A Triad of Serum Response Factor and the GATA and NK Families Governs the Transcription of Smooth and Cardiac Muscle Genes*

Received for publication, December 12, 2001

Published, JBC Papers in Press, December 14, 2001, DOI 10.1074/jbc.M111824200

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Serum response factor and the (CCAT(T))_6(GG) (CarG) box interact to promote the transcription of c-fos and muscle genes; this tissue-specific activity may require co-regulators for serum response factor. The α_1 integrin promoter contains two cis-elements besides the CarG box: a TAAT sequence, a consensus binding site for homeoproteins, and a GATA-binding box. As a candidate TAAT-binding factor, we cloned an NK family homeobox gene, Nkx-3.2, which is expressed mainly in smooth muscle tissues and skeletal structures. Nkx-3.2, serum response factor, and GATA-6 were co-expressed only in the medial smooth muscle layer of arteries. These three transcription factors formed a complex with their corresponding cis-elements and cooperatively transactivated smooth muscle genes, including α_1 integrin, SM22α, and caldesmon. Cardiac muscle-specific members of the NK and GATA families exist, and the triad of Nkx-2.5, serum response factor, and GATA-4 also transactivated the cardiac atrial natriuretic factor gene, which contains a CarG-like box, a GATA-binding box, and an NK-binding element. Our findings demonstrate that smooth and cardiac muscle have a shared transcriptional machinery and that the GATA and NK families confer muscle specificity on the serum response factor/CARG interaction.

The vertebrate muscle system consists of skeletal, cardiac, and smooth muscles derived from the mesoderm. After differentiation, all three muscle types are equipped with the contractile apparatus for force generation. Actin and myosin are essential components in the contractile apparatus, and their interactions are regulated by muscle type-specific regulatory proteins such as tropomyosin, troponins, caldesmon, and myosin light chain kinase. Interestingly, most of the muscle type-specific contractile proteins have tissue- or phenotype-dependent isofoms. This is true of actin (1), myosin (2), tropomyosin (3, 4), and caldesmon (5), and changes in their expression are regulated at the transcriptional level in a cellular context-dependent manner (6, 7). Transcriptional regulation that defines muscle phenotype has been studied extensively, but no general scheme explaining muscle specificity has been proposed. Thus far, an interaction between serum response factor (SRF) and the CarG box has been identified as a core machinery in the transcription of several muscle-specific genes, including the skeletal α-actin (8), caldesmon (9), cardiac α-actin (10), α_1 integrin (11), SM22α (12), smooth muscle myosin heavy chain (13), smooth muscle α-actin (14), calponin (15), atrial natriuretic factor (16), and β-tropomyosin (17) genes.

SRF, a member of the MADS domain family, was originally identified as a transcription factor that binds to the serum response element (an alias of the CarG box) within the promoter region of c-fos (18). However, SRF is expressed at high levels in skeletal, cardiac, and smooth muscles (19), and more recent studies demonstrated that targeted disruption of the mammalian SRF gene leads to malformation of the mesoderm (20). These observations suggest that a variety of biological roles for SRF might be directed by co-regulators. In fact, ternary complex factor is a co-regulator of SRF in c-fos transcription (21). A homeoprotein that is specific for cells of the cardiac muscle lineage, Nkx-2.5/Csx (22, 23), is a reported co-regulator of SRF in the transcription of the genes for cardiac α-actin (10) and atrial natriuretic factor (24). In addition, GATA-4, a member of the GATA family, is coordinately involved in the transcription of cardiac genes with Nkx-2.5/Csx (25, 26). These findings demonstrate the importance of the SRF/CarG interaction and suggest that auxiliary factors may also be necessary for muscle-specific transcription.

α_1 Integrin is localized exclusively to SMCs, and its expression is SMC phenotype-dependent; the SRF/CarG interaction is critical for its transcription (11). Here, we identified two additional cis-elements, a TAAT sequence and a GATA box, in α_1 integrin’s promoter region. As a candidate TAAT-binding transcription factor, we cloned an NK family homeobox gene, Nkx-3.2, which is expressed mainly in smooth muscle tissues and skeletal structures and co-localizes with SRF and GATA-6 in the medial SMC layer of arteries. The triad of Nkx-3.2, SRF, and GATA-6 formed a complex with their corresponding cis-elements and strongly activated the transcription of smooth muscle genes, including SM22α and caldesmon as well as α_1 integrin. Interestingly, both the NK and GATA families contain cardiac muscle-specific isofoms, Nkx-2.5/Csx and GATA-4, and their partial involvement in cardiac gene transcription has been reported as described above. Atrial natriuretic factor

* This work was supported by Grants-in-Aid for Research on Brain Science from the Ministry of Health and Welfare of Japan (to K. S.) and Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (to K. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ The abbreviations used are: SRF, serum response factor; MADS, MCM1/Agamous/Deficiens/SRF; SM22α, smooth muscle cell; CarG, (CCAT(T))_6(GG); HD, homeodomain; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; ANF, atrial natriuretic factor; wt, wild-type; aa, amino acid(s); E, embryonic day.
(ANF) is a well-characterized cardiac gene that contains three putative cis-elements, a GATA and CArG-like box and an NK family binding consensus sequence. Here, we demonstrate that Nkx-2.5/Csx, SRF, and GATA-4 dramatically activated ANF transcription and suggest that the cooperative interactions between the NK, SRF, and GATA families are also involved in the transcription of cardiac muscle genes. In this study, we hypothesize for the first time that there is a shared transcriptional machinery for both smooth and cardiac muscle cells.

MATERIALS AND METHODS

**Plasmid Constructs—**Chicken α1 integrin (−258 to +281; Ref. 11), chicken caldesmon (−913 to +63; Ref. 9), mouse SM22α (−441 to +62; Ref. 12), and rat atrial natriuretic factor (−145 to −4; Ref. 24) promoter regions were amplified by PCR and subcloned into the pGL3 basic vector (Promega). Mutations were introduced using the Quikchange site-directed mutagenesis kit (Stratagene). The expression vector of wild-type human SRF (pSG5-SRF) was a kind gift of Dr. M. Seiki (Tokyo University). Full-length human SRF and the NH2 terminus, MADS domain, and COOH terminus of human SRF were subcloned into the pGEX-6P-1 vector (Amersham Biosciences, Inc.). Homeobox genes expressed in smooth muscle tissues were cloned from a chicken gizzard cDNA library (17). Full-length chicken Nkx-3.2 (wt; aa 1–274) and the NH2-terminal deletion (ΔN; aa 149–274), COOH-terminal deletion (ΔC; aa 1–208), homeodomain deletion (ΔH; aa 1–148 and 209–274), homeodomain alone (HD; aa 149–208), and homeodomain plus the NH2-terminal-flanking 10 amino acids (10–HD; as 129–208) of chicken Nkx-3.2 were generated by PCR and subcloned into pCS2+MT. Similarly, Nkx-3.2 derivatives were introduced into the pGEX-6P-2 plasmid. The full-length open reading frames of chicken Nkx-2.5 (GenBank™ accession number X91838) and GATA-4, -5, and -6 (GenBank™ accession numbers U11887, U11888, and U11889) were amplified from cDNA and inserted into pCS2+MT and pcDNA-3.1-FLAG vectors, respectively. Full-length chicken GATA-6 (wt; aa 1–387) and the NH2 terminus (N; aa 1–178), COOH terminus (C; aa 291–387), and ZF1-ZF2-basic domain (ZFB; aa 179–270) of chicken GATA-6 were generated by PCR and subcloned into pCS2+MT. Similarly, Nkx-3.2 derivatives were introduced into the pGEX-6P-2 plasmid. All of the plasmid constructs were verified by sequencing.

**DNase I Footprinting—**Solid-phase DNase I footprinting was performed (27) using nuclear protein extracts prepared from large arteries, including the aorta and gizzard, of chicken embryos at E15 (17). A sense primer positioned from 223 to 1 integrin promoter region was end-labeled with [γ-32P]ATP, and an antisense primer positioned from +24 to +47 was biotinylated, respectively.

**Miscellaneous Techniques—**In situ hybridization, promoter assay, EMSA, pull-down assay, and immunoprecipitation were carried out as described elsewhere (17). Primary cardiomyocytes were prepared from neonatal Sprague-Dawley rats as described previously (28), except that the original enzyme solution was replaced with Blendzyme 3 (Rosh). EMSA probes of α1 integrin were prepared as follows. A distal probe (TAAT-CaRg) positioned from −230 to −172 and a proximal probe (CaRg-GATA) positioned from −191 to −111 were subcloned into pBS-SKII(+) vector (Stratagene) and excised with BamHI and PstI digestion. Cell lysates for immunoprecipitation were prepared using 0.5% Triton X-100 and 0.1% SDS as detergents.

RESULTS

Identification of Putative cis-Elements in the α1 Integrin Promoter Region—DNA footprinting analysis revealed three putative cis-elements that bind to nuclear proteins prepared from vascular and visceral SMCs in the α1 integrin promoter region (indicated by FP1–FP3 in Fig. 1). FP1 corresponds to the repetitive TAAT core sequence, which is a consensus-binding site for homeoproteins (29). FP2 and FP3 mapped to the CaRg box and GATA binding sequences, respectively. Of these, we previously identified the CaRg box sequence in the α1 integrin promoter region as an SRF-binding cis-element. These findings suggest that at least one homeoprotein and GATA family protein are putative co-regulators for SRF in the transcription of smooth muscle genes.

Cloning of Chicken Nkx-3.2 cDNA—To isolate the smooth muscle-related homeobox genes, we designed a degenerate oli-
are expressed in vascular and visceral smooth muscle tissues, we performed in situ hybridization of chicken embryos at E15 (Fig. 2A). The Nkx-3.2 transcripts were located exclusively in skeletal structures and smooth muscle tissues, including smooth muscle in the esophagus, crop, gizzard, and hindgut. In addition to Nkx-3.2, Barx1b transcripts accumulated in the SMC layer of the digestive tract (17). The Hox cluster genes were distributed throughout the whole body, and Gax was highly expressed in striated muscles as well as in SMCs (data not shown). Thus, the Nkx-3.2 and Barx1b homeoproteins seem to be good candidates for trans-factors that bind to the TAAT core sequence. However, Barx1b did not bind to the AT-rich sequence of the β-tropomyosin promoter (17), nor did it bind to the TAAT motifs of the α1 integrin promoter (data not shown). Therefore, we focused on Nkx-3.2 as a TAAT-binding partner.

The Nkx-3.2 clone encoded an open reading frame of 276 amino acid residues (GenBank™ accession number AB042507) (Fig. 2B). A search for homology in the GenBank™ data base shows that the homeodomain of Nkx-3.2 was significantly similar to that of Drosophila bagpipe (GenBank™ accession number L17133), mouse Bapx1 (GenBank™ accession number NM_007524), human BAPX1 (GenBank™ accession number AF005260), and chicken NKX3.2 (GenBank™ accession number AF138905) (Fig. 2C). The identities were 86%, 100%, 100%, and 100%, respectively. The deduced primary structure of our clone was essentially same as that of chicken NKX3.2, but there were differences in amino acid residues 103–132, and 2% of the Nkx-3.2 nucleotide sequence was mismatched with the reported cDNA sequence (GenBank™ accession number AF138905). It is possible that our clone and NKX3.2 are distinct avian orthologues of Drosophila bagpipe, so we designated this clone as chicken Nkx-3.2. The NH2-terminal sequences of mouse Bapx1 and human BAPX1 are relatively conserved but are distinct from those of Drosophila bagpipe and chicken Nkx-3.2. The homeodomain of chicken Nkx-3.2 also showed 70% identity with a member of the NK homeoprotein family, mouse Nkx-2.5 (GenBank™ accession number X75415). Indeed, chicken Nkx-3.2 contained an NK domain downstream of the homeodomain and a TN domain in its NH2 terminus (30). As we reported previously (17), the TN domain consensus overlaps completely with the FIM domain of Barx family. Furthermore, a sequence of 10 residues upstream of the homeodomain was highly conserved between mouse Bapx1, human BAPX1, and chicken Nkx-3.2.

Co-localization of Nkx-3.2, SRF, and GATA-6 mRNAs in the Vascular SMC Layer—The GATA family is another candidate for SRF co-regulators in α1 integrin transcription, and only GATA-6 is reported to be expressed in vasculature (31). To compare the fine localization of Nkx-3.2, SRF, and GATA-6, we performed in situ hybridization of chicken arteries (Fig. 3). α1 Integrin, SM22α, and GATA-6 transcripts were strictly located in the medial SMC layer of branch from thoracic aorta at E15 and ascending aorta at posthatched day 7. SRF and Nkx-3.2 were detected at high levels in the medial SMC layer but were also expressed in the adventitia and the endothelium/intima (Fig. 3A). In the gizzard SMC and glandular layers, SRF and Nkx-3.2 were highly expressed. GATA-6 was totally absent in the gizzard SMC layer and was expressed only in the glandular layer (Fig. 3B). Thus, our data indicate that the expression of these three transcription factors overlaps only in the medial SMC layer of the arteries, where smooth muscle genes are expected to be expressed.

Cooperative Transactivation of the α1 Integrin Gene by Nkx-3.2, SRF, and GATA-6—We examined the possibility that Nkx-3.2 and GATA-6 are involved in the transcription of α1 integrin as SRF co-regulators (Fig. 4). In this experiment, we used 10T1/2 cells to prevent possible interference by endogenous smooth muscle-specific transcription factors. When only the α1 integrin reporter gene was transfected, it showed no significant promoter activity. Co-transfection with Nkx-3.2 resulted in 5-fold activation. SRF or GATA-6 expressed alone resulted in activation rates of less than 3-fold. When the cells were co-transfected with combinations of SRF and Nkx-3.2, GATA-6 and Nkx-3.2, or SRF and GATA-6, the promoter activities
reached 9-, 19-, and 5-fold, respectively. A 45-fold activation was achieved when all three factors, Nkx-3.2, SRF, and GATA-6, were co-transfected. In contrast, transfection of other homeoproteins expressed in smooth muscle tissues, Gax or MHox, with or without SRF and GATA-6 did not show significant effects. These results indicate that Nkx-3.2 and GATA-6 specifically transactivated the \( /H_9251 \) integrin promoter in combination with SRF (Fig. 4A). Three variants of the \( /H_9251 \) integrin reporter gene containing a mutated TAAT sequence (mTAAT), CArG box (mCArG), or GATA box (mGATA) had almost no promoter activity, even in the presence of all three transcription factors, indicating the critical involvement of these sequences as cis-elements (Fig. 4B). We further examined the effectiveness of three GATA family proteins in this cis-element-dependent activation. When equimolar levels of GATA proteins were expressed with Nkx-3.2 and SRF, GATA-4 and -5 showed 56% and 76% of the promoter activity seen with GATA-6 (Fig. 4B).

Finally, we confirmed whether the coordinated transactivation by a triad consisting of Nkx-3.2, SRF, and GATA-6 also worked for other smooth muscle gene promoters. Sequence analysis revealed that the chicken caldesmon promoter contains a GATA box (−314) immediately upstream of the CArG box (−309) and a repetitive TAAT sequence (−544). The mouse SM22\( \alpha \) promoter also contains a GATA box and TAAT core sequence at −478 and −508, respectively. As expected, the promoter activities of chicken caldesmon and SM22\( \alpha \) significantly increased (by 22 and 28-fold, respectively) when this
A triad of transcription factors was co-expressed with the promoter construct. In contrast, the human c-fos promoter containing a serum response element flanked by an ets domain failed to exhibit cumulative activation in the presence of the triad (Fig. 4C).

Complex Formation between Nkx-3.2, SRF, and GATA-6 with Their Corresponding cis-Elements—We examined the interactions between Nkx-3.2, SRF, and GATA-6 with their corresponding cis-elements. Because the promoter region spanning three cis-elements is so long for an EMSA probe to reveal a complex formation, we divided the region into two parts as shown in Fig. 1. First, we analyzed the interaction between Nkx-3.2 and SRF with a 32P-labeled TAAT-CArG probe (Fig. 5A). SRF formed a complex with the probe (lane 1, black arrowhead), and this complex was displaced by an excess amount of the unlabeled DNA probe (lane 2). The complex was further supershifted by anti-SRF antibody (lane 3) but not by anti-GST antibody or normal rabbit IgG (data not shown), confirming a specific complex formation. Because GST-fused full-length Nkx-3.2 was easily degraded during purification, we used GST-
Domains of GATA-6 are essential for the physical interaction of the wild-type GATA-6 fusion protein with SRF, as shown by the supershift of the complex ( lane 16) but not by anti-GST antibody or normal antibody ( lane 17). When increasing amounts of SRF were added to a fixed amount of GST-Nkx-3.2-C, a new, slowly migrating complex (asterisk) appeared in an SRF dose-dependent manner ( lanes 9–11). This newly formed complex was also displaced by an excess amount of unlabeled probe (data not shown) and supershifted by anti-SRF or anti-GST antibody, respectively ( lanes 12 and 13). Mobility shift of the complex was not influenced in the presence of normal rabbit IgG (data not shown).

Next, we examined the interaction between SRF and GATA-6 with the 32P-labeled CArG-GATA probe (Fig. 5B). SRF bound to the probe ( lane 14, black arrowhead), and an excess amount of unlabeled probe displaced the complex formation ( lane 15). This complex was also supershifted with anti-SRF antibody ( lane 16) but not by anti-GST antibody or normal rabbit IgG (data not shown). Because the molecular weight of the wild-type GATA-6 fusion protein is similar to that of SRF, we used a truncated mutant of GATA-6 containing only the zinc finger and basic domains (GST-GATA-ZFB). In the following experiment, we confirmed that the zinc finger and basic domains of GATA-6 are essential for the physical interaction with SRF (Fig. 6). GST-GATA-ZFB bound to the 32P-labeled CArG-GATA probe (Fig. 5B, lane 18, white arrowhead), and the complex was specifically displaced by an excess amount of unlabeled probe ( lane 19). This complex was supershifted by the anti-GST antibody (Fig. 5B, lane 20) but not by anti-SRF antibody or normal rabbit IgG (data not shown). In this experiment, a new, slowly migrating complex was also seen (Fig. 5B, asterisk) when increasing amounts of SRF were added to a fixed amount of GST-GATA-ZFB ( lanes 23–25). This complex formation was inhibited by an excess amount of unlabeled probe (data not shown). The complex was supershifted by anti-GST or anti-SRF antibody ( Fig. 5B, lanes 26 and 27) but was not affected in the presence of normal rabbit IgG (data not shown). Thus, we demonstrated the formation of a ternary complex of SRF and Nkx-3.2 or GATA-6 with the TAAT-CArG or CArG-GATA probes, respectively.

Binding Domains of Nkx-3.2, SRF, and GATA-6—To define the binding domains of Nkx-3.2, SRF, and GATA-6 interactions, we performed a series of pull-down assays. First, we analyzed the Nkx-3.2 and SRF interaction. GST-fused full-length SRF (GST-SRF-wt) or the NH2 terminus (GST-SRF-N), MADS domain (GST-SRF-MADS), or COOH terminus (GST-SRF-C) of SRF was immobilized on a glutathione-Sepharose 4B gel matrix and then incubated with in vitro-translated 35S-labeled chicken Nkx-3.2 (Fig. 6A). 35S-labeled Nkx-3.2 bound to GST-SRF-wt and GST-SRF-MADS (Fig. 6A, lanes 1 and 4), but not to GST-SRF-N or GST-SRF-C ( lanes 2 and 3). We further investigated the domain of Nkx-3.2 that interacts with SRF ( Fig. 6B). GST-fused full-length Nkx-3.2 (GST-Nkx-wt) or the NH2 terminus (GST-Nkx-N), COOH terminus (GST-Nkx-C), homeodomain alone (GST-Nkx-HD), or the homeodomain plus 10 NH2-terminal-flanking residues (GST-Nkx-10+HD) of Nkx-3.2 was immobilized on a glutathione-Sepharose gel matrix that had been incubated with 35S-SRF. GST-Nkx-wt, GST-Nkx-HD, and GST-Nkx-10+HD bound to the 35S-SRF (Fig. 6B, lanes 1, 4, and 5), but GST-Nkx-N and GST-Nkx-C did not ( lanes 2 and 3). These results indicate that the homeodomain of Nkx-3.2 and the MADS domain of SRF are required for the interaction.

We then studied the interaction between SRF and GATA-6. The GST-fused variants of SRF described above were incubated with 35S-GATA-6 (Fig. 6C). GST-SRF-wt and GST-SRF-MADS bound to 35S-GATA-6 (Fig. 6C, lanes 1 and 4), but GST-SRF-N and GST-SRF-C did not ( lanes 2 and 3). To elucidate the domain of GATA-6 required for SRF binding, we performed similar experiments (Fig. 6D). 35S-labeled full-length GATA-6 (GATA-wt) and the NH2-terminal deletion (GATA-ΔN), COOH-terminal deletion (GATA-ΔC), COOH terminus (GATA-C), or zinc fingers plus basic domain (GATA-ZFB) of GATA-6 were incubated with GST-SRF-wt immobilized on a Sepharose gel matrix. GATA-wt, GATA-ΔN, GATA-ΔC, and GATA-ZFB were pulled-down with GST-SRF-wt ( Fig. 6D, lanes 1, 2, 3, and 5), but GATA-C was not ( lane 4). These results indicate that the MADS domain of SRF and the ZFB domain of GATA-6 directly interact with each other.

To confirm the physical interactions between three transcription factors in vivo, we performed immunoprecipitation assays (Fig. 6E). FLAG-SRF, FLAG-GATA-6, and myc-Nkx-3.2 expression vectors were co-transfected into 10T1/2 cells. The cell lysates were incubated with an anti-SRF rabbit polyclonal antibody, and the SRF complex was precipitated using protein A-Sepharose. Transcription factors thus precipitated were identified with an anti-FLAG or anti-myc monoclonal antibody. FLAG-GATA-6 and myc-Nkx-3.2 were successfully precipitated with SRF. Thus, Nkx-3.2, SRF, and GATA-6 clearly interacted with each other in living cells.

Minimum Domains of Nkx-3.2, SRF, and GATA-6 Required for Transcriptional Activation of α1 Integrin—We investigated the minimum domains of Nkx-3.2, SRF, and GATA-6 required for the cooperative activation of the α1 integrin gene using 10T1/2 cells (Fig. 7). We examined the effects of chicken Nkx-3.2 mutants on the α1 integrin promoter activity. Nkx-wt, Nkx-ΔC, or Nkx-10+HD in cooperation with SRF and GATA-6 activated the promoter activity, but Nkx-ΔN, Nkx-ΔHD, and Nkx-HD did not. Interestingly, Nkx-10+HD retained the same activity as Nkx-wt, and Nkx-ΔC further increased the promoter activity about 2-fold. We then analyzed the functional domains of GATA-6 in the α1 integrin transcription. GATA-wt and GATA-ΔC activated the promoter activity, but GATA-N, GATA-C, GATA-ZFB, and GATA-ΔN did not. These results indicate that the homeodomain plus the NH2-terminal-flanking 10 residues of Nkx-3.2 and the ZFB domain plus the NH2 terminus of GATA-6 synergistically activate the SRF-dependent transcription of α1 integrin.

Cardiac Muscle Genes Also Use the SRF, GATA, and NK Triad in Their Transcriptional Machinery—The existence of cardiac muscle-specific members of the GATA and NK families, GATA-4 and Nkx-2.5, prompted us to test our hypothesis that the transcriptional machinery used for the expression of smooth and cardiac muscle genes is conserved. ANF is one of the most extensively characterized cardiac muscle genes, and its promoter region contains a GATA box, a CArG-like box, and an NK-binding element (Fig. 8A). First, we examined the promoter activity of the ANF gene in neonatal rat cardiomyocytes (Fig. 8B). When a mutation was introduced into the GATA box, CArG box, or NK-binding element, the promoter activity declined to 10%, 27%, or 23% of wild-type, respectively. Co-transfection with Nkx-2.5, SRF, or GATA-4 alone resulted in 2.4-, 1.4-, and 1.8-fold activation. However, a 6.7-fold activation was achieved when Nkx-3.2, SRF, and GATA-6 were co-transfected. These results suggest that the three cis-elements are essential for ANF transcription in...
cardiomyocytes and that GATA-4 and Nkx-2.5 are co-regulators of SRF.

Finally, we examined the contribution of the three transcription factors using non-muscle 10T1/2 cells (Fig. 8C). The ANF reporter gene failed to exhibit remarkable promoter activity in the absence of any member of the transcription factor triad. Co-transfection with Nkx-2.5, SRF, or GATA-4 alone resulted in a 17-, 4-, or 8.3-fold increase in transcription, respectively. When the cells were co-transfected with SRF, Nkx-2.5, and GATA-4, however, the promoter activity reached 145-fold. When GATA-4 was replaced with GATA-5 or GATA-6, the resultant activation rate was 123 and 129-fold, respectively. In contrast, transfection of Gax or MHox, which are endogenously expressed in the heart, showed no significant effects. These results suggest that Nkx-2.5 and GATA-4 serve as SRF co-regulators in cardiac muscle, just as Nkx-3.2 and GATA-6 do in smooth muscle.

**DISCUSSION**

Each muscle type-specific transcription has been studied independently, but no consensus mechanisms governing the transcription of muscle genes have been documented. However, it has been reported that SRF expression is essential for the development of mesoderm-derived muscles (20) and that SRF
plays a central role in muscle gene transcription. SRF is also ubiquitously distributed in non-muscle cells and is involved in the transcription of a wide variety of genes. Taken together, these observations have led to the suggestion that co-regulators for SRF would confer cellular context awareness on the transcription. Furthermore, several smooth muscle genes, including \( \alpha \)-smooth muscle actin and SM22\(_{a}\), are transiently transcribed during cardiogenesis (32). This phenomenon suggested that closely related transcriptional machineries exist in both smooth and cardiac muscles. Footprinting analysis of the \( \alpha_1 \) integrin promoter revealed three major binding sites of nuclear proteins prepared from vascular and visceral SMCs (Fig. 1). The putative cis-elements were a repetitive TAAT sequence, a CArG box, and a GATA box, in order from 5' to 3'.

TAAT is a consensus binding sequence for homeodomain transcription factors (29), and many muscle gene promoters contain it. Thus far, two kinds of homeoproteins have been reported as SRF co-regulators. Grueneberg et al. (33) reported enhancement of the DNA binding activity of SRF by a paired class human homeoprotein, Phox1. MHome, a mouse homologue of Phox1, binds to an AT-rich element in the muscle creatine kinase enhancer, and its expression is restricted to the mesoderm (34). Nkx-2.5/Csx, a member of the NK family of homeoproteins, is another co-regulator of SRF. Nkx-2.5/Csx is thought to be a mammalian orthologue of Drosophila tinman, which is critical for the development of the heart, visceral mesoderm, and body wall muscles (35). Targeted disruption of murine Nkx-2.5/Csx arrests cardiac development at the linear heart tube stage (36). Chen and Schwartz (10) reported that Nkx-2.5/Csx and SRF physically associate with each other and activate the transcription of cardiac \( \alpha \)-actin gene containing three CArG boxes and a typical TAAT core sequence. However, expression of Nkx-2.5/Csx is restricted to a cardiac muscle lineage and is absent in SMCs. Several studies have been performed to identify the homeobox genes expressed in SMCs, and Hox cluster genes (37, 38) and Gax (39) have been cloned. However, Hox cluster genes such as Hox A5, Hox B3, Hox B4, and Hox B5 are widely distributed in a variety of tissues (Fig. 2), and Gax is also expressed in cardiac and skeletal muscles.

Here, we cloned an Nkx-3.2 cDNA and found it to be exclusively expressed in the smooth muscle tissues and skeletal structures (Figs. 2 and 3). Sequence alignment revealed that chicken Nkx-3.2 appears to be an avian orthologue of Drosophila bagpipe (Fig. 2). Drosophila tinman and bagpipe are members of the NK family, and bagpipe-deficient fly embryos fail to develop a midgut (35). It has been reported that mouse BapX1 is transiently expressed in the embryonic stomach (40) and chicken NKX3.2 has also been detected in the gizzard and hindgut at E7 (41). We showed here that Nkx-3.2 is highly expressed in the arteries, digestive tract, and skeletal structures of chicken embryos, and its expression in chicken arteries persists after hatching (Figs. 2 and 3). Thus, Nkx-3.2 shows smooth muscle-related expression at embryonic and postnatal stages.

Bapx1-deficient mice were recently generated and characterized (42, 43). These mice show skeletal dysplasia and asplenia. Tribioli et al. (43) found that failure of skeletal formation in Bapx1-deficient mice is a consequence of impaired chondrogenesis. Spontaneous calcification is frequently seen in the media of aged human arteries, the so-called Monckeberg's sclerosis (44). The mechanism of calcification is still unclear, but the distribution of Nkx-3.2 in the smooth muscle and skeleton may imply a close linkage between SMCs and chondrocytes. Whereas chicken Nkx-3.2 is also expressed in smooth muscle tissues, no abnormal phenotype was reported in the tissues of Bapx1-deficient mice. One possible explanation for this discrepancy is that there are other NK or Barx1 (described below) family members in SMCs that can compensate for the loss of Bapx1. A similar discrepancy was observed in Nkx-2.5/Csx-deficient mice. Nkx-2.5/Csx was not essential for mesodermal cells to enter the cardiac muscle lineage, and functional redundancy between Nkx-2 family members was observed (36). Another explanation is that the smooth muscle tissues of mutant mice seem to be normal in appearance, but subtle differences such as an immature phenotype might exist. A third possibility is that mammalian Bapx1 is different from Drosophila bagpipe. Bapx1 might have lost its essential role in the gut but acquired new functions in the skeleton and spleen during evolution. Because the NH\(_2\)-terminal sequences of mammalian Bapx1 and chicken Nkx-3.2 are quite different, they may be discrete paralogues.

Among the ternary transcription factors, the GATA family shows functional redundancy in its target genes. The GATA family consists of hematopoietic (GATA-1, -2, and -3) and non-hematopoietic members (GATA-4, -5, and -6), and there is substantial overlap in the distribution profiles of the latter group (45). Consistent with this, all the nonhematopoietic GATA members transactivated both \( \alpha_1 \) integrin and ANF genes in co-operation with SRF and the NK family (Figs. 4 and 8). Morin et al. (16) also examined the synergistic activation of
the ANF promoter by GATA-member or GATA-related transcription factor in visceral SMCs.

As described above, several investigators have partially reported physical interactions between Nkx-2.5/Csx and SRF or GATA-4 and their cooperative function in the transactivation of cardiac muscle genes (10, 16, 25, 26). However, no one has verified a single complex formation of this triad and confirmed their physiological significance in the tissue-specific transcription of smooth and cardiac muscle genes. In this study, we provided the first demonstration of direct interactions of SRF, Nkx-3.2, and GATA-6 with their corresponding cis-elements (Figs. 5 and 6). The three transcription factors formed complexes both in vitro and in vivo, and the homeodomain of Nkx-3.2, the MADS domain of SRF, and the ZFB domain of GATA-6 were essential for their interactions. In addition to these binding domains, other domains took part in gene transcription (Fig. 7). Only the homeodomain of Nkx-3.2 failed to show cooperative transactivation. However, the addition of the short NH₂-terminal-flanking sequence, which is highly conserved between chicken Nkx-3.2, mouse Bapx1, and human BAPX1 (Fig. 2), caused the promoter activity to recover to the same level as that of wild-type Nkx-3.2. This conserved short sequence of Nkx-3.2 could play a critical role in the cooperative transactivation. Chen and Schwartz (10) reported that a Nkx-2.5/Csx mutant lacking the COOH-terminal domain further increases the promoter activity of cardiac α-actin. Consistent with their finding, Nkx-3.2ΔC also accentuated α₁ integrin promoter activity. Therefore, the COOH terminus of the NK family appears to work as an inhibitory domain in muscle-specific transcription. In the case of GATA-6, the ZFB domain was essential for its interaction with SRF but did not activate the transcription of α₁ integrin. The cooperative activation was recovered only when a GATA-6 derivative including both the NH₂ terminus and the ZFB domain was used. This finding is consistent with the results of Morrissy et al. (46), who demonstrated two independent transcriptional activation domains in the NH₂ terminus of GATA-4. In short, the MADS domain of SRF, the ZFB domain plus the NH₂ terminus of GATA-6, and the homeodomain plus the short NH₂-terminal-flanking sequence of Nkx-3.2 are essential for their interactions and cooperative transactivation.

We recently cloned another smooth muscle lineage-dependent homeobox gene, Barx1b, from a smooth muscle cDNA library. Barx1b, a member of Barx family, interacts physically with SRF and activates β-tropomyosin gene transcription in visceral SMCs (17). We found that a FIL domain consensus sequence (XXXXIL) located at the NH₂ terminus of Barx family member genes is also in the TN domain of the NK family. These findings suggest that the Barx and NK families are closely related homeobox genes. Barx1b forms a ternary complex with the SRF-CARG probe but does not directly bind to the probe by itself. It also failed to bind with α₁ integrin EMSA probes (data not shown). In contrast to Nkx-3.2, Barx1b does not need its own DNA-binding sequence but accomplishes its cooperative transactivation through the interaction with SRF bound to the CARG box. Consistent with this, co-expression of SRF and Barx1b activated the α₁ integrin promoter but did so less efficiently than the Nkx-3.2/RF/GATA-6 triad (<10-fold activation compared with mock; data not shown). Thus, smooth muscle genes seem to properly use at least two types of transcriptional machinery: one is composed of the Nkx-3.2/RF/GATA-6 triad, and another is the Barx1b/RF binary combination.

In conclusion, the SRF/CARG interaction serves as the core machinery of smooth and cardiac muscle gene transcription, and the NK and GATA families work as co-regulators for SRF.
in specific tissues. According to our model, one of the criteria required for transcription is a high level of SRF expression. The second and third criteria are co-expression of the appropriate isoforms of the NK and GATA families. When these three criteria are satisfied, the transcription of smooth and cardiac muscle genes is activated. The fourth element, such as MEF2 family or chromatin remodeling factors, might exist because smooth and cardiac muscle gene transcriptions are completely exclusive in respective tissues in which the SRF/NK/GATA triad is similarly expressed. However, there is still a missing link in skeletal muscle gene transcription, in which the MyoD family plays definitive roles in the commitment of myoblasts to myotubes (47). Although the MyoD family has been investigated extensively, the details of skeletal muscle gene transcription remain unclear. For example, the CArG box works as a cis-element in the skeletal α-actin (8) and myosin light chain IA (48) genes, but whether a homeodomain protein and/or GATA (-like) zinc finger proteins co-regulate the SRF/CArG interaction in skeletal muscle cells is not known. Future studies are required to elucidate the precise mechanism of the transcription of skeletal muscle genes.

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A Triad of Serum Response Factor and the GATA and NK Families Governs the Transcription of Smooth and Cardiac Muscle Genes

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J. Biol. Chem. 2002, 277:7308-7317.
doi: 10.1074/jbc.M111824200 originally published online December 14, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M111824200

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