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Recruitment of the Tinman Homolog Nkx-2.5 by Serum Response Factor Activates Cardiac α-Actin Gene Transcription

CHING YI CHEN AND ROBERT J. SCHWARTZ*

Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

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We recently showed that the cardiogenic homeodomain factor Nkx-2.5 served as a positive acting accessory factor for serum response factor (SRF) and that together they provided strong transcriptional activation of the cardiac α-actin promoter, depending upon intact serum response elements (SREs) (C. Y. Chen, J. Croissant, M. Majesky, S. Topouz, T. McQuinn, M. J. Frankovsky, and R. J. Schwartz, Dev. Genet. 19:119–130, 1996). As shown here, Nkx-2.5 and SRF collaborated to activate the endogenous murine cardiac α-actin gene in 10T1/2 fibroblasts by a mechanism in which SRF recruited Nkx-2.5 to the α-actin promoter. Activation of a truncated promoter consisting of the proximal α-actin SRE1 occurred even when Nkx-2.5 DNA-binding activity was blocked by a point mutation in the third helix of its homeodomain. Investigation of protein-protein interactions showed that Nkx-2.5 was bound to SRF in the absence of DNA in soluble protein complexes retrieved from cardiac myocyte nuclei but could also be detected in coassociated binding complexes on the proximal SRE1. Recruitment of Nkx-2.5 to an SRE depended upon SRF DNA-binding activity and was blocked by the dominant negative SRF_{pm1} mutant, which allowed for dimerization of SRF monomers but prevented DNA binding. Interactive regions shared by Nkx-2.5 and SRF were mapped to N-terminal/helix I and helix II/helix III regions of the Nkx-2.5 homeodomain and to the N-terminal extension of the MADS box. Our study suggests that physical association between Nkx-2.5 and SRF is one way that cardiac specified genes are activated in cardiac cell lineages.

The embryonic appearance of cardiac primordia requires commitment of pluripotent mesodermal stem cells to the cardiac lineage, activation of subsets of unlinked cardiac lineage-specified genes encoding proteins required for the cardiac myocyte differentiation, and morphogenetic elaboration into the cardiac organ. Novel insights have come from *muscle segment homeobox* (*msh*) genes from *Drosophila melanogaster*, whose expression is restricted primarily to mesodermal cell types during insect development. One of the *Drosophila* homeobox-containing *msh*-2/NK-4 genes, *tinman*, was expressed in the developing dorsal vessel, an insect version of a heart (6). Mutations in *tinman* did not effect mesoderm invagination or dorsal spreading, but dorsal vessel formation in the *Drosophila* embryo was blocked and visceral mesoderm was virtually absent (3, 5). A second homeobox gene, *bagpipe* or NK-3, closely linked to *tinman* in the *Drosophila* genome and expressed in segmental clusters of mesodermal cells, appeared to be a mesodermal determination factor downstream of *tinman*. The absence of *bagpipe* led to deficiencies of midgut musculature in fly larvae (3). Interestingly, another invertebrate NK-2 class homeodomain factor from *Caenorhabditis elegans*, *ceh-22*, was shown to be required for the expression of the pharyngeal myogenic program and played a key role in muscle-specific activity of the myosin heavy chain gene, *myo-2* (55).

A murine Nkx-2.5 homeobox gene (45), also named cardiac-specific homeobox (36), was identified as one of the potential vertebrate homologs of *tinman*. In mouse embryos, Nkx-2.5 transcripts were localized to early cardiac cell progenitors, prior to cardiogenic differentiation, and continued to be expressed in cardiac myocytes throughout development. Nkx-2.5 was also detected in the pharyngeal endoderm immediately adjacent to the cardiac mesoderm, tongue muscle, and visceral muscle in the stomach and the spleen. Superimposed upon the appearance of Nkx-2.5 in cardiac progenitor cells was the sequential activation of the cell type-restricted α-cardiac actin and myosin heavy-chain genes (45). Recently, a homozygous knockout of Nkx-2.5 caused embryonic death, probably elicited by the failure to fold the heart tube into a chambered heart and by the lack of myocardial cell expansion associated with ventricular trabeculation (46). Although these mice die at 9 days of embryonic development, the expression of several cardiogenic restricted genes marking the differentiation of early cardiac myocytes did not appear to be affected by the Nkx-2.5 mutant. The lack of a more severe phenotype might not be surprising when one considers that there are at least six murine Nkx-2 genes in which the expression pattern of only a few genes have been characterized (40, 45, 60). In addition, the coexpression of Nkx-2.3 and Nkx-2.6 (46) in the developing cardiovascular system of higher vertebrate embryos suggests a potential role for genetic redundancy of Nkx-2 loci during cardiogenesis.

We recently determined that Nkx-2.5 homeodomain DNA-binding targets included a preferred Nkx-2.1-binding sequence, TNAAGTG, and a second subset of sequences that contained a 5′-GTTAATTC-3′ core similar to the binding sites of the *Antennapedia* class of homeodomains (10). These targets also resembled the AT-rich central core of the serum response element (SRE) [$CC(A/T)_6GG$], which serves as an efficient binding site for serum response factor (SRF) (76), and we recently showed that certain SREs are efficient Nkx-2.5-binding sites (9). For example, subsets of conserved cardiac α-actin SREs served as both high-affinity (SRE_2 and SRE_3) and intermediate-strength (SRE_1 and SRE_4) binding targets of bacterially expressed Nkx-2.5. SRF is highly conserved throughout evolution, and its DNA-binding/dimerization domain of 50 amino acids, termed the MADS box, bears striking

* Corresponding author. Mailing address: Department of Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-6649. Fax: (713) 798-7799.

homology to yeast transcription factors MCM1 and ARG80 and several plant proteins which also have similar DNA sequence-binding specificity (67). Several studies (12, 24, 75) have indicated that SRF-binding sites play a primary role in regulating early response genes such as c-*fos* and *egr-1* and the myogenic restricted α-actin genes (13, 41, 51, 54, 77). In fact, SRF marginally activated the avian skeletal α-actin promoter in transient-transfection experiments (42). We also recently demonstrated that a dominant-negative SRF mutant, SR-Fpm1, which allowed for dimerization with the wild-type SRF but inhibited DNA binding (33, 63), blocked the avian skeletal α-actin promoter activity in terminally differentiated muscle cells (15). Although SRF appeared to play a primary role in regulating the α-actin genes, it was still unclear how a purported ubiquitous factor like SRF could provide tissue-specific transcriptional activity (41, 42, 52).

Studies regarding the regulation of the c-*fos* gene by SRF have led to the identification of several SRF accessory factors including SAP-1, Elk-1, and Phox-1 (17, 23, 26, 62, 65, 66). All these SRF accessory factors appeared to potentiate the transcriptional activity of SRF on the c-*fos* SRE, although the mechanisms are somewhat different. For example, Elk-1 formed a ternary complex with the SRF MADS box on the c-*fos* SRE, which required a 30-amino-acid peptide from Elk-1 and a purine-rich sequence (AGGA) at the 5′ end of the c-*fos* SRE (69, 70). The same ternary complex was also shown to be required for induction of *egr-1* following antigen receptor cross-linking in B lymphocytes (49). Phox-1 interacts with SRF to enhance the exchange of SRF with its binding site in the c-*fos* gene. This function of the homeodomain does not require direct DNA-binding activity. The interactions between Phox-1 and SRF may also recruit Elk-1 to the c-*fos* SRE (27). Hypothetically, interactions between SRF and various Hox proteins suggested a mechanism through which cells can coordinate intrinsic information on cell specificity with the response to external regulatory signals and a mechanism which can control cell-type-specific expression and differentiation in both a positive and negative manner (27). In this regard, MHox (16), the murine counterpart of Phox-1 (27) which promotes growth factor signaling through the c-*fos* SRE, could not transactivate the cardiac α-actin promoter in the presence of SRF in fibroblasts, as shown here.

We showed that Nkx-2.5 is composed of both transcriptional activation and inhibitory domain regions (10). Nkx-2.5 served as a modest transcription activator in transfection assays when analyzed on reporter genes carrying multimerized binding sites. However, the activity of Nkx-2.5 was stimulated about 50-fold respectively when its C-terminal inhibitory domain was deleted (10). This finding hints that association with other factors under certain conditions might transduce Nkx-2.5 into an operative transcriptional activator. Indeed, we showed that the cardiogenic homeodomain factor such as Nkx-2.5 could serve as the positive acting accessory factor for SRF, which resulted in strong transcriptional activation of the cardiac α-actin promoter, which was shown to be dependent upon intact SREs (9). Here, we demonstrated that Nkx-2.5 and SRF were capable of activating the endogenous α-cardiac actin gene in nonmyogenic fibroblasts. We then asked how this mechanism involving the synergistic transactivation of the cardiac α-actin gene by Nkx-2.5 and SRF was mediated. Even though Nkx-2.5 can bind weakly to some SREs, we found that activation of a minimal promoter consisting of a single SRF-binding site was dependent upon SRF. When Nkx-2.5-binding activity was blocked by a point mutation in the third helix of the homeodomain, SRF was still capable of recruiting mutated Nkx-2.5 to cardiac α-actin promoter DNA. Nkx-2.5 was also bound to SRF in the absence of DNA in protein complexes extracted from cardiac myocyte nuclei. We observed that short peptides from the Nkx-2.5 protein (amino acids 122 to 162 and 162 to 203) and SRF (amino acids 142 to 171), mapped by mutagenesis, were sufficient to mediate Nkx-2.5 and SRF protein-protein interactions. These protein associations probably stabilized mutual inclusive binding of both factors over SREs in the cardiac α-actin promoter. Our study suggests that Nkx-2.5 physical association with SRF is one way that cardiac lineage-specified genes are activated in cardiac and muscle cell lineages.

MATERIALS AND METHODS

Recombinant plasmids. A 330-bp fragment of the chicken cardiac α-actin promoter and immediate upstream region, previously defined by French et al. (21) as a *Sma*I-*Bst*EII fragment from −315 to +15, relative to the transcription start site, was subcloned into the *Hin*dIII site of the pGL2-basic luciferase vector (Promega) to obtain an α-CA-LUC reporter construct. CaSRE1-TATA-LUC was constructed by digesting α-CA-LUC with *Bgl*II and *Nco*I to remove the promoter sequences from −310 to −100. An Nkx-2.5 expression vector was constructed by inserting an *Eco*RI fragment containing the full-length Nkx-2.5 cDNA into *Eco*RI of pEMSV driven by the murine sarcoma virus long terminal repeat. The pEMSV-Nkx-2.5pm plasmid was obtained by insertion of a *Pst*I fragment isolated from pCGN-Nkx-2.5pm (10) into the *Pst*I site of pEMSV-Nkx-2.5. The SRF expression vectors (pCGN-SRF and pCGN-SRFpm1) driven by the cytomegalovirus promoter were kindly provided by Ron Prywes. pEMSV-MHox encoding the full-length MHox cDNA (16) was kindly provided by Eric Olson.

Human SRF was bacterially expressed as a glutathione-*S*-transferase (GST) fusion protein by isolation of a 1.6-kb cDNA fragment from pCGN-SRF (33) inserted in frame in the GST coding region of the pGEX-4T-3 vector (Pharmacia). GST-SRF(1–412) and GST-SRF(1–171) were constructed by cutting GST-SRF(1–508) with *Sph*I and *Xho*I and with *Stu*I and *Xho*I, respectively, blunted with T4 DNA polymerase in the presence of deoxynucleoside triphosphates, and ligated with T4 DNA ligase. GST-SRF(142–508), GST-SRF(142–412), and GST-SRF(142–171) were constructed by digesting GST-SRF(1–508), GST-SRF(1–142), and GST-SRF(1–171) with *Eco*RI and *Sma*I to remove amino acids 1 to 141 of SRF, blunted, and religated. GST-SRF(46–244) was obtained by digesting GST-SRF(1–508) with *Sma*I and *Bgl*II and processed as described above.

Maltose-binding protein-Nkx-2.5 (MBP–Nkx-2.5) mutant constructs subcloned into the *Xba*I and *Hin*dIII sites of pMAL-c2 vector were described previously (10). In addition, MBP–Nkx-2.5(122–160), MBP–Nkx-2.5(162–203), and MBP+12aa were obtained by digesting the MBP–Nkx-2.5(122–203) plasmid with *Kpn*I, *Xmn*I plus *Kpn*I, and *Xba*I plus *Kpn*I and were blunted, and then the plasmids sealed by ligation. In the case of MBP–Nkx-2.5(122–160), a 12-amino-acid (CRRQSATSWPAC) polypeptide downstream from the authentic Nkx-2.5 polypeptide was obtained as a result of the construction. MBP+12aa, which served as a control, encoded the MBP plus the additional 12 amino acids of MBP–Nkx-2.5(122–160).

Transfection assays into C3H10T1/2 fibroblast cell cultures. Growing C3H10T1/2 mouse fibroblasts were transfected with a total of 6 μg of DNA composed of reporter constructs (2 μg) in the presence of pEMSV-Nkx-2.5 (2 μg), pCGN-SRF (0.3 μg), or both, and CMV–β-gal (1 μg), an internal control, balanced with the parental expression vector in the presence of 9 μl of DEAE-dextran (25 mg/ml). Luciferase and β-galactosidase reporter gene activity was assayed 24 to 36 h later as previously described (10, 43). Experimental data are presented as the mean of three independent transfection assays done in duplicate and normalized to β-galactosidase activity.

Reverse transcriptase PCR analysis for cardiac α-actin transcripts. Replicating 10T1/2 fibroblasts were cotransfected with pSV-Neo (0.1 μg) and pCGN (2 μg), pCGN-Nkx-2.5 (2 μg), or pCGN-SRF (2 μg) by using Lipofectamine (GIBCO). Transfected cells were grown in a G418-containing medium (400 μg/ml) for 2 to 3 weeks, and drug-resistant colonies were pooled and expanded for further analysis. Expression of endogenous cardiac lineage-restricted genes was analyzed by reverse transcriptase PCR, as recommended by the manufacturer (Life Technologies, Inc.), with gene-specific primers. First-strand cDNA synthesis was performed with 1 μg of total RNA, 200 U of Moloney murine leukemia virus RNase H reverse transcriptase, and 50 pmol of 3′ gene-specific primer 1 in a final reaction volume of 20 μl. After incubation for 60 min at 37°C, the reverse transcriptase reaction was terminated by boiling for 5 min. The reverse transcriptase reaction mixture was then adjusted to a final volume of 100 μl containing 1× PCR buffer by the addition of 10× PCR buffer, 100 pmol of a 5′ gene-specific primer, 50 pmol of 3′ gene-specific primer 1, and 2.5 U of *Taq* DNA polymerase (Promega). The amplification sequence consisted of an initial denaturation at 94°C for 5 min followed by 30 cycles (cardiac α-actin) or 20 cycles (glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) of denaturation at 94°C for 45 s, annealing at 62°C for 45 s, and extension at 72°C for 1 min. A final extension at 72°C for 5 min was also performed. The authenticity of the amplified

products was determined by a second PCR with ^{32}P-labeled 3′ gene-specific primer 2, which was analyzed on an 8% polyacrylamide gel and subjected to autoradiography. The primers used were 5′-GCTCCCAGCACCATGAAG-3′ (5′ primer), 5′-GGCTAAGAGAGAGACATC-3′ (3′ primer 1), and 5′-ATCTC AGAAGCACTTGCGGTG-3′ (3′ primer 2) for cardiac α-actin and 5′-ACCAC AGTCCATGCCATCAC-3′ (5′ primer) and 5′-TCCACCACCCTGTTGCTGT A-3′ (3′ primer) for GADPH.

DNA EMSA. Double-stranded oligonucleotides corresponding to the avian cardiac actin SRE1 (nucleotides [nt] −96 to −74), SRE2 (nt −136 to −112), SRE3 (nt −168 to −146), and SRE4 (nt −212 to −190), the avian skeletal actin SRE1 (nt −96 to −74), the human c-*fos* SRE (nt −314 to −292), and a consensus Nkx-2.1-binding sequence, which were described previously (8, 10, 75), were synthesized and used for EMSA.

Nuclear extract preparation and affinity purification of bacterially expressed GST-SRF and MBP–Nkx-2.5 derivative proteins. Primary chicken cardiomyocytes were prepared from 12-day-old chicken ventricles as described previously (54). Nuclear extracts were prepared by the method of Hennighausen and Lubon (31). All procedures were performed in a cold room (4°C) and with ice-cold reagents. The isolated cardiomyocytes were washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in 5 packed-cell volumes of 0.3 M sucrose in ice-cold buffer A [10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.6), 10 mM KCl, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg of leupeptin per ml, 2 μg of aprotinin per ml, 2 μg of pepstatin per ml). Cells were homogenized in a Dounce glass homogenizer with a B pestle by applying 10 strokes without and 2 strokes with 0.4% Nonidet P-40 (NP-40). The nuclei were then pelleted by centrifugation at 2,500 × *g* at 4°C and washed twice with buffer A containing 0.3 M sucrose but no NP-40. The nuclear pellet was resuspended by 10 strokes of an all-glass Dounce homogenizer in 2.5 packed nuclear volumes of buffer B (400 mM NaCl, 10 mM HEPES-KOH [pH 7.6], 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol, 0.5 mM PMSF). The resuspended nuclei were slowly swirled for 30 min at 4°C and subjected to centrifugation at 13,000 rpm for 30 min in an Eppendorf centrifuge. The supernatants were dialyzed at 4°C against 100 volumes of 1× binding buffer (50 mM KCl, 15 mM HEPES [pH 7.6], 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 5% glycerol) for 4 h. The nuclear extracts were cleared by centrifugation at 13,000 rpm for 5 min, and the protein concentration was determined with a Bio-Rad kit. To express SRF and Nkx-2.5 in bacteria, BL21(DE3) bacteria were transformed with GST-SRF and MBP–Nkx-2.5 constructs. Fusion protein expression was induced by isopropyl-β-D-thiogalactopyranoside IPTG (0.5 mM) in growing bacteria for 1 h. The cells were then harvested, resuspended in PBS, sonicated, and centrifuged for 10 min at 4°C, and supernatants were subjected to affinity purification. GST-SRF fusion proteins were purified with glutathione-Sepharose 4B beads (Pharmacia), and MBP–Nkx-2.5 fusion proteins were purified with amylose resins (New England BioLabs) as specified by the manufacturer. The glutathione-bound GST-SRF fusion proteins were eluted by incubation with 10 mM reduced glutathione (Boehringer Mannheim) in 50 mM Tris-Cl (pH 8.0). The MBP–Nkx-2.5 fusion proteins were released from amylose resins by washing with 10 mM maltose in PBS. The purity and quantity of purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining.

Immunoprecipitation of the SRF–Nkx-2.5 complex from transfected primary avian cardiomyocytes. Primary chicken cardiomyocytes plated at a density of 10^6 per 35-mm dish were transfected with 4 μg of pCGN-Nkx-2.5 (HA epitope tagged) by the lipofection method. At 48 h after transfection, the cells were washed twice with ice-cold PBS, scraped from the dish into ice-cold PBS supplemented with 10 mM EDTA, and collected by centrifugation. The cell pellet was resuspended in low-stringency buffer (EBC buffer; 50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% NP-40, 2 μg of aprotinin per ml, 2 μg of leupeptin per ml, 2 μg of pepstatin per ml, 1 mM PMSF), rocked for 15 min at 4°C, and centrifuged at 12,000 rpm for 5 min. Supernatant (500 μg of total protein) was incubated with 4 μg of anti-HA antibody, 15 μl of the affinity-purified anti-SRF antibody against amino acids 404 to 418 of human SRF, or 7 μl of anti-YY1 antiserum in 500 μl of NETN buffer (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 0.05% NP-40). The samples were rocked at 4°C for 2 h, 30 μl of protein G PLUS/protein A-agarose beads (Oncogene Science) was added, and the mixture was incubated for another 2 h. The beads were pelleted at 12,000 rpm for 10 s and washed three times in 1 ml of NETN buffer. The beads were resuspended in 30 μl of SDS sample buffer and boiled for 5 min. The supernatant was separated by SDS-PAGE (10% polyacrylamide) and subjected to Western blot (immunoblot) analysis. Western blotting was performed by standard procedures, and proteins were detected with alkaline phosphatase-conjugated secondary antibodies.

Binding of in vitro-translated ^{35}S-labeled Nkx-2.5 and bacterially expressed MBP–Nkx-2.5 fusion proteins to the immobilized GST-SRF fusion proteins. GST-SRF derivatives were immobilized on glutathione-agarose beads, treated with DNase I to remove DNA contamination, and used for in vitro protein interaction assays. In vitro-translated ^{35}S-labeled Nkx-2.5 protein was produced in rabbit reticulocyte lysates (Promega) from RNAs transcribed in vitro from *Hin*dIII-linearized pBluescript–Nkx-2.5 with T3 RNA polymerase. Portions (10 μl) of glutathione-agarose beads bearing equal amounts of either GST or the fusion proteins (1 to 2 μg) were mixed with ^{35}S-labeled Nkx-2.5 (10 μl) or MBP–Nkx-2.5 derivatives (300 ng) in 200 μl of binding buffer (100 mM NaCl, 40 mM HEPES [pH 7.9], 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 0.05% NP-40). The binding-reaction mixtures were gently rocked on a rotating wheel at room temperature for 1 h. The beads were then washed four times with 600 μl of the same buffer, resuspended in Laemmli sample buffer, and analyzed by SDS-PAGE. ^{35}S-labeled Nkx-2.5 protein was visualized by fluorography and quantified with Betagen. The retained MBP–Nkx-2.5 derivatives were analyzed by immunoblotting with anti-MBP antiserum (New England BioLabs) and an alkaline phosphatase-conjugated anti-rabbit antibody (Promega). To investigate if SRF could selectively associate with the Nkx-2.5 protein in cell lysate, 10T1/2 cells were transfected with 3 μg of either pCGN or pCGN-Nkx-2.5 plasmid with Lipofectamine (10 μl). At 48 h after transfection, the cells were lysed in EBC buffer. The cell lysates were then incubated with GST-SRF resin for 2 h at 4°C as described above. The bound proteins were analyzed by SDS-PAGE and protein immunoblot analysis with an anti-HA monoclonal antibody.

DNA affinity chromatography assay. Purified bacterial GST-SRF (1 μg) was incubated at room temperature for 30 min with MBP–Nkx-2.5 fusion proteins (1 μg) in 200 μl of DNA-binding solution (50 mM NaCl, 15 mM HEPES [pH 7.6], 1 mM EDTA, 0.5 mM DTT, 5% glycerol) containing bovine serum albumin (BSA; 5 mg/ml), poly(dI-dC) (5 μg), and synthetic biotin-labeled (labeled at one end of the top strand) cardiac actin SRE1 oligonucleotide (2 pmol). Then streptavidin gel (10 μl; Boehringer Mannheim), which had been preincubated with DNA-binding solution containing BSA (10 mg/ml), was added to the binding-reaction mixtures, which were then gently rocked at room temperature for 1 h. The streptavidin gel was washed three times with 1 ml of DNA-binding solution, resuspended in Laemmli sample buffer, split into two equal fractions, and analyzed by SDS-PAGE followed by immunoblotting with anti-MBP or anti-GST (Santa Cruz Biotechnology, Inc.) antiserum.

RESULTS

Coexpression of Nkx-2.5 and SRF activates the avian cardiac α-actin promoter and endogenous cardiac α-actin gene activity in 10T1/2 fibroblasts. SRF alone can induce modest activation of the skeletal α-actin promoter in differentiation blocked myoblasts (42), and, like the closely related cardiac α-actin promoter, both promoters required intact SREs for activity (13, 41, 52, 54). Additional cofactors might be required to drive these actin promoters optimally, as well as to specify cell type-restricted expression. Recently, we showed that ectopic expression of Nkx-2.5 and SRF could stimulate the cardiac α-actin promoter activity in C3H10T1/2 (10T1/2) fibroblasts (9) but failed to activate other SRE-laden promoters such as those for the skeletal α-actin and c-*fos* genes (data not shown). We asked if another homeodomain factor such as MHox, a murine homolog of human Phox-1 (16, 26), could substitute for Nkx-2.5 in cotransfection assays. As shown in Fig. 1, MHox transfections with the cardiac α-actin promoter did not stimulate reporter gene activity. MHox cotransfections with SRF failed to coactivate the cardiac actin promoter and inhibited SRF-stimulated promoter activity. These results were in contrast to the Nkx-2.5- and SRF-codirected 15-fold increase in cardiac α-actin promoter activity under identical conditions (Fig. 1). Thus, the failure of MHox, a paired-like homeodomain factor, to stimulate the cardiac α-actin promoter demonstrates that homeodomain factors are not functionally equivalent. These results suggest that Nkx-2.5 and SRF cotransactivation was restricted to the cardiac α-actin promoter and that the transcriptional synergy on the cardiac actin promoter with SRF may be limited to a specific class of homeodomain factors.

To investigate whether expression of Nkx-2.5 and SRF was capable of activating the endogenous cardiac α-actin gene, Nkx-2.5 and SRF expression constructs were transfected into 10T1/2 fibroblasts to obtain pools of stable transfectants. Specific gene activation was analyzed by reverse transcriptase PCR with gene-specific primers. As shown in Fig. 2, cardiac α-actin gene transcripts were detected in the Nkx-2.5- plus SRF-transfected pooled fibroblasts, whereas cardiac α-actin mRNA was not detected in the parental 10T1/2 fibroblasts, control fibroblasts transfected with the empty pCGN expression vector, or

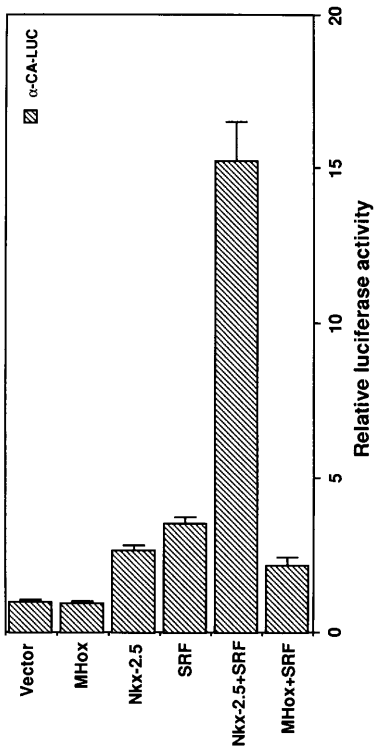

FIG. 1. MHox, a murine homolog of human Phox-1, fails to coactivate the cardiac actin promoter and inhibits SRF-stimulated promoter activity. α-CA-LUC was cotransfected with an Nkx-2.5 or MHox expression vector alone or in combination with SRF into 10T1/2 cells. MHox, a paired-liked homeodomain factor, was ineffectual in transactivating the cardiac α-actin promoter, while Nkx-2.5 and SRF caused about a 15-fold increase in cardiac α-actin promoter activity under identical conditions. Results are means of two independent duplicate transfection experiments normalized to β-galactosidase activity, an internal standard. The relative value of each promoter obtained with an empty expression vector was set at 1.

fibroblasts transfected with only either pNkx-2.5 or pSRF. These results show that transcripts emanating from the endogenous cardiac α-actin gene can be increased by elevated levels of Nkx-2.5 and SRF and lend further support to the notion that Nkx-2.5 and SRF might be endogenous activators of cardiac α-actin gene transcription.

Endogenous Nkx-2.5-like SRE-binding activity could be detected in cardiac nuclear extracts and in Nkx-2.5-transfected C2C12 cells. How might Nkx-2.5 and SRF collaborate to stimulate cardiac α-actin gene activity? We conducted EMSA with cardiac cell nuclear extracts prepared from 12-day-old primary chicken cardiomyocytes to examine the distribution of cardiac nuclear protein complexes that bound cardiac actin SREs. SRF-binding complexes were previously detected on four SREs, as demonstrated by Moss et al. (54) and shown in Fig. 3A, and endogenous SRF bound efficiently to the proximal SRE1 and the distal SRE4 which adhered to the consensus SRE sequence [CC(A/T)$_6$GG]. The most rapid migratory complex was detected on the second and third SREs, which correlated with YY1-binding activity. Other specific binding complexes, including an intermediate migrating doublet, were also detected with the cardiac actin SRE2 oligonucleotide (Fig. 3A, lane 3; Fig. 3B, lane 1). The identity of these complexes was further investigated by a competition EMSA in which the binding complexes formed with a cardiac actin SRE2 probe were challenged with various cold competitors (Fig. 3B). The fastest complex was efficiently inhibited by a self competitor or a skeletal actin SRE1 (SkSRE1) oligonucleotide which has been shown to be a strong YY1-binding site (28, 41), suggesting that it was contributed by YY1. The two intermediate-mobility complexes were only slightly inhibited by a 200-fold excess of SkSRE1 oligonucleotide; however, they were efficiently inhibited by a self competitor or an Nkx-2 consensus oligonucleotide that was believed to be recognized by the Nkx-2 class of homeodomain proteins (10), suggesting that these two complexes were most probably contributed by Nkx-2-related homeodomain factors. The Nkx doublet observed in the EMSA of cardiac cell extracts might be due to related Nkx-2.3 and/or Nkx-2.6 proteins that are expressed in embryonic cardiac myocytes. Since we do not yet have a reliable antibody that unequivocally recognizes avian Nkx-2.5, we suggest, on the basis of specific competition and migratory patterns, that the endogenous shift appears to be Nkx-2.5 like. Indeed, one of the intermediate-mobility complexes comigrated with the binding complex formed with the murine Nkx-2.5 protein that was enriched in C2C12 cells transfected with a murine HA-epitope-tagged Nkx-2.5 expression construct (Fig. 3C, lane 1). Lower levels of a comigrating shifted species were observed in the EMSA of an extract taken from C2C12 cells mock transfected with the empty plasmid expression vectors (pCGN; Fig. 3C, lane 2). YY1- and SRF-binding complexes were present in both transfected and control C2C12 cell extracts.

To examine the SRE-binding preferences of the Nkx-2.5 protein expressed in vivo, EMSA mixtures with nuclear extracts prepared from Nkx-2.5-transfected C2C12 nuclear extracts were incubated with a labeled CaSRE2 probe and competed against the four cardiac actin SRE oligonucleotides. Binding competition against SRE2 occurred at a competition/probe molar ratio of 50 and revealed that the c-*fos* SRE competed efficiently for Nkx-2.5 binding as well as SRE2 did, whereas cold SRE1, SRE3, and SRE4 oligonucleotides did not show significant competition for Nkx-2.5 binding (Fig. 3D). These results suggested that endogenous Nkx-2.5-binding activity preferentially bound SRE2 and the c-*fos* SRE sites at low protein inputs. Since cellularly expressed Nkx-2.5 was HA epitope tagged (HA–Nkx-2.5), specific Nkx-2.5-binding activity was confirmed by being diminished selectively by antibodies

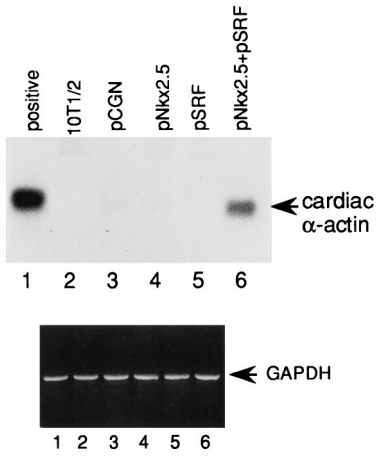

FIG. 2. Nkx-2.5 and SRF activate the endogenous cardiac α-actin gene in 10T1/2 fibroblasts. (A) Activation of the endogenous α-cardiac actin mRNA by overexpression of Nkx-2.5 and SRF in 10T1/2 cells. Steady-state cardiac actin mRNA levels expressed in a variety of stable transfectants (indicated above each lane) were analyzed by reverse transcriptase PCR. A positive control was represented by RNA isolated from an adult mouse heart. The GAPDH gene served as an internal control.

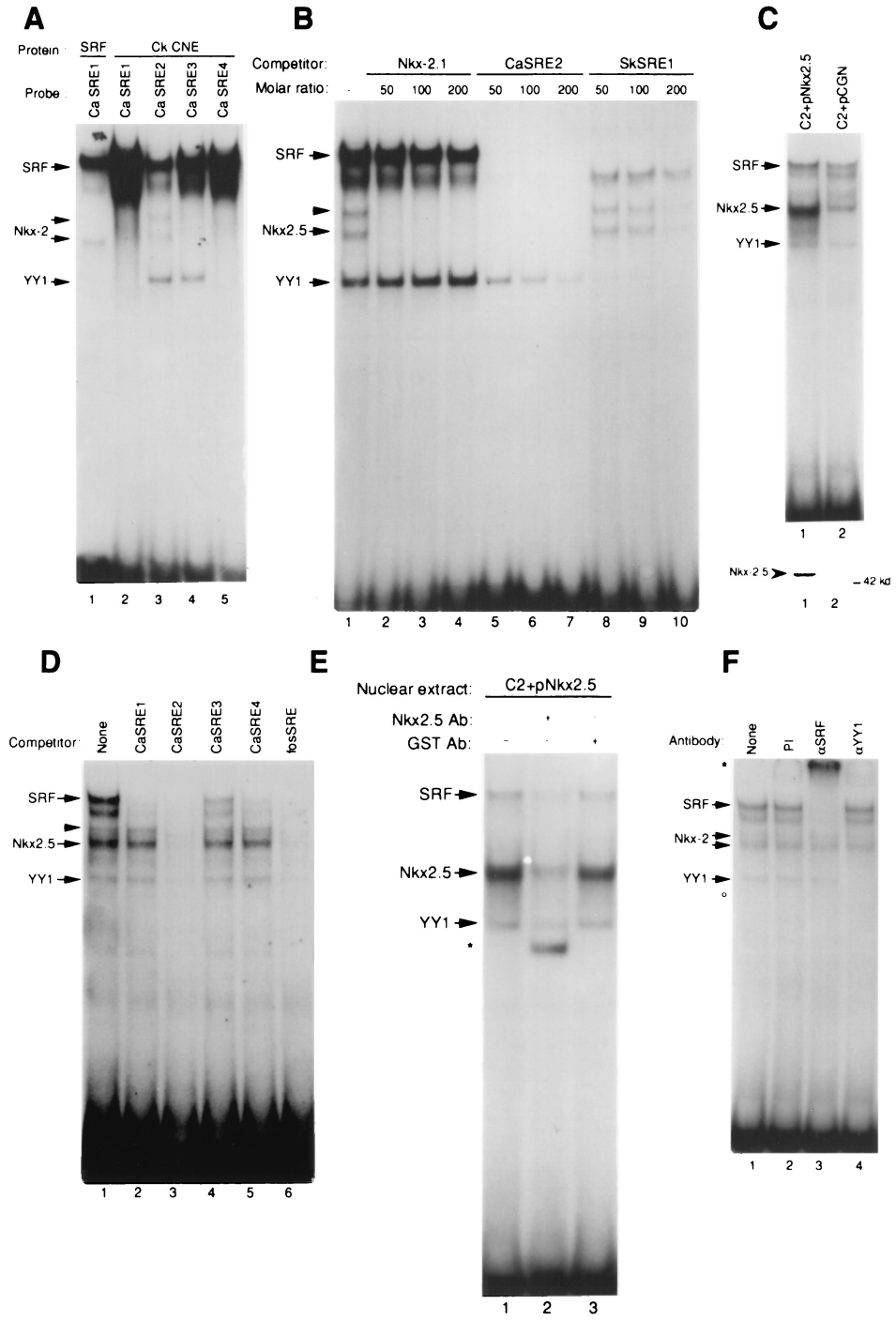

FIG. 3. Binding of the endogenous Nkx-2.5 protein to SREs. (A) Bacterially expressed SRF (lane 1) and chicken nuclear extracts (Ck CNE) (10 μg; lanes 2 to 5) were used in an EMSA with cardiac actin ^{32}P-end-labeled paired SRE oligonucleotides. (B) Specific binding of distinct nuclear proteins to the cardiac actin SRE2. Cardiac nuclear extracts (10 μg) were incubated with a labeled cardiac actin SRE2 oligonucleotide. Various unlabeled oligonucleotides were added to the EMSA mixtures at three competitor/probe molar ratios, indicated above each lane. The identity of each protein-DNA complex is indicated on the left (arrows). A binding complex which may represent an Nkx-2-related homeodomain factor is indicated by an arrowhead. (C) Comparison of Nkx-2.5-transfected C2C12 cell (C2+pNkx-2.5; 5 μg) and control C2C12 cell (C2+pCGN; 5 μg) nuclear extracts in EMSA with a CaSRE2 probe. Expression of the HA epitope-tagged Nkx-2.5 protein in C2C12 cells (20 μg; lane 1) and control C2C12 cells (20 μg; lane 2) is shown in the bottom panel by immunoblotting with anti-HA antibodies. (D) Cardiac actin SRE2 probe-binding complexes from cardiac nuclear extracts were challenged with unlabeled competitors (indicated above each lane) at a 50-fold molar excess. The Nkx-2.5-binding activity was significantly reduced by the addition of CaSRE2 and c-*fos* SRE oligonucleotides. (E) C2C12-transfected DNA-binding complexes containing expressed HA epitope-tagged Nkx-2.5 (lane 1) were challenged with anti-HA antibodies (Ab) (lane 2) and with control anti-GST antibodies (lane 3). (F) C2C12 DNA-binding complexes were challenged with preimmune (PI) sera (lane 2), anti-human SRF antibodies (lane 3), and anti-YY1 antibodies (lane 4).

TABLE 1. Conservation of the Nk-2 homeodomain DNA-binding targets

Homeobox gene	Target gene	Sequence[a]
Nkx-2.1	Rat thyroglobulin A	−160 TACTCAAGTA −151
	B	−133 GACTCAAGTA −123
	C	−69 CAGTCAAGTG −59
	Human surfactant protein B 5′SPB-f1	−101 CCCTCCAGGT −110
	3′SPB-f1	−100 CTCTGAAGAG −91
Ceh-22	*C. elegans* Myo-2 enhancer B207	CGCTAAAGTG
Nkx-2.5	Consensus	TNAAGTG C(A/T)TAATTN
	Avian cardiac α-actin SRE2	−95 CATTCATGGG −85

[a] Sequences are numbered relative to the start sites of transcription from their respective genes. Sources for Nk-2 binding sequences are as follows: rat thyroglobulin gene (14), mouse surfactant protein B gene (7), *C. elegans* Myo-2 gene (55), Nkx-2.5 consensus (10), and avian cardiac α-actin gene (8, 64).

raised against the flu antigen (Fig. 3E). In Fig. 3F, a supershift with anti-SRF confirmed the identity of SRF-shifted complexes while selective inhibition of YY1 binding complexed with anti-YY1 antibodies corroborated the identity of YY1.

Comparisons of the strong SRE2-binding sequences with well-documented Nk-2 binding factors were made, as shown in Table 1. The consensus Nkx-2.1-binding sequence, 5′-CCACT CAAGT-3′, was quite similar to the SRE2 5′-CCATTCATG G-3′, thus resembling a preferred Nkx-2.5 site rather than the weaker 5′-TTAATT-3′ binding core. In addition, CaSRE2 served as a binding site for at least three distinct nuclear factors, namely, SRF, Nkx-2.5, and YY1, which were present in nuclear extracts prepared from chicken cardiomyocytes and Nkx-2.5-transfected cells.

Nkx-2.5 associated with SRF independently of DNA binding. We asked if the synergy displayed by Nkx-2.5 and SRF transactivation of a downstream target gene, such as cardiac α-actin, was mediated at the level of protein-protein associations. In a previous study, Phox-1–SRF–DNA interactions (26) could not be observed by ternary-complex formation during native gel electrophoresis. Similarly, the formation of a ternary complex on an SRE with SRF and Nkx-2.5 was either transient or unstable and could not be observed under EMSA conditions (reference 9 and data not shown). Thus, the GST pull-down assay of Shore and Sharrocks (70) was used to investigate the interaction of Nkx-2.5 with SRF in solution. A fusion protein between SRF and the carboxyl terminus of GST was expressed in bacteria, purified, and immobilized onto glutathione-agarose beads. The immobilized GST-SRF or GST was incubated with in vitro-translated and ^{35}S-labeled Nkx-2.5 protein and washed with the incubation buffer. The bound material was eluted and analyzed by SDS-PAGE. As shown in Fig. 4A, the ^{35}S-labeled Nkx-2.5 protein was retained by the GST-SRF resin either in the absence (lane 3) or in the presence (lanes 4 and 5) of the cardiac α-actin SRE oligonucleotide but not by the GST resin (lane 2). However, another control, the ^{35}S-labeled MyoD, was not retained by the GST-SRF resin (lane 6), which indicated that the Nkx-2.5 protein specifically interacted with SRF.

Could cellular lysates containing expressed Nkx-2.5 protein selectively bind with SRF? Immobilized GST-SRF was incubated with cell lysate from 10T1/2 cells, which had been transfected with a Nkx-2.5 expression vector expressing the HA epitope-tagged Nkx-2.5 protein. After extensive washings, the bound material was eluted and analyzed by SDS-PAGE and immunoblotting with an anti-HA monoclonal antibody. As shown in Fig. 4B, GST-SRF selectively bound Nkx-2.5 protein (lane 4) whereas GST polypeptides failed to retain Nkx-2.5 (lane 5). Since these results indicated that SRF and Nkx-2.5 were able to establish protein-protein contacts in solution, we then asked if Nkx-2.5 and SRF could form complexes in vivo. The HA epitope-tagged mouse Nkx-2.5 protein (HA–Nkx-2.5) was expressed in primary chicken cardiomyocytes by transient transfection. Affinity-purified polyclonal rabbit antiserum to a peptide (amino acids 404 to 418) conserved among all known vertebrate SRFs was used to immunoprecipitate SRF and any associated proteins from the transfected cardiac myocyte cell extracts. The resulting immunoprecipitates were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and subjected to protein immunoblot analysis with the anti-HA monoclonal antibody. The SRF antiserum coimmunoprecipitated HA–Nkx-2.5 from the transfected cardiomyocytes (Fig. 4C, lane 5); however, the YY1 antiserum failed to coimmunoprecipitate HA–Nkx-2.5 (lane 6). The authenticity of the immunoreactive species corresponding to HA–Nkx-2.5 was demonstrated by (i) an immunoreactive species present in the Nkx-2.5-transfected 10T1/2 cell extracts (lane 2) and chicken cardiac cell extracts (lane 3) but absent in nontransfected chicken cardiac cell extracts (lane 1) and (ii) an immunoreactive species in the precipitates precipitated with the anti-HA antibody (lane 4). Taken together, our results indicated that Nkx-2.5 and SRF form complexes in cardiomyocytes.

Protein-protein interactions between Nkx-2.5 and SRF mapped to the basic region of SRF and the homeodomain of Nkx-2.5. Several mutated proteins were made to define SRF domain that interacts with Nkx-2.5. Removal of the C-terminal (amino acids 171 to 508) and N-terminal 142 amino acids of SRF retained the ability of SRF to interact with Nkx-2.5 (Fig. 5A, lanes 4 to 6). However, removal of amino acids 46 to 244, which overlaps the MADS box of SRF, impaired the capability of SRF to bind Nkx-2.5 (lane 7). Comparison of the binding activities of the SRF mutants SRF(1–171), and SRF(142–508) with SRF(46–244) suggested that amino acids 142 to 171 of SRF was required for Nkx-2.5 binding. Use of an additional truncated SRF mutant (shown in Fig. 5B) revealed that the polypeptide between amino acids 142 and 171 of SRF was responsible for associating with Nkx-2.5. We next wanted to identify the domain of Nkx-2.5 that was required for SRF interaction. The wild-type and truncated Nkx-2.5 were expressed as bacterial proteins fused to MBP. MBP–Nkx-2.5 fusion proteins were purified by amylose chromatography and incubated with GST-SRF(1–508) immobilized on glutathione-agarose beads. The bound proteins were analyzed by SDS-PAGE and blotted with antiserum against MBP. All the N-terminal and C-terminal truncations of Nkx-2.5 protein, which retained the homeodomain region (amino acids 122 to 203) (45), were able to interact with SRF (Fig. 6A, lanes 3 to 14). As a control, MBP did not interact with SRF (lane 2), indicating that amino acids 122 to 203 of the Nkx-2.5 protein was necessary and sufficient for the Nkx-2.5 to contact SRF.

The homeodomain of a homeobox gene can be divided into four subdomains (37), the N-terminal arm, helix 1, helix 2, and helix 3. Recent nuclear magnetic resonance and X-ray-crystallographic studies on the protein-DNA complex of three homeodomains have shown that the N-terminal arm and the third

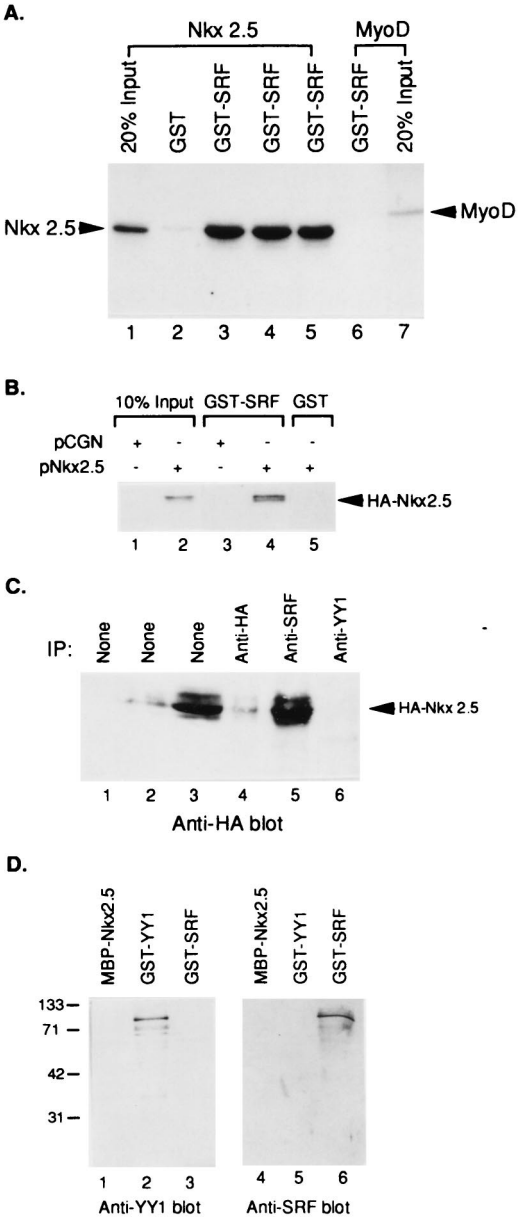

FIG. 4. Physical association between SRF and Nkx-2.5 in the absence of DNA. (A) SRF was expressed as a GST fusion protein (GST-SRF) in bacteria and immobilized onto glutathione-agarose beads. In vitro-translated ^{35}S-labeled Nkx-2.5 (lanes 1 to 5) or MyoD (lanes 6 and 7) was incubated with GST (lane 1) or GST-SRF resin in the absence (lane 3) or presence (lanes 4 [0.1 pmol] and 5 [0.5 pmol]) of a double-stranded cardiac actin SRE2 oligonucleotide. Labeled proteins (20%) are also shown (lanes 1 and 7). After binding, the bound proteins were analyzed by SDS-PAGE (12% polyacrylamide) followed by fluorography. (B) Cell lysates (300 μg), prepared from 10T1/2 cells transfected with either a pCGN empty vector (lane 3) or an Nkx-2.5 expression vector (lanes 4 and 5), expressing an HA epitope-tagged Nkx-2.5 protein, were incubated with GST-SRF resin (lanes 3 and 4) or GST resin (lane 5) and processed for binding reactions as described in Materials and Methods. The bound proteins were analyzed by SDS-PAGE followed by immunoblotting with an anti-HA monoclonal antibody. Cell lysates (10%) were loaded in lanes 1 and 2. (C) Association of Nkx-2.5 with SRF in vivo. Primary chicken cardiomyocytes were transfected with pCGN–Nkx-2.5, and extracts (500 μg) from these transfected cardiomyocytes were then immunoprecipitated with anti-HA (lane 4), anti-SRF (lane 5), or anti-YY1 (lane 6) antisera. Products of the immunoprecipitations (IP) were resolved by SDS-PAGE and subjected to protein immunoblot analysis with the anti-HA antibody. Lane 1 contains the extracts (50 μg) from untransfected cardiomyocytes as a negative control. Lane 2 contains extracts (10 μg) from 10T1/2 cells transfected with pCGN–Nkx-2.5 plasmid as an indication of HA-tagged Nkx-2.5 protein. Lane 3 represents 10% of the extracts used for lanes 4 to 6. The weaker interaction of HA–Nkx-2.5 with anti-HA antibody in lane 2 was due to loading of less cell extract (10 μg). (D) Specific interactions of anti-YY1 and anti-SRF antibodies with their cognate proteins. Affinity-purified bacterial proteins, MBP–Nkx-2.5 (50 ng; lane 1), GST-YY1 (50 ng; lane 2), and GST-SRF (50 ng; lane 3), were separated by SDS-PAGE and probed with anti-YY1 (lanes 1 to 3) or anti-SRF (lanes 4 to 6) antiserum used for immunoprecipitation in panel C. These antisera did not cross-react with the Nkx-2.5 protein.

helix of the homeodomain make specific contacts with bases in the minor and major grooves of DNA, respectively (35, 57, 80). The roles of the first and second helices are yet to be defined, although they are likely to mediate interactions with other proteins (27, 81). Therefore, which subdomains of the Nkx-2.5 homeodomain bind to SRF? Several truncations of the Nkx-2.5 homeodomain, which all contained MBP and a 12-amino-acid spacer, were constructed such that MBP–Nkx-2.5(122–160) encompassed the N-terminal arm and helix I of the homeodomain and MBP–Nkx-2.5(162–203), contained helix II and III of the homeodomain. These Nkx-2.5 homeodomain mutants were incubated with the immobilized GST-SRF(142–171), and the bound proteins were visualized by immunoblotting with anti-MBP antiserum. MBP–Nkx-2.5(122–160) bound to SRF as well as MBP–Nkx-2.5(122–203) did (Fig. 6B, lane 6). A significant amount of MBP–Nkx-2.5(162–203) was retained by the GST-SRF resin, although it appeared to bind less efficiently to SRF than did the other mutants (lane 4). The control protein, MBP plus the 12 amino acids, was not bound by SRF (lane 8). Our results suggest that two separable subdomains of the Nkx-2.5 homeodomain, the N-terminal arm or the first helix and the second or third helix, mediate protein-protein interactions with SRF.

Nkx-2.5 and SRF synergistically transactivate a reporter gene consisting of a single cardiac α-actin SRF-binding site. It was also important to determine the biological significance of protein-protein interactions shared between Nkx-2.5 and SRF proteins. For this purpose, transient-transfection experiments were performed with 10T1/2 cells by using a reporter gene (CaSRE1-TATA-LUC), which contains the avian cardiac α-actin promoter sequence from −100 to +15, consisting of a single SRE1 element upstream from the TATA box. This promoter was cotransfected with various combinations of Nkx-2.5 and SRF expression constructs into 10T1/2 fibroblasts (Fig. 7A). When an Nkx-2.5 or SRF expression vector was cotransfected with the reporter, a three- to fourfold increase in activity was observed. However, the luciferase activity was elevated about 15- to 20-fold with the combination of the Nkx-2.5 and SRF expression vectors. Inclusion of a DNA-binding-defective Nkx-2.5 mutant (Nkx-2.5pm), which was incapable of binding DNA (Fig. 7B) because of an Asp-to-Gln substitution in the DNA recognition helix III of the homeodomain but still retained the ability to bind to GST-SRF resin (data not shown), activated the minimal promoter together with the SRF expression vector to the same extent as did the wild-type Nkx-2.5. These data demonstrated that binding of Nkx-2.5 to any cryptic sites in the plasmid could not be responsible for the transcriptional synergy. The dominant-negative SRF (SRFpm1), which does not bind to SREs, did not support functional cooperation with the Nkx-2.5 protein, indicating that DNA-binding activity of SRF to SRE is primary and necessary for the cotransactivation activity. These results were consistent with our previous results, in which we showed that cotransactivation by SRF and Nkx-2.5 was dependent upon a single intact SRE1 (9). The SRE1 conversion from CCAAATAAGG to CCAAAGATCT, which eliminated paired G contacts required for SRF binding, prevented the cotransactivation of this promoter construct (9).

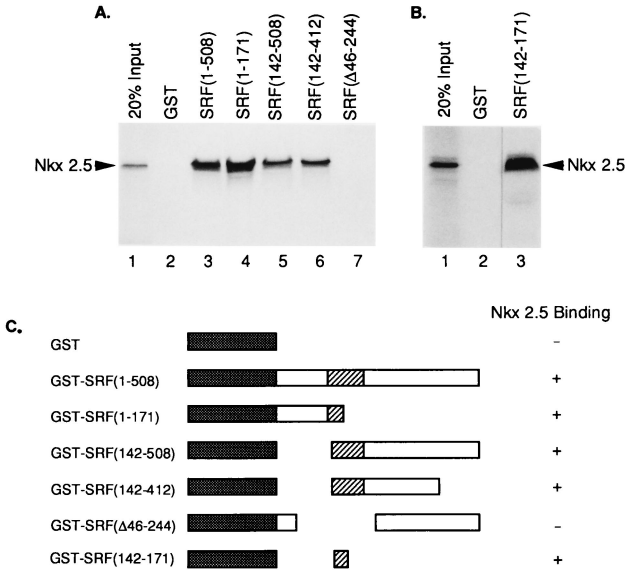

FIG. 5. Identification of the Nkx-2.5 interaction domain of SRF. (A and B) The same amounts of various truncated forms of GST-SRF fusion proteins (1 μg) were treated as in the binding reactions described in Fig. 6A, but in the absence of SRE2 oligonucleotide. (C) Schematic diagram of the SRF deletion mutants and a summary of the observed binding activity. The hatched boxes represent the MADS box and dimerization domain (amino acids 133 to 222) of SRF.

The synergy between SRF and Nkx-2.5pm on the test promoter suggested that DNA-bound SRF could interact with the Nkx-2.5 protein in the absence of Nkx-2.5 DNA-binding activity. Thus, DNA affinity chromatography, in which SRF–Nkx-2.5 complexes were loaded onto a biotin-labeled cardiac actin SRE1 oligonucleotide immobilized on a streptavidin gel, was used to further explore this possibility (Fig. 7C). While the wild-type Nkx-2.5 protein was retained by SRE oligonucleotide, perhaps as a result of its ability to bind DNA (lane 4), Nkx-2.5pm was not retained by the gel (lane 5), indicating its incapability of binding to SRE oligonucleotide. However, when Nkx-2.5pm was incubated with SRF in the presence of SRE, it was retained by the streptavidin gel as well as the wild-type Nkx-2.5 protein (lanes 7 and 8) was, suggesting that binding of Nkx-2.5pm to the gel occurred through its interaction with SRF. As controls, MBP did not interact with SRE-SRF complexes (lane 6) and MBP–Nkx-2.5 did not interact with the streptavidin gel in the absence of SRE either with or without SRF (lanes 1 and 2). Taken together, these results show persuasively that SRF can recruit Nkx-2.5 to the promoter in the absence of Nkx-2.5-binding activity. Such protein-protein interactions might generate a ternary complex that synergistically activates transcription, thus providing further support that the Nkx-2.5/SRF interaction indeed takes place in vivo.

DISCUSSION

The developmental programs in vertebrate cardiac muscle, *Drosophila* cardiac and visceral muscle, and *C. elegans* pharyngeal muscle all occur without myogenic basic helix-loop-helix factors related to MyoD (18, 38, 50). The recent homologous recombination knockouts of the endogenous murine Nkx-2.5 gene caused embryonic death (46), suggesting that Nkx-2.5 was essential for normal heart morphogenesis and that this gene is a component of a genetic pathway required for myogenic specialization of the ventricles. The regulatory role of Nkx-2.5 appears to be conserved across vertebrate species, in which the expression pattern of *Xenopus laevis XNkx-2.5* also coincided with the appearance of cardiac progenitor cells, which converged to form the presumptive heart, and continued to be expressed in differentiated heart tissue (74). An attractive hypothesis from the analysis of these NK-2 homologs is that these

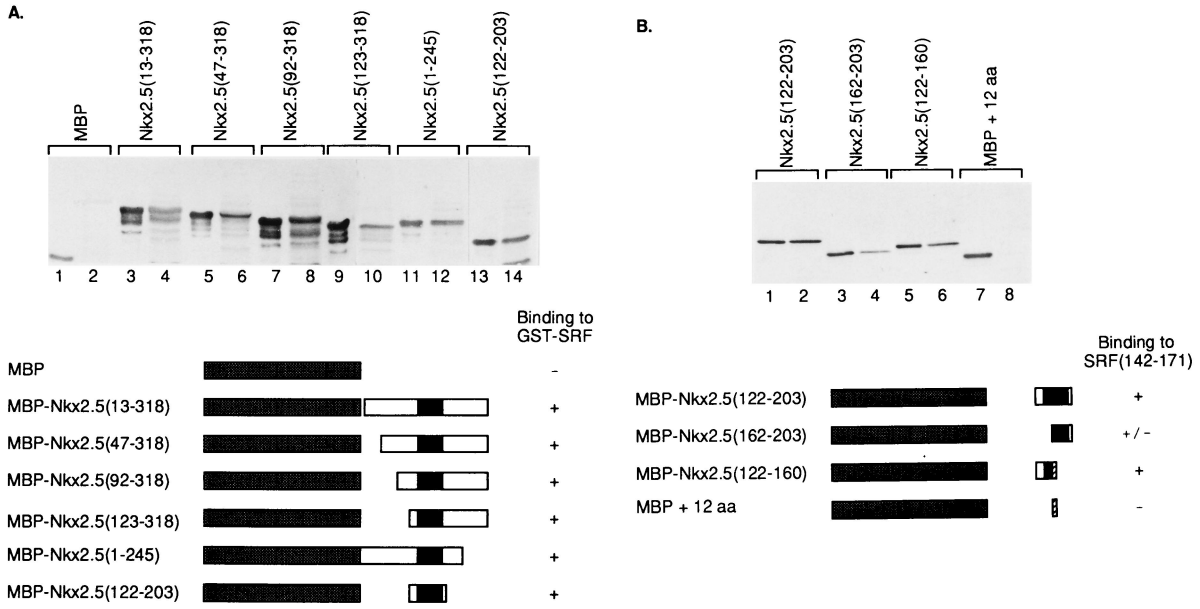

FIG. 6. Identification of the SRF interactive domain of Nkx-2.5. (A) Various truncations of affinity purified MBP–Nkx-2.5 protein (300 ng) were incubated with GST-SRF resin (1 μg). The bound proteins were visualized by immunoblotting with an anti-MBP antiserum. Odd-numbered lanes contain 20% of input proteins, and even-numbered lanes contain the bound MBP–Nkx-2.5 proteins. A schematic diagram of the Nkx-2.5 deletion derivatives used and summary of the interaction results obtained is shown below the gel. (B) Various truncations of the MBP–Nkx-2.5 homeodomain were incubated with 1 μg of GST-SRF(142–171) resin. The homeodomain is indicated by the solid boxes; hatched boxes indicate an additional unrelated 12 amino acids resulting from the construction.

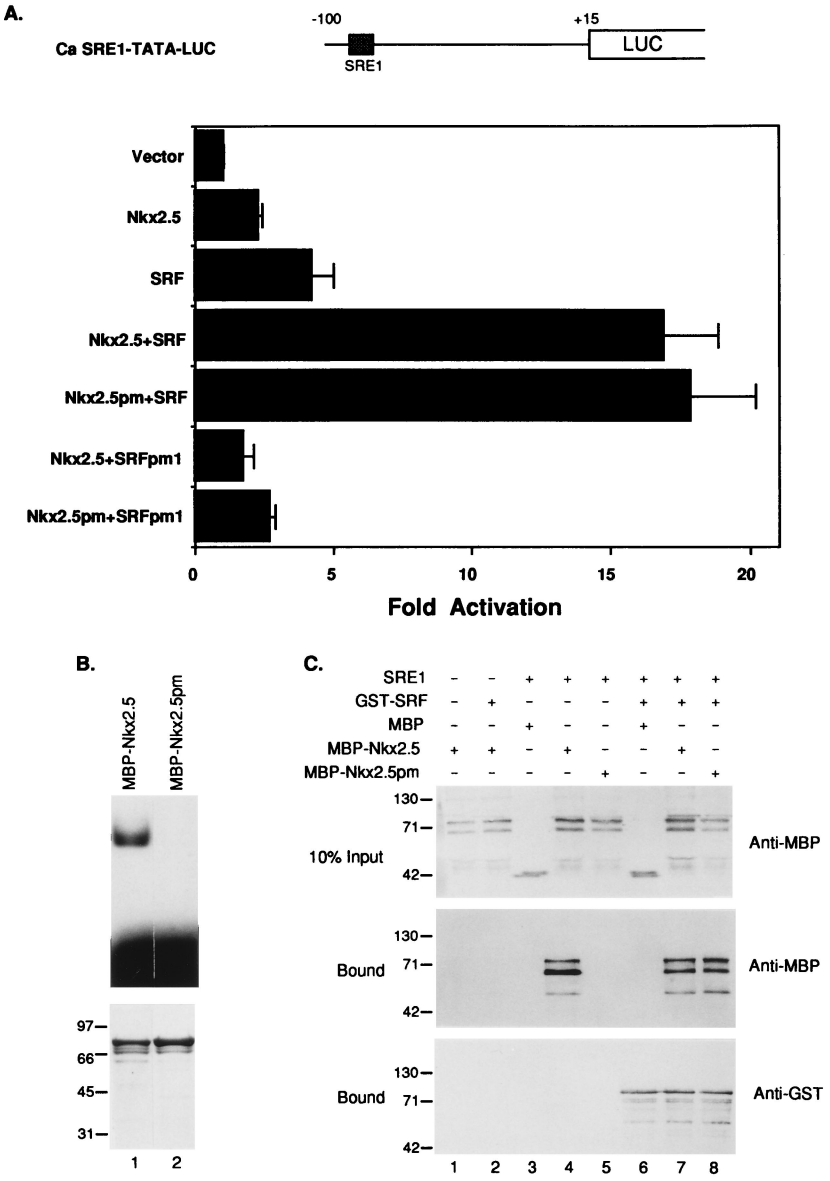

FIG. 7. Transcriptional synergy between Nkx-2.5 and SRF on a minimal cardiac α-actin SRE1-containing promoter. (A) A reporter gene (shown at the top of the figure), containing the avian cardiac α-actin promoter from −100 to +15, was cotransfected with various combinations of expression constructs (indicated on the left) into 10T1/2 cells. Values are presented as fold activation, in which the luciferase activity obtained with empty expression vectors was set at 1, and are representative of three independent triplicate transfection experiments. (B) Nkx-2.5pm does not bind to SRE. Purified bacterial MBP–Nkx-2.5 or MBP–Nkx-2.5pm (250 ng) was used in EMSA with a labeled cardiac actin SRE2 oligonucleotide (top panel). The purified proteins (750 ng) were also analyzed on an SDS-polyacrylamide gel and detected by Coomassie blue staining (bottom panel). (C) Recruitment of Nkx-2.5 by SRF to SRE in the absence of Nkx-2.5 DNA binding. Purified MBP (lanes 3 and 6), MBP–Nkx-2.5 (lanes 4 and 7), and MBP–Nkx-2.5pm (lanes 5 and 8) were incubated with GST (lanes 3 to 5) or GST-SRF (lanes 6 to 8) in the presence of biotin-labeled cardiac actin SRE1 oligonucleotide and streptavidin gel. The bound protein was analyzed by SDS-PAGE and immunoblotting. As controls, streptavidin gel and MBP–Nkx-2.5 were also incubated with (lane 2) or without (lane 1) SRF in the absence of biotin-labeled SRE1. The top panel shows 10% input of each MBP fusion probed with anti-MBP antiserum, and the bottom two panels show bound proteins probed with anti-MBP or anti-GST antiserum. The doublet of MBP–Nkx-2.5 proteins was due to degradation of MBP fusions while expressed in bacteria.

homeodomain factors function in a phylogenetically conserved pathway in muscle cell types that do not utilize the MyoD family. Ectopic expression of Nkx-2.5 in 10T1/2 fibroblasts demonstrated that downstream targets such as the cardiac α-actin gene was not directly activated by Nkx-2.5 alone but required the collaboration of additional factors, such as SRF (9). Whether the vertebrate Nkx-2.5 or other Nkx-2-related genes with SRF are sufficient to play the primary role in heart specification and serve as regulators of other downstream cardiac genes remains to be determined. We believe that the MADS box family-related *Drosophila* dMEF-2 (44) and vertebrate MEF-2C (47) genes and the zinc finger-containing GATA-4 factor (2, 25) are also well suited to be high in the hierarchical order of regulatory factors that, in combination with Nkx-2.5 and SRF, may specify the cardiac cell lineage.

We observed that Nkx-2.5, together with SRF, elicited robust activation of the α-cardiac actin promoter in 10T1/2 fibroblasts. Previous analysis of the cardiac α-actin promoter mutants indicated that maximal transcriptional activity required multiple intact upstream SREs (54). The deletion mu-

tant which removed three of the four SREs caused a 70% reduction in the overall cotransfection activity in primary myoblast cell culture (64). The proximal SRE1 appeared to play the central role in regulating SRF–Nkx-2.5-dependent promoter activity, as shown in Fig. 7A and demonstrated by the complete loss of promoter function by the mutated SRE1 site in cardiac myocytes (54) and in the cotransfection assays with SRF–Nkx-2.5 in 10T1/2 fibroblasts (9). The avian cardiac α-actin promoter SRE2 was among the most efficient binding sites for Nkx-2.5 but was also among the weakest SRF-binding sites (9, 11). Pollock and Treisman (59) have shown that the affinity of SRF for $CC(A/T)_6GG$ elements can be reduced up to 10-fold by mutations that introduce deletions in the central $(A/T)_6$ core or by substitutions of the $(A/T)_6$ core with CG base pairs. Comparison of the strong SRE2 binding sequence was made with well-documented NK-2-binding factors, as shown in Table 1. Alignment of the binding sequences of the *C. elegans ceh*-22 factor (54), the 5′ and 3′ lung surfactant promoter factor SPB-f1-binding sites (7), and consensus TTF-1 or Nkx-2.1 site (29) with the avian cardiac actin SRE2 (8, 64) revealed remarkable sequence similarities. The consensus Nkx-2.1-binding sequence 5′-CCACTCAAGT-3′ was quite similar to SRE2 5′-CCATTCATGG-3′, thus resembling a preferred Nkx-2.5 site rather than the weaker 5′-TTAATT-3′ binding core. Interestingly, the placement and organization of the cardiac actin α-promoter SREs are conserved across the evolution of birds and mammals, in which the first and fourth SREs adhere to the strict $CC(A/T)_6GG$ consensus sequence while all of the SRE2 elements contained a single G · C base pair substitution in the $(A/T)_6$ core (73). Thus, conservation of a G · C base pair substitution in these SREs might have played an ancient regulatory role to enhance Nkx-2.5 binding to the cardiac α-actin promoter.

Although we were unable to observe the co-occupation of the SRF–Nkx-2.5 complex on a single SRE by gel mobility shift assays, their mutually inclusive binding to SRE suggested that it is possible that these factors reside simultaneously on SREs. The recent elucidation of the X-ray crystal structure of the SRF core bound to DNA provides an explanation (58). A novel DNA-binding motif, a coiled-coil, formed by the MADS box αI helices (amino acids 153 to 179) lies parallel to and on top of a narrow DNA major groove, making contacts with the phosphate backbone on an SRE half site, while an unstructured N-terminal extension from the αI helix (amino acids 132 to 152) makes critical base contacts in the minor groove. Dimerization of the MADS box occurs above the αI helix by a structure composed of two β-sheets in the monomer that interact with the same unit in its partner. A second αII helix in the C-terminal portion of the MADS box, stacked above these β-sheets, completes this stratified structure and is involved with Elk-1 binding. Bending of the DNA toward the protein allows the N-terminal part of each α-helix to make groove contacts at the edge of the SRE, thus leaving an unobstructed major groove in the center of the SRE and allowing for simultaneous interaction of the SRF with homeodomain proteins such as Phox-1 and Nkx-2.5.

Comparison of the solvent-exposed amino acid side chains on helices 1 and 2 of Phox-1 with those of Nkx-2.5 indicated that amino acid residues in the first helix required for interaction with SRF are conserved but the amino acid residues in the second helix are divergent (data not shown). Possibly, sequence differences in the second helix provide subtle differential interactions with the subdomain regions of the MADS box to affect protein interactive specificity, affinity to the DNA-binding site, and perhaps even growth factor signaling. For example, the DNA-binding activity was required for Phox-1 function in vivo, but the DNA-binding activity of Nkx-2.5 to SRE was not necessary for its function (Fig. 7). Coassociation of Nkx-2.5 and SRF on the SRE might be stabilized through a short peptide encompassing the amino terminus and helix 1 and perhaps helix 2 within the homeodomain of Nkx-2.5, which was shown to be sufficient to mediate protein-protein contacts in solution with a portion of the unstructured N-terminal extension motif (amino acids 142 to 171) of SRF. Since homeodomain proteins appear to bind target sequences as monomers rather than dimers (20, 68), the co-occupation of the SRE probably occurs through the mutual interaction of an Nkx-2.5 monomer with an SRF dimer. This view was confirmed by cross-linking experiments done in solution with truncated SRF and Nkx-2.5 proteins (data not shown).

How is specific gene transcription generated from SRF-homeodomain complex interactions? In *S. cerevisiae*, MCM1 functions by recruitment of an array of homeodomain accessory factors to activate and repress genes in a cell-type-specific and temporal pattern (32, 34). Whether MCM1 acts as an activator or repressor of transcription depends on the identity of an associated factor. Therefore, we believe that in analogous manner to MCM1, SRF serves to mediate accessory factor interactions with certain classes of homeodomain factors. For example, under cellular growth conditions which spur cell replication but inhibit cell differentiation processes, SRF, Phox-1, and Elk-1 were shown to form a tertiary complex on the c-*fos* SRE, which was stabilized by Elk-1 5′ DNA contact sites (AGGA). The region in SRF necessary for the formation of the ternary complex with Elk-1 was mapped to the C-terminal half of the MADS box (amino acids 175 to 217) (68, 69), in which dimerization of SRF was a prerequisite for the formation of the Elk-1–SRF complex. Shore and Sharrocks (70) suggested that Elk-1 interacted either with a composite surface involving components from both halves of the SRF dimer or with a motif which is presented only upon dimerization. In contrast, Nkx-2.5 (and perhaps Phox-1) interacted with the N-terminal subdomain regions of the MADS box. While the formation of the SRF–Phox-1–Elk-1 tertiary complex on the c-*fos* SRE activated the c-*fos* promoter under appropriate extracellular signals, we did not observe that Phox-1 and Elk-1 activated α-actin promoter activity (data not shown). Possibly, during early cardiogenesis, paired homeodomain genes such as Phox-1 (MHox) (16) or S8 (56) form nonproductive inhibitory complexes with SRF on the α-actin promoter SREs. The high level of S8 expression in the endocardial cushions (56) in fact correlates well with regions of the heart which do not express Nkx-2.5, α-actin genes, or other contractile genes, thus perhaps playing a repressive role to block contractile activity in regions of the heart that will form septations and valves. Interactions between Nkx-2.5 and SRF might actually compete through the same interactive subdomains with Phox-1 for SRF MADS box binding. The outcome of Nkx-2.5 and SRF interactions may simply exclude Phox-1 and Elk-1 from the complex, allowing for activation of the α-actin promoter in cardiac cell lineages. Consistent with this idea, Nkx-2.5 blocked the serum-inducible expression of a minimal c-*fos* SRE-containing promoter in transient-transfection assays (4).

Although it is generally assumed that SRF plays a role as a constitutive factor during its association with accessory factors, we have shown that SRF-binding activity actually increased dramatically following the ending of the cell replication, primarily as a result of changes in the cellular content of SRF in primary myoblasts (42). Surveys of early avian (15) and murine (data not shown) embryos also indicated tissue-restricted expression of SRF which substantially increased the cellular mass of SRF in cardiac myocytes. We have also observed expression

of SRF coinciding with the reported expression patterns of Nkx-2.5 in the early lateral-plate mesoderm, pharyngeal endoderm, and paired heart tube during early avian (15) and murine (4) embryogenesis. In primordial cardiac myocytes, cardiac α-actin gene activation may also require increased SRF cell content in combination with the coappearance of Nkx-2.5 to foster cooperative transfactor complex formation. Building up of SRF–Nkx-2.5 complexes might inhibit negative acting factors such as YY1, and/or inhibitory SRF–Phox-1 complexes, allowing for saturation of the multiple SREs with positive acting SRF complexes to activate the promoter. We believe that for similar reasons, it is also necessary to express SRF in these transfection assays to increase the cellular levels of SRF in 10T1/2 fibroblasts, which might outcompete inhibitory complexes. Conversely, the Nkx-2.5–SRF complexes that are activated in nonreplicating cardiac myocytes might serve to repress the c-*fos* promoter by forming nonproductive complexes through its SRE, similar to the inhibitory activity of MyoD exerted upon the c-*fos* SRE (78). Therefore, SRF might be able to mediate accessory-factor interactions with certain homeodomain factors that either activate or repress transcription.

How does the formation of the Nkx-2.5–SRF complex activate transcription? The interaction of Nkx-2.5 with one of the putative transcriptional inhibitory domains of SRF (amino acids 142 to 171) (33) may expose the C-terminal activation domain of SRF and become accessible to a protein factor such as the RAP74 subunit of TFIIF (82). Particularly, the C-terminal domain of SRF is able to interact with its N terminus (61). On the other hand, interaction of SRF with the homeodomain of Nkx-2.5 may cause similar effects and potentiate the transcriptional activation activity of Nkx-2.5. We previously identified a strong transcriptional activation domain and a transcriptional inhibitory domain in the N terminus (amino acids 1 to 122) and the C terminus (amino acids 203 to 318), respectively, and suggested that potential hydrophobic interactions between the inhibitory and activation domains might block access of the highly charged moiety with transcription initiation factors (10). Although these regions are distinct from the homeodomain to which the interaction domain of Nkx-2.5 with SRF was mapped, the interactions between the Nkx-2.5 homeodomain and SRF may cause a conformational change in Nkx-2.5 protein structure, which in turn may relieve an inhibitory constraint and transduce Nkx-2.5 into an operative transcriptional activator. This is consistent with the data that the homeodomain region of Nkx-2.5 alone fails to cooperate with SRF to activate the cardiac actin promoter in transfection assays (9), although this domain is sufficient for mediating protein contacts with SRF.

In addition to SRF, MEF-2 protein, another member of MADS family, was shown to interact with other regulatory factors (53). The recent demonstration that MEF-2 and myogenic basic helix-loop-helix proteins associate on DNA raises the possibility that these two classes of myogenic transcriptional factors collaborate to induce muscle-specific transcription of MEF-2-dependent genes. That certain MEF-2 sites also bind the mesodermal homeodomain protein MHox suggests that additional possibilities for cross talk between MEF-2 proteins and other regulators. Therefore, a common property of MADS proteins could be their ability to cooperate with other transcriptional regulators to control gene expression.

Finally, homeodomain factors in general are unlikely to achieve their specificity of action in vivo only on the basis of the DNA-protein interaction (30). An emerging global view of how homeodomain proteins provide specific regulatory activity appears through protein-protein interactions. For example, in *D. melanogaster*, mutants of the *fushi tarazu* (*ftz*) protein lacking the homeodomain DNA-binding region retain significant biological activity, suggesting that protein-protein interactions may be required for some *ftz* biological activity (1, 19). Also, the *Drosophila* paired class homeodomain proteins were able to exhibit cooperative dimerization on the DNA through homeodomain-homeodomain interaction (79). Another example comes from transcriptional activation of the human HOXD9 promoter by the HOXD9 protein, which was antagonized by the HOXD8 protein. The inhibitory activity of HOXD8 was not affected by deletion of the homeodomain helix 2/3 region, suggesting a mechanism mediated by protein-protein touching, and the amino-terminal and helix 1 region of HOXD8 was able to interact with the homeodomain of HOXD9 (81). Finally, other examples come from investigation of the POU homeodomain protein, Oct-1, which interacted with the viral transactivator, VP16. The interaction domains of Oct-1 with VP16 was mapped to the helix 1/2 region (39, 71, 72), which is somewhat analogous to SRF–Phox-1 (27) and SRF–Nkx-2.5 interactions. Because most HOX and HOM proteins have similar DNA-binding specificities, protein-protein interactions between different HOX proteins or between these proteins and other transcription factors could be a general mechanism for the regulation of HOX functional specificity (30, 48). The fact that HOXD8-HOXD9, Oct-1-VP16, Phox-1–SRF, and Nkx-2.5–SRF interactions all required the amino-terminal arm/helix 1/helix 2 region led us to propose a hypothesis that the amino terminus and helix 3 are responsible for homeodomain-DNA interactions (for a review, see reference 22) whereas helix 1/2 probably mediates protein-protein interactions of HOX genes among themselves and with other protein factors, which might be an essential requirement for regulating their specificity of action as activators, coactivators, or repressors.

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