/CARG element (arrows). These complexes do not form in the presence of a 50-fold excess of unlabeled NKE/CARG element (lane 5); however, they are not inhibited by excess GATA protein-binding sites (lane 7). B, lane 1, WT Nkx3-1 probe only; lane 2, Nkx2-5 homeodomain binding (100 ng); lane 3, Nkx3-1 homeodomain binding (400 ng); lane 4, Nkx3-1 + self-competitor; lane 5, Nkx3-1 + nonspecific competitor (competes slow complex, not fast); lane 6, NKE/CARG/SRE1 mutant probe only; lane 7, Nkx2-5 (100 ng); lane 8, Nkx3-1 (400 ng); lane 9, Nkx3-1 + Nkx3-1 homeodomain binding competitor (competes fast complex, not slow); and lane 10, Nkx3-1 + nonspecific GATA competitor. Note the NS (denoted with the asterisk) as nonspecific shifted band that did not compete with any binding site including unlabeled probe fragment. C shows an autoradiogram of a DNase I footprinting experiment in which the purified Nkx3-1 fusion protein was incubated with a DNA probe representing −108 to +15 of the avian SMGA gene. The probe was analyzed in parallel reactions as detailed under “Material and Methods” containing 0, 0.25, 0.5, or 1 μg of protein and displayed on 6% polyacrylamide sequencing gels. Position of probe protection is indicated by the arrows, and the positions of DNase I enhancements are shown by the arrowheads. The negative numbers refer to nucleotide positions to the gene and were determined by running Maxam and Gilbert sequence reactions of the labeled fragment run in adjacent wells of the same gel. D and E show experiments utilizing purified GST-SRF fusion protein. D is an electrophoretic mobility shift assay of SRF (100 ng) with the NKE/CARG element-labeled DNA probe. The position of the SRF shift (lane 2) is shown to the left of the autoradiogram, which is effectively competed by excess, unlabeled fragment (lane 3) but not with excess DNA housing GATA-binding sites (lane 4). E shows the protection of the SMGA promoter fragment (~188 to +15) by added SRF protein (0.2–0.8 μg). The protected region of the probe fragment (nucleotides −93 to −76) is shown between the arrows to the right of the panel.
Nkx3-1 binding (data not shown). Thus, SRF binding to the Nke/CArG was facilitated by the Nkx3-1 homeodomain.

**Nkx3-1 Associated with SRF Independent of DNA Binding—** We did not observe the formation of higher order complexes in gel shift experiments with Nkx3-1 homeodomain and SRF proteins incubated together with the NKE/CArG probe. Similar results have been observed for interactions of SRF with other proteins such as Phox1 (48, 56, 57) and Nkx2-5 (41–43, 50), indicating that the formation of a ternary complex if they exist is transient or unstable and could not be resolved under the binding and gel electrophoresis conditions employed in our experiments. We wanted to determine if the synergy shared between Nkx3-1 and SRF in the activation of SMGA transcription was attributable to protein-protein associations; therefore, we employed coimmunoprecipitation assays to test if these two factors are capable of interacting in vivo. Cellular lysates were derived from NIH 3T3 cells following transfection of the cells with vectors expressing HA epitope-tagged SRF or Nkx3-1. For these experiments, we utilized conditions that maximized the expression of the HA-tagged protein, and control experiments showed that immunoreactive, HA-tagged protein was easily detected by Western assay (Fig. 1E). The Nkx3-1 expression vector contained the entire coding sequence of the protein cloned in-frame with the HA epitope so that we could test for protein interactions utilizing the complete Nkx3-1 protein.

Equivalent protein content of lysates from cells that were overexpressing SRF or Nkx3-1 were incubated in the presence of an SRF antibody, and antibody-SRF complexes were then collected by precipitation with protein A/G-agarose beads. The SRF antibody was derived from a peptide antigen representing amino acids 486–505 of the human SRF polypeptide, and we have demonstrated previously that it reacts specifically with the native and bacterially expressed protein (40–44, 50). The collected material was then analyzed by SDS-PAGE, immunoblotted, and probed with an anti-HA epitope monoclonal specific antibody. As shown in Fig. 5, a band corresponding to HA-tagged Nkx3-1 was recognized by the HA epitope antibody only when SRF is also present in the lysate, indicating that these two proteins are capable of forming complexes via protein-protein interactions.
The MADS Box and C-terminal Domains of SRF and the Homeobox Domain of Nkx3-1 Was Required for Coactivation of the SMGA Promoter—We asked if specific domains of these factors are important for functional coactivation of the SMGA promoter. The MADS box domain of SRF has been demonstrated to convey gene regulatory activity through associations with other factors, as well as being the DNA binding domain of the molecule (42, 43, 55, 58). The SRFpm1 molecule contains three amino acid substitutions (Arg to Leu at position 143, Lys to Ala at position 145, and Leu to Gly at position 146) that interrupt DNA binding but do not alter the ability of the protein to dimerize (58). Cotransfection of the SRFpm1 mutant blocked SRF-mediated transcriptional activation of α-skeletal and α-cardiac actin promoters (43, 46). Moreover, the SRFpm1 mutant protein has been demonstrated to inhibit transcription from the SMGA promoter in differentiated smooth muscle (46) and inhibit the expression of multiple smooth muscle marker proteins (calponin, SM22α, and SMMA) in developing coronary smooth muscle cells (39). Substitution of SRFpm1 for native SRF in our cotransfection experiments completely abolished the Nkx3-1/SMRF-mediated transcriptional activation of the SMGA promoter (Fig. 5B). Therefore, these data indicate that coactivation of the SMGA promoter by SRF and Nkx3-1 required SRF DNA binding activity. We next asked whether the transactivation domain within the C-terminal region of the molecule was necessary for the Nkx3-1/SMRF-mediated transcriptional activation of SMGA. Coexpression of an SRF molecule truncated at amino acid 338, lacking the C-terminal activation domain (41, 60), with native Nkx3-1 reduced promoter activity from the SMGA −176-bp construct by ~75% (Fig. 4B), indicating that the coactivation of SMGA transcription by SRF and Nkx3-1 is directed, at least in part, through the C-terminal transactivation domain of SRF.

Although there is significant homology between mNkx3-1 and bagpipe within their homeodomains, which leads to the classification of mNkx3-1 as an NK3 homologue (10, 11, 14), these proteins do not exhibit significant homologies at other segments or domains. This differs from the relationships shown for other Drosophila nk genes and their mammalian counterparts, such as tinman and nkg2-5, which have homologous segments outside the homeodomain thought to be important for protein-protein interactions and perhaps transactivation activities (6, 41, 42). To test if there were specific regions of Nkx3-1 that were important for its transcriptional activation activity, we generated mutant Nkx3-1 proteins and assayed their activity upon the SMGA −176 promoter fragment singularly and in the presence of SRF (Fig. 6). When the 53 amino acids on the C-terminal side of the homeodomain were deleted from the
mNkx3-1 and SRF Coactivate γ-SM Actin Promoter

In Drosophila, the NK3 homedomain protein bagpipe is expressed in the visceral mesoderm region of the embryo and has been implicated in the development of visceral and gonadal mesoderm (8, 9). Although the expression pattern of the vertebrate bagpipe homologue Nkx3-1 has been described (10, 11, 14), there is little information regarding molecular targets accorded by this developmentally and androgen-regulated transcription factor. Previous studies (17–21, 37, 39, 40, 51, 61) demonstrated that the SMGA isoform is a marker for visceral as well as vascular mesoderm differentiation. We asked if a vertebrate homologue of bagpipe, murine Nkx3-1, could regulate SMGA gene activity. As observed in cotransfection experiments, we found that the SMGA promoter was transactivated by SRF with mNkx3-1. The transcriptional activation elicited by Nkx3-1 was significant and specific since this factor was not capable of stimulating transcription from a vector bearing response elements derived from the cardiac α-actin gene. Furthermore, another Nkx factor, Nkx2-5, could not substitute for Nkx3-1 in transactivating the SMGA promoter (Fig. 1). Our results showed that the initial 176 bp of the SMGA promoter was sufficient for the synergistic activation of the SMGA promoter by SRF and Nkx3-1. This short segment of the promoter is highly conserved across vertebrate evolution (21) and plays a role in cell-specific transcription of the gene (21, 40, 61). Although both CArG/SRE motifs within the 176-bp promoter segment influenced SMGA transcription, CArG/SRE1 was demonstrated to be necessary for the synergistic Nkx3-1/SRF transactivation, and the proteins were found to bind with adjacent but overlapping motifs surrounding and including this motif (Fig. 3). Analysis of this novel and vital binding motif, designated as an NKE/CArG element, supports earlier studies that predicted a role for its involvement in directing transcription of the SMGA gene (21, 40). Therefore, our present studies substantiate SMGA as a Nkx3-1 regulatory target and define a mechanism by which this homeodomain is capable of controlling gene transcription. As Nkx3-1 is expressed within the somatic mesoderm in early embryos (10, 11) and in differing tissues throughout adulthood (11, 14), our data set a paradigm by which this gene product may regulate other genes and directly influence developmental gene programs.

Promoter deletion analyses have previously demonstrated two flanking regions of the SMGA gene that convey a smooth muscle-specific, positive transcriptional response (21). These two regions, called the smooth muscle specifier and the smooth muscle modulator domains (21), are heavily dependent upon SRF for their positive transcriptional activity (37, 40). Furthermore, the proximal segment of the smooth muscle specifier domain (sequences –200 bp flanking the gene) containing 2 CArG/SRE motifs was observed to bind SRF complexes in smooth muscle cell nuclear lysates as well as purified SRF (40). We demonstrated here that both of the CArG/SRE motifs within the initial 200 bp flanking the SMGA gene are capable of SRF binding, albeit with differing affinities. CArG/SRE1 of the SMGA promoter weakly bound SRF alone. This binding was facilitated by the presence of the Nkx3-1 homeodomain via binding to a DNA sequence within and adjacent to CArG/SRE1 of the SMGA promoter as shown in Fig. 3. The Nkx3-1-binding site adjacent to CArG/SRE1 on the SMGA promoter, 5’-CTA-

molecule (Nkx3-1 ΔCT), there was a 15-fold increased activity of the mutant protein on the SMGA promoter compared with the native protein (Fig. 6A). These data indicate that the C terminus of Nkx3-1 contains a transcriptional repressor domain, similar to that located in the C-terminal domain of Nkx2-5 (42, 43). Overexpression of the Nkx3-1 homeodomain resulted in 7-fold greater transcriptional activity upon the SMGA promoter than observed for the wild type mNkx3-1 molecule. Lysates of transfected cells analyzed by Western blotting demonstrated that the mutant proteins were adequately expressed in these experiments (Fig. 6B). Thus, the DNA binding activity of the Nkx3-1 homeodomain (Fig. 3) is capable of transducing a significant transcriptional response. The Nkx3-1 mutant containing only the homeodomain was sufficient to elicit a measurable transcriptional response in experiments where the Nkx3-1 proteins were coexpressed with native SRF (Fig. 6B). In addition, these cotransfection assays indicate that the C-terminal region of the mNkx3-1 molecule may play an important inhibitory role that is relieved by the association with SRF. Taken together, our experiments show that regulatory regions in addition to the SRF and Nkx3-1 DNA binding domains are necessary for the transcriptional coactivation of SMGA promoter.

DISCUSSION

The inhibitory C-terminal domain of Nkx3-1 activity is required for SMGA promoter coactivation with SRF. CMV promoter expression vector pCGN was utilized to encode HA epitope-tagged fusion Nkx3-1 wild type and mutant proteins. Expressed mutant proteins are Nkx3-1 ΔNT, a deletion of amino acids 1–124, Nkx3-1 ΔCT, a deletion of amino acids 184–237 and the homeodomain alone (deletion of amino acids 1–124 and 184–237). These vectors were cotransfected with the SMGA –176 promoter-luciferase reporter construct with (C) or without (A) added SRF expression vector in CV-1 cells. A, the fold activity was calculated by comparing the luciferase activity in cells transfected with the various Nkx3-1 mutant protein vectors with that obtained from overexpressing the native mNkx3-1 protein (Nkx3.1 WT). The amount of luciferase activity generated from native mNkx3-1 (WT) was assigned a value of 1.0, and the activities of the mutant proteins were compared with the wild type activity. B represents a Western blot of cellular lysates derived from cells transfected with empty CMV promoter vector (lane 1), native mNkx3-1 (WT, lane 2), Nkx3-1 ΔHOM (lane 3), Nkx3-1 ΔCT (lane 4), Nkx3-1 ΔNT (lane 5), and Nkx3-1 homeodomain (HOM, lane 6) probed with an anti-HA epitope monoclonal antibody. Antibody-antigen complexes (shown by the arrows to the left of the autoradiogram) were revealed by chemiluminescence as described previously. C, wild type (WT) and mutant mNkx3-1 vectors (0.8 μg) were assayed with added pCGN-SRF (0.2 μg) expression vector as described previously. In these experiments the fold activity was calculated from luciferase activities in lysates of cells transfected with SRF and Nkx3-1 compared with the activity measured with empty pCGN vector.

| Protein | Fold Activity |
|---------|--------------|
| WT      | 1.0          |
| ΔNT     | 0            |
| ΔCT     | 15           |
| ΔHOM    | 7            |
| ΔNT     | 0            |
| HOM     | 1            |

FIG. 6. The inhibitory C-terminal domain of Nkx3-1 activity is required for SMGA promoter coactivation with SRF. CMV promoter expression vector pCGN was utilized to encode HA epitope-tagged fusion Nkx3-1 wild type and mutant proteins. Expressed mutant proteins are Nkx3-1 ΔNT, a deletion of amino acids 1–124, Nkx3-1 ΔCT, a deletion of amino acids 184–237 and the homeodomain alone (deletion of amino acids 1–124 and 184–237). These vectors were cotransfected with the SMGA –176 promoter-luciferase reporter construct with (C) or without (A) added SRF expression vector in CV-1 cells. A, the fold activity was calculated by comparing the luciferase activity in cells transfected with the various Nkx3-1 mutant protein vectors with that obtained from overexpressing the native mNkx3-1 protein (Nkx3.1 WT). The amount of luciferase activity generated from native mNkx3-1 (WT) was assigned a value of 1.0, and the activities of the mutant proteins were compared with the wild type activity. B represents a Western blot of cellular lysates derived from cells transfected with empty CMV promoter vector (lane 1), native mNkx3-1 (WT, lane 2), Nkx3-1 ΔHOM (lane 3), Nkx3-1 ΔCT (lane 4), Nkx3-1 ΔNT (lane 5), and Nkx3-1 homeodomain (HOM, lane 6) probed with an anti-HA epitope monoclonal antibody. Antibody-antigen complexes (shown by the arrows to the left of the autoradiogram) were revealed by chemiluminescence as described previously. C, wild type (WT) and mutant mNkx3-1 vectors (0.8 μg) were assayed with added pCGN-SRF (0.2 μg) expression vector as described previously. In these experiments the fold activity was calculated from luciferase activities in lysates of cells transfected with SRF and Nkx3-1 compared with the activity measured with empty pCGN vector.
AGT-3' (minus strand) is identical in structure to the consensus Nkx2 core-binding sequences 5'-TNAAGTG-3' (41, 52, 62). Nkx3-1 has been shown to bind synthetic oligonucleotides bearing the Nkx2 core-binding sequence (11); therefore, our studies demonstrate that naturally occurring binding sites for vertebrate NK3 proteins fit the general NKE consensus. Our results differ from the studies that evaluated the role of Nkx2-5/SRF-facilitated binding and activation of the cardiac α-actin gene, where both proteins bound the same CArG/SRE motif (41–43, 50), because there is the juxtaposition of an NKE with a CArG/SRE element within the SMGA proximal promoter. However, we did observe a weak, secondary binding of Nkx3-1 to the SRE sequence, and our data cannot formally eliminate the possibility that this binding is required for facilitated SRF binding. In addition, Nkx3-1/SRF-dependent SMGA gene regulation is mediated by sequences that are highly conserved among species, suggesting a strict maintenance of this regulatory paradigm throughout evolution for cell-specific gene transcription.

Although smooth muscle-specific expression of SMGA is strongly dependent upon SRF activity (40), SRF appears to cooperate with other factors to regulate SMGA transcription. The interaction of SRF with accessory factors has been demonstrated in a number of systems. Studies of c-fos gene regulation has led to the identification of several accessory factors, including SAP-1, Elk-1, and Phox-1 (48, 63–65). These factors appear to be expressed ubiquitously and together with SRF potentiate the transcriptional activity of the c-fos gene, although the exact regulatory mechanisms are somewhat distinct. Additionally, SRF interaction with cell-specific regulatory factors has been characterized. MCM1, the yeast SRF homologue, is influenced by the interactions with an array of accessory factors that either activate or repress genes in a cell-specific and temporal pattern (66). In vertebrates, the cardiac-specific homeodomain Nkx2-5 makes specific physical interactions with SRF producing an enhanced transcription of cardiac-specific genes (41–43, 53). Similarly, SRF interaction with skeletal muscle cell-restricted basic helix-loop-helix proteins of the MyoD family may directly influence the expression of muscle-specific genes (47) including SRF itself (44, 60). In many of these systems, SRF is regarded as providing the platform for the recruitment of accessory factors, thus affecting the regulatory pattern. It is clear that SRF binds avidly to multiple sites within the SMGA promoter, any of which may provide the opportunity for SRF-dependent interactions (21, 37, 40). Nkx3-1 facilitated the binding of SRF to the SMGA CArG/SRE1 and surrounding sequences to affect the synergistic activation of SMGA promoter. The fact that SRF binding was required for this activation (Fig. 5) and that SRF activation of an SMGA promoter in the presence of a mutated high affinity SRE (CArG/SRE2) required the coexpression of Nkx3-1 (Fig. 2) correlates well with Nkx3-1-dependent facilitated SRF binding to the SMGA promoter. Thus, SRF interactions with accessory proteins may be more complex than previously anticipated and may depend to a greater extent upon the composition of regulatory elements as a mechanism to ensure cell-specific gene activation.

Combinatorial synergy of Nkx3-1 and SRF for the regulation of SMGA transcription might be achieved at several levels including enhanced protein complex interaction, changes in DNA conformation, and/or release of a repressor protein activity. The native polypeptides are able to form protein-protein interactions in the absence of DNA binding (Fig. 5) which may serve to alter protein complex conformations and enhance the interaction of other protein domains with general transcription factors. This appears to be an operational mechanism for regulation of the α-skeletal actin gene via the interaction of SRF, TEF-1, and Sp1 (67). Alternatively, since a mutation of the NKE sequence of the Nke/CArG element did not totally obstruct activation of the −176 SMGA promoter fragment (Fig. 2), it is possible that protein-protein interactions allow activation without Nkx3-1 binding. Phox, a human homologue of Mhox, interacts with SRF to enhance the exchange of SRF with its binding site in the c-fos promoter and does not require specific homeodomain DNA binding activity (48, 51, 68). However, Mhox and chicken HOXB4 (an avian Dip paralogue), like Nkx2-5 (Fig. 1), was incapable of activating the SMGA −176 promoter in the presence of SRF (data not shown). Thus, SRF is capable of interacting with homeodomain factors in a way that alters the activity of the SRF molecule.

We noted an appearance of new DNase I-sensitive cleavage sites with Nkx3-1 binding upon the SMGA promoter fragment with or without SRF present (Figs. 3 and 4), indicating that the binding of the Nkx3-1 homeodomain alters the conformation of the DNA 5’ to its binding site. This might provide the means for enhancing SRF binding to the relatively weak CArG/SRE1 motif, in essence making this motif more attractive for SRF binding and thus facilitating binding. A variety of homeodomain (69–71) and MADS box proteins, including SRF (58, 72), are capable of bending DNA, thereby increasing the specificity or affinity of DNA binding. DNA bending and the formation of looping structures appears to be a general mechanism for bringing transcriptional activator complexes together, providing a mechanism for cooperativity among complexes separated along the DNA by some distances (73–76). Therefore, the altered chromatin structure induced by way of Nkx3-1 binding the NKE/CArG element might allow DNA bending that brings complexes bound along the SMGA promoter together for appropriate cell-specific transcription. It is also possible that Nkx3-1/SRF complexes compete for binding with negative acting trans-factors. Synergistic activation of the α-cardiac actin via Nkx2-5/SRF complexes includes the displacement of the negative acting factor YY1 from SRE2 of the promoter (50). Analysis of the SMGA promoter identified CarG/SRE1 as a potential YY1-binding site (21) which might function to repress SMGA synthesis. When the distal CarG/SRE was removed from the SMGA −176 promoter fragment, it was not activated by Nkx3-1/SRF complexes. In these experiments, a high affinity SRF which is an integral motif of the positive acting SMGA specifier was removed from the test promoter. Because of the dependence upon SRF for SMGA transcription (40), the removal of a positive acting cis element (CarG/SRE2) may allow the dominance of negative regulatory influences, such as YY1, over the positive acting Nkx3-1-SRF complex.

It is clear that cell-specific transcription of the SMGA gene requires complex interactions directed by multiple cis-acting elements. One way of stimulating the SMGA promoter involves the cooperative interactions of SRF and the vertebrate NK3 homologue Nkx3-1 upon a specific segment of the promoter, the NKE/CArG element. Therefore, SMGA gene activation may require increased levels of SRF with the appearance of Nkx3-1 to foster cooperative trans-factor complex formation. Consistent with this hypothesis, we have recently demonstrated that the developmentally regulated expression of SRF is a key determinant of SMGA gene regulation during smooth muscle myogenesis (40). In addition, the appearance of Nkx3-1 within the smooth muscle component of visceral organ vessels such as the kidney (14) corresponds with the onset of SGMA expression detected by in situ hybridization analyses (17). Therefore, the combinatorial interaction of SRF with Nkx3-1 may directly influence the cell-specific activation of the SMGA gene. Moreover, Nkx3-1/SRF complexes may be an important part of the regulatory machinery of other genes during embryogenesis and in adult tissues. Nkx3-1 is expressed in somites, brain, blood
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REFERENCES

1. Gehring, W. J., Affolita, and T. Burglin T. (1994) Annu. Rev. Biochem. 63, 117–140
2. Khorasanizadeh, S., and Rastinejad, F. (1999) Curr. Biol. 9, R456–R458
3. Mannervik, M. (1999) Science 287, 3631–3639
4. Doevendans, P. A., and Van Bilsen, M. (1996) Mol. Cell. Biol. 16, 194–200
5. Holland, P. W. H., and Garcia-Fernande, J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 266–270
6. Li, L., Miano, J., Mercer, B., and Olson, E. N. (1996) J. Cell. Biol. 135, 1123–1130
7. Schroter, H., Muller, C. G., Meese, K., and Nordheim, A. (1990) J. Biol. Chem. 265, 6372–6384
8. MacLellan, W. R., Lee, T. C., Schwartz, R. J., and Schneider, M. D. (1994) J. Biol. Chem. 269, 272, 1731–1734
9. Kerppola, T. K. (1998) Structure 6, 549–554
10. Perez-Martin, J., and Espinosa, M. (1993) Structure 2, 171–178
11. Wolberger, C. (1999) Annu. Rev. Biochem. 68, 3671–3698
12. Galvagni, F., Lestingi, M., Cartresi, E., and Olivier, S. (1997) Mol. Cell. Biol. 17, 1731–1734
13. Becker, J. C., Nikroo, A., Brablett, T., and Reisfeld, R. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9727–9731
14. Kerpola, T. K (1998) Structure 6, 549–554
15. Perez-Martin, J., and Espinosa, M. (1993) Science 260, 805–807
16. Van der Vliet, J. C., and Verijzer, C. P. (1993) BioEssays 15, 25–32
17. Bhattacharjee, R., Donjacour, A. A., Sciavolino, P. J., Kim, M., Desai, N., Young, P., Norton, C. R., Gridley, T., Cardiff, R. D., Cunha, G. R., Abate-Shen, C., and Shen, M. M. (1999) Genes Dev. 13, 966–977
18. Durocher, D., Charron, F., Warren, R., Schwartz, R. J., and Nemer, M. (1997) Mol. Cell. Biol. 17, 3318–3326
19. West, A. G., Shore, P., and Sharrocks, A. D. (1997) Mol. Cell. Biol. 17, 2876–2887
20. Kim, J. H., Jahnansen, F. E., Robertson, N., Catino, J. J., Prywes, R., and Kumar, C. C. (1994) J. Biol. Chem. 269, 13740–13743
21. Belaguli, N. S., Zhou, W., Trich, T. H., Majesky, M. W., and Schwartz, R. J. (1999) Mol. Cell. Biol. 19, 4582–4591
22. Qian, J., Kumar, A., Szucsick, J., and Lessard, J. (1996) Dev. Biol. 180, 135–144
23. Guaszi, S., Price, M., DeFelice, M., Danaste, G., Matti, M. G., and DiLauro, R. (1990) EMBO J. 9, 3631–3639
24. Dalton, S., and Treisman, R. (1992) J. Biol. Chem. 267, 597–612
25. Pollock, R., and Treisman, R. (1991) Genes Dev. 5, 2327–2341
26. Schreiber, H., Muller, C. G., Meeke, E., and Nordheim, A. (1999) EMBO J. 18, 1123–1130
27. Herskowitz, I. (1989) Science 342, 749–757
28. MacLellan, W. R., Lee, T. C., Schwartz, R. J., and Schneider, M. D. (1994) J. Biol. Chem. 269, 16754–16760
29. Cserjesi, P., Lillly, B., Bryson, L., Wang, Y., Sassoon, D. A., and Olson, E. N. (1992) Development 115, 1087–1101
30. Knesevici, V., DeSanto, R., Schughart, K., Huffstadt, A., Chiang, C., Mahon, A. K., and Mackem, S. (1997) Development 124, 4523–4536
31. Nelson, H. B., and Laughon, A. (1996) New Biol. 2, 171–178
32. Wolberger, C. (1999) Annu. Rev. Biophys. Biomol. Struct. 28, 39–56
33. Galvagni, F., Lestingi, M., Cartresi, E., and Olivier, S. (1997) Mol. Cell. Biol. 17, 1731–1734
34. Becker, J. C., Nikroo, A., Brablett, T., and Reisfeld, R. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9727–9731
35. Kerpola, T. K (1998) Structure 6, 549–554
36. Perez-Martin, J., and Espinosa, M. (1993) Science 260, 805–807
37. Van der Vliet, J. C., and Verijzer, C. P. (1993) BioEssays 15, 25–32
38. Bhatia-Gaur, R., Donjacour, A. A., Sciavolino, P. J., Kim, M., Desai, N., Young, P., Norton, C. R., Gridley, T., Cardif, R. D., Cunha, G. R., Abate-Shen, C., and Shen, M. M. (1999) Genes Dev. 13, 966–977