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Activation of Cardiac Gene Expression by Myocardin, a Transcriptional Cofactor for Serum Response Factor

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Summary

Serum response factor (SRF) regulates transcription of numerous muscle and growth factor-inducible genes. Because SRF is not muscle specific, it has been postulated to activate muscle genes by recruiting myogenic accessory factors. Using a bioinformatics-based screen for unknown cardiac-specific genes, we identified a novel and highly potent transcription factor, named myocardin, that is expressed in cardiac and smooth muscle cells. Myocardin belongs to the SAP domain family of nuclear proteins and activates cardiac muscle promoters by associating with SRF. Expression of a dominant negative mutant of myocardin in *Xenopus* embryos interferes with myocardial cell differentiation. Myocardin is the founding member of a class of muscle transcription factors and provides a mechanism whereby SRF can convey myogenic activity to cardiac muscle genes.

Introduction

The mechanisms that control skeletal muscle gene expression have been defined in considerable detail (reviewed in Hauschka, 1994), but relatively little is known of the mechanisms that regulate cardiac and smooth muscle genes. A common feature of many muscle genes is their dependence on a *cis*-acting sequence known as a CArG box ($CC(A/T)_6GG$), which serves as the binding site for serum response factor (SRF) (reviewed in Shore and Sharrocks, 1995). SRF belongs to the MADS (MCM1, Agamous, Deficiens, SRF) box family of transcription factors, which includes MEF2, a muscle-enriched transcription factor required for differentiation of skeletal, cardiac, and smooth muscle cell types (Black and Olson, 1998). The MADS domain mediates homodimerization and DNA binding, and recruits a variety of transcriptional cofactors that influence DNA binding affinity, transcriptional activity, and target gene specificity (reviewed in Treisman, 1994). A hallmark of MADS domain proteins is their ability to activate different sets of genes by interpreting various intracellular signals and cell identities through combinatorial associations with accessory factors.

SRF is expressed in a wide range of cell types and was first identified by its ability to confer serum inducibility to the c-*fos* promoter (Norman et al., 1988). Growth factor responsiveness of the c-*fos* promoter is mediated by the association of SRF with members of the ternary complex factor (TCF) family of Ets domain transcription factors, which serve as targets for mitogen-activated protein kinase signaling (Shaw et al., 1989; Gille et al., 1992; Marais et al., 1993). Association of TCF with the MADS box of SRF is dependent on binding of SRF to a CArG box, referred to as the serum response element (SRE), and on weak contacts between TCF and specific sequences immediately flanking the SRE.

CArG boxes are also required for expression of many skeletal and cardiac muscle genes and every smooth muscle-specific gene analyzed to date (reviewed in Reecy et al., 1998). The requirement of SRF for expression of growth factor-inducible and muscle-specific genes is paradoxical since growth factor signaling represses muscle gene expression. The mutually exclusive expression patterns of these two sets of SRF-regulated genes has led to the suggestion that SRF cooperates with myogenic accessory factors to selectively activate muscle target genes. Indeed, SRF has been shown to cooperate with the cardiac-restricted zinc finger transcription factor GATA4 (Belaguli et al., 2000; Morin et al., 2001) and the homeodomain protein Nkx2.5 (Chen and Schwartz, 1996), but these factors are relatively weak activators and they do not form a stable ternary complex with SRF on DNA.

Here, we describe a novel transcriptional cofactor of SRF, called myocardin, discovered using a bioinformatics approach to identify unknown cardiac-specific genes. Myocardin belongs to the SAP (SAF-A/B, Acinus, PIAS) domain family of nuclear proteins, which regulate diverse aspects of chromatin remodeling and transcription. Myocardin is highly expressed in embryonic cardiac and smooth muscle lineages before becoming restricted to the myocardium after birth. Myocardin is an extraordinarily potent transcriptional activator that activates CArG box-dependent cardiac promoters by forming a ternary complex with SRF. Through expression in *Xenopus* embryos of a dominant negative myocardin mutant that can associate with SRF but cannot activate transcription, we show that myocardin is required for myocardial cell differentiation in vivo. The association of SRF with myocardin provides a mechanism whereby SRF can convey myogenic activity to cardiac target genes.

Results

Cloning of Myocardin by a Bioinformatics-Based Screen In Silico

In an effort to identify unknown cardiac-specific genes, we searched expressed sequence tag (EST) databases for novel sequences found only in cardiac cDNA librar-

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A

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MDSSVKEAIKGTEVSLSKAADAFAFEDDSSRDGLSPDQARSEDPQGSTGSTPDIKSTEAPLDTIQDLTPG   70
                                                          basic
SESDKNDAASQPGNQSDPGKQVLGPLSTPIPVHTAVKSKSLGDSKNRHKKPKDPKPKVKKLKYHQYIPPD  140
QKAEKSPPPMDSAYARLLQQQQLFLQLQILSQQQQQQQQQQQQQQQQQQQQQRFSYPGMHQTHLKEPNEQ  210
                                                               SAP
MARNPNPSSTPLSNTPLSPVKNSISGQTGVSSLKPGPLPPNLDDLKVSELRQQLRIRGLPVSGTKTALVD  280
RLRPFQDCAGNPVPNFGDITTVTFPVTPNTLPSYQSSPTGFYHFGSTSSSPPISPASSDLSAAGSLPDTF  350
                                                     Leucine Zipper-like
TDASPGFGLHASPVPACTDESLLSSLNGGSGPSEPDGLDSEKDKMLVEKQKVINQLTWKLRQEQRQVEEL  420
RMQLQKQKSSCSDQKPLPFLATTIKQEDVSSCPFAPQQASGKGQGHSSDSPPPACETAQLLPHCVESSGQ  490
THVLSSTFLSPQCSPQHSPLGGLKSPQHISLPPSPNNHYFLASSSGAQRENHGVSSPSSSQGCAQMTGLQ  560
SSDKVGPTFSIPSPTFSKSSSAVSDITQPPSYEDAVKQQMTRSQQMDELLDVLIESGEMPADAREDHSCL  630
QKIPKIPGSSCSPTAIPPKPSASFEQASSGGQMAFGHYANDSDEHLEVLLNSHSPIGKVSDVTLLKIGSE  700
EPPFDSIMDGFPGKAAEDLFSAHELLPGPLSPMHAQLSPPSVDSSGLQLSFTESPWETMEWLDLTPPSST  770
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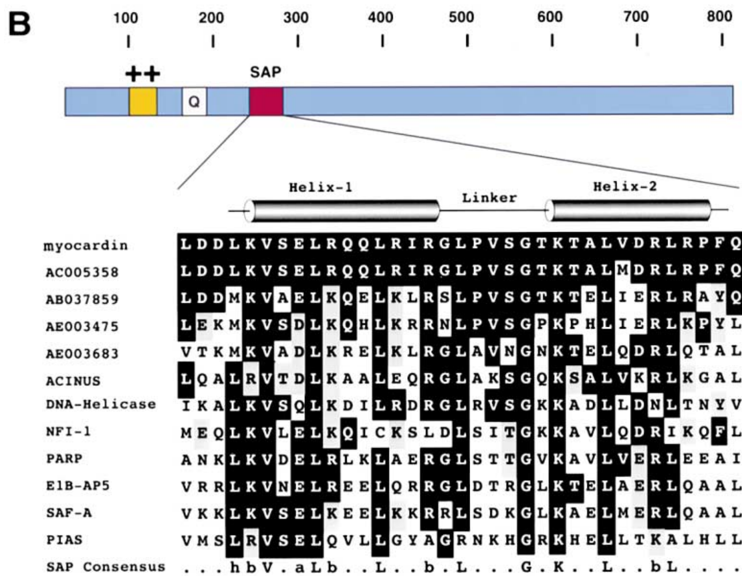


Figure 1. Deduced Amino Acid Sequence and Homology of Myocardin with other SAP Domain Proteins

(A) Deduced amino acid sequence of mouse myocardin (accession number AF384055).

(B) Homology of myocardin with SAP domain proteins. AC005358 is a human genomic sequence that likely represents the ortholog of mouse myocardin. AB037859 is a human EST sequence for a myocardin-related gene. AE003475 and AE003683 are *Drosophila* genomic sequences. The two predicted α helices of the SAP domain are shown at the top. a, acidic; b, basic; h, hydrophobic.

ies. Sequences that did not correspond to known genes were then used as probes for in situ hybridization to E9.5 mouse embryos and for Northern blot analysis of adult mouse tissues, in order to further confirm their possible cardiac-specific expression. One of the cDNAs identified in this screen corresponded to a 3′ untranslated region, which we used to isolate full-length cDNAs. The extended cDNA sequence encoded a novel protein of 807 amino acids (Figure 1A), which we named myocardin because of its specific expression in the adult myocardium and its essential role in myocardial gene expression in vivo.

Myocardin contains a SAP domain (Figure 1B), found in a variety of proteins that influence nuclear architecture and transcription (reviewed in Aravind and Koonin, 2000). Other notable features of myocardin include a basic region, an extended amphipathic α helix resembling a leucine zipper, and a stretch of glutamine (Q) residues (Figure 1A).

The SAP domain is named for the nuclear scaffold attachment factors A and B (SAF-A and -B), which recognize chromosomal regions known as scaffold or matrix attachment regions (SARs/MARs), Acinus, a caspase-3-activated protein required for apoptotic chromatin condensation, and PIAS, an inhibitor of STAT-mediated gene activation. The SAP domain is a 35 amino acid motif containing two predicted amphipathic helices separated by an intervening region with an invariant glycine residue. The two helices, which contain several highly conserved positively charged residues, have been predicted to resemble helices-1 and -2 of the homeodomain (Kipp et al., 2000). However, SAP domain proteins do not contain a third helix as is found in homeodomain proteins. Myocardin does not show homology to other known proteins outside of the SAP domain. However, we identified additional mouse, human, and *Drosophila* sequences encoding proteins with homology to the SAP and basic domains of myocardin (Figure 1B).

Myocardin Is Highly Expressed in Developing Cardiac and Smooth Muscle

Northern blot analysis of adult mouse tissues revealed multiple myocardin transcripts specifically in adult heart.

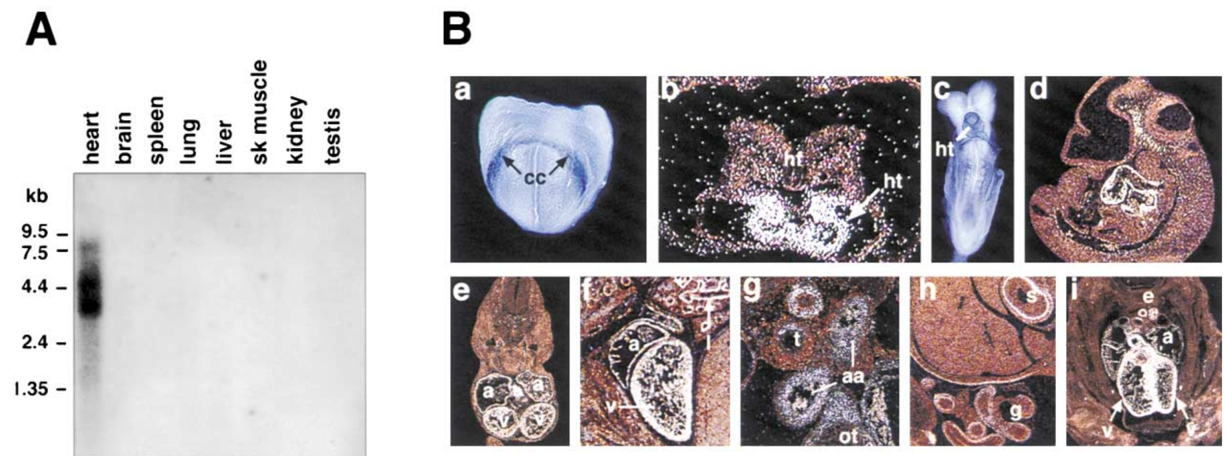


Figure 2. Expression of Myocardin Transcripts in Embryonic and Adult Mouse Tissues

(A) An adult mouse tissue Northern blot (Clontech) probed with a myocardin cDNA fragment.

(B) Myocardin transcripts were detected by whole-mount (a and c) or section (b and d–i) in situ hybridization to mouse embryos. (a) E7.75. Myocardin expression is localized to the cardiac crescent (cc). (b) E8.0. Transverse section shows myocardin expression specifically in the heart tube (ht) beneath the head folds (hf). (c) E8.0. Myocardin expression is detected throughout the linear heart tube. (d) E10.5. Saggital section shows myocardin expression localized to the heart. A subset of head mesenchyme cells also express myocardin at a low level. (e) E11.5. Transverse section shows myocardin expression in atrial (a) and ventricular (v) chambers. (f–h) E13.5. (f) Sagittal section shows myocardin expression in atrial and ventricular chambers and in adjacent pulmonary smooth muscles of the branches of the segmental bronchus of the lung (l). (g) Transverse sections through the outflow tract vasculature at E13.5 show myocardin expression in the smooth muscle cell layers of the outflow tract (ot), trachea (t), and aortic arch arteries (aa). (h) Myocardin expression in smooth muscle of the stomach (s) and gut (g). (i) E15.5. Transverse section shows myocardin expression in the heart and esophagus (e).

There was no detectable expression of myocardin in any other adult tissue examined (Figure 2A).

In situ hybridization to staged mouse embryos showed that myocardin transcripts were first detected in the cardiac crescent at E7.75 (Figure 2B, a), concomitant with expression of the homeobox gene *Nkx2.5*, the earliest known marker for cardiogenic specification (Lints et al., 1993). Thereafter, myocardin transcripts were detected specifically in the linear heart tube at E8.0 (Figure 2B, b and c) and throughout the developing atrial and ventricular chambers until birth (Figure 2B, d–f and i).

Myocardin is also expressed in a subset of embryonic vascular and visceral smooth muscle cells. At E13.5, myocardin expression was evident within smooth muscle cells lining the walls of the esophagus and aortic arch arteries, as well as the pulmonary outflow tract (Figure 2B, f and g). Expression in these smooth muscle cell types was still apparent, but was diminished, by E15.5 (data not shown). Myocardin expression was also detected in smooth muscle cells within the lung and gut (Figure 2B, f and h), as well as in head mesenchyme (Figure 2B, d), which may serve as a source of smooth muscle precursors. Myocardin was not expressed at detectable levels in skeletal muscle.

Myocardin Is a Highly Potent Transactivator

As a first step toward determining the function of myocardin, we examined the subcellular distribution of the protein in transfected COS cells. Myocardin protein was localized predominantly to the nucleus and showed a punctate intranuclear staining pattern with exclusion from nucleoli (Figure 3A).

Because myocardin was localized to the nucleus, we tested whether it possessed transcriptional activity by fusing it to the DNA binding domain of yeast GAL4. As shown in Figure 3B, myocardin fused to the GAL4 DNA binding domain potently activated a GAL4-dependent reporter in transfected COS cells. Residues 50–537 or portions of this region showed no transcriptional activity in this assay, whereas the region from residues 541–807 was approximately 60-fold more active than the full-length protein. This suggests that the amino-terminal portion of myocardin may mask the activation domain or mediate protein-protein or protein-DNA interactions that compete for activation through the GAL4 DNA binding site. Further carboxy-terminal deletions resulted in diminished transcriptional activity, suggesting that the activation domain is distributed over a relatively extended region.

Myocardin Activates Transcription through SRF Binding Sites

To identify potential target genes for myocardin, we tested a series of cardiac muscle gene regulatory regions linked to a luciferase reporter for their responsiveness to myocardin in transfected COS cells. Myocardin strongly transactivated the promoters for the *SM22, atrial natriuretic factor (ANF), myosin light chain (MLC)-2V*, and α-*MHC* genes, as well as the enhancer for the *Nkx2.5* gene (Figure 4A). In contrast, myocardin failed to activate the *HRT2* promoter or the *dHAND* enhancer, which are active in subsets of cardiac muscle cells. The *cytomegalovirus* (CMV) or *E1b* promoters, which show no tissue specificity, also were not activated by myocardin, indicating that myocardin does not act as a general transcriptional activator.

The *SM22* promoter was the most responsive to myocardin, being upregulated by several thousand-fold.

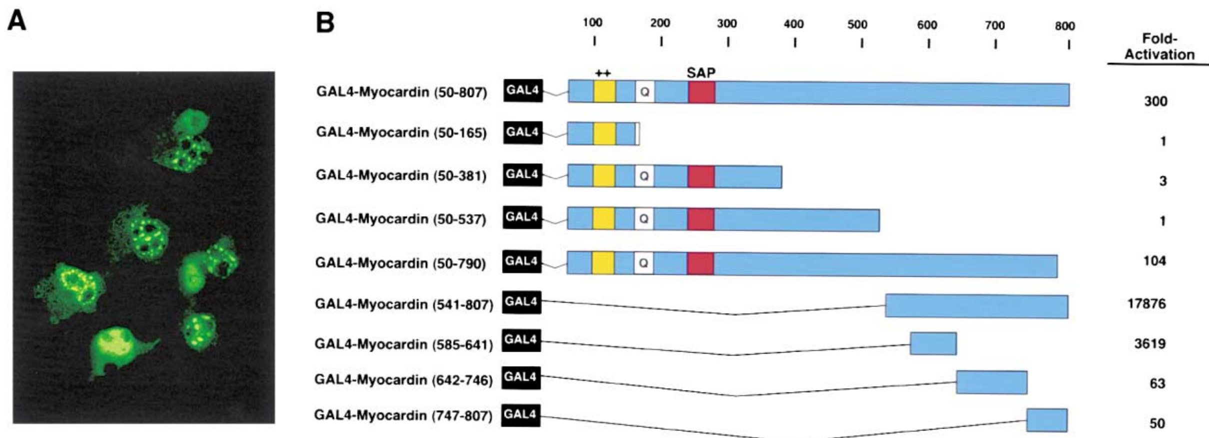


Figure 3. Nuclear Localization and Transcriptional Activity of Myocardin

(A) Subcellular location of myocardin protein in transfected COS cells, detected by immunostaining.

(B) COS cells were transiently transfected with expression vectors encoding the indicated regions of myocardin fused to GAL4 (1–147) and the pL8G5-luciferase reporter, which contains binding sites for the GAL4 DNA binding domain. Luciferase activity is expressed as fold-increase above that with the GAL4 expression plasmid without a cDNA insert.

SM22 encodes a troponin-related protein expressed in developing cardiac, smooth, and skeletal muscle cells during early embryogenesis, before becoming restricted specifically to the smooth muscle lineage (Li et al., 1996). Transcription of *SM22* in cardiac and smooth muscle cells in vivo is dependent on two CArG boxes in the promoter, referred to as CArG-near (at $-150/-141$) and CArG-far (at $-273/-264$), that bind SRF (Li et al., 1997; Kim et al., 1997). Although these sites are essential for *SM22* transcription, SRF transactivates the *SM22* promoter only about 10-fold in nonmuscle cells, suggesting that an additional SRF cofactor may be required for full activity of the *SM22* promoter in muscle cells.

To investigate the potential requirement of the *SM22* CArG boxes for responsiveness to myocardin, we tested whether mutations in either CArG box in the context of the 1343 bp promoter impaired responsiveness to myocardin. Mutation of the distal CArG box (CArG-far) reduced responsiveness to myocardin by 5-fold, but this mutant promoter was still activated several hundred-fold. In contrast, mutation of the proximal CArG box (CArG-near) almost completely eliminated the ability to respond to myocardin, and mutation of both CArG boxes completely abolished activation by myocardin (Figure 4B).

The *ANF* promoter also contains two CArG boxes that are required for transcriptional activity in cardiomyocytes (Hines et al., 1999). As with the *SM22* promoter, mutation of the distal CArG box severely reduced activation by myocardin, and a promoter with both CArG boxes mutated was unable to respond to myocardin (Figure 4B). In contrast to the extreme sensitivity of the *SM22* and *ANF* promoters to myocardin, the c-*fos* promoter, which contains a single essential CArG box (Gilman et al., 1986), was not activated by myocardin (Figure 4A).

To determine whether the CArG box was sufficient to confer transcriptional responsiveness to myocardin, we tested whether myocardin could transactivate reporter genes containing four tandem copies of *SM22* CArG-near or the c-*fos* SRE linked to the E1b promoter. These reporters were transactivated several hundred-fold by myocardin, whereas SRF was only able to activate expression by 8-fold (Figure 4C). Together, these results demonstrate that myocardin is a much more potent transactivator than SRF, and that myocardin acts preferentially through multiple CArG boxes.

As shown in Figure 4D, myocardin was extremely sensitive to the level of SRF, such that at low concentrations of SRF expression plasmid, myocardin and SRF synergistically activated *SM22* transcription, whereas at higher concentrations of SRF, transcriptional activation by myocardin was reduced. Inhibition of myocardin-dependent transcription by excess SRF could be relieved by increasing the amount of myocardin. We conclude from these results that the ratio of SRF to myocardin is important for transcriptional activation by myocardin, and that exceeding an optimal ratio with an excess of SRF results in attenuation of myocardin activity.

Domain Mapping of Myocardin

To further define the mechanism for myocardin-dependent transcription, we analyzed the transcriptional activity of a series of amino- and carboxy-terminal deletion mutants (Figure 5A). Deletion of the first 66 residues (mutant NΔ66) did not impair transcriptional activity of myocardin with either the *SM22* or *ANF* promoters. In contrast, further amino-terminal deletion to residue 140 (mutant NΔ140), which eliminates the basic region, resulted in a complete loss in transcriptional activity. The loss in activity of this mutant appears to be due to deletion of the basic region because an internal deletion mutant (Δbasic) lacking only the basic region was also unable to activate transcription. All further N-terminal deletions up to amino acid 615, as well as an internal deletion of the Q-rich domain (mutant ΔQ), also eliminated transcriptional activity (Figure 5A).

Deletion of residues 585–807 (mutant CΔ585) or 381–807 (CΔ381) also abolished all transcriptional activity. These results are consistent with the results of GAL4 fusions, which indicated the existence of a transcription

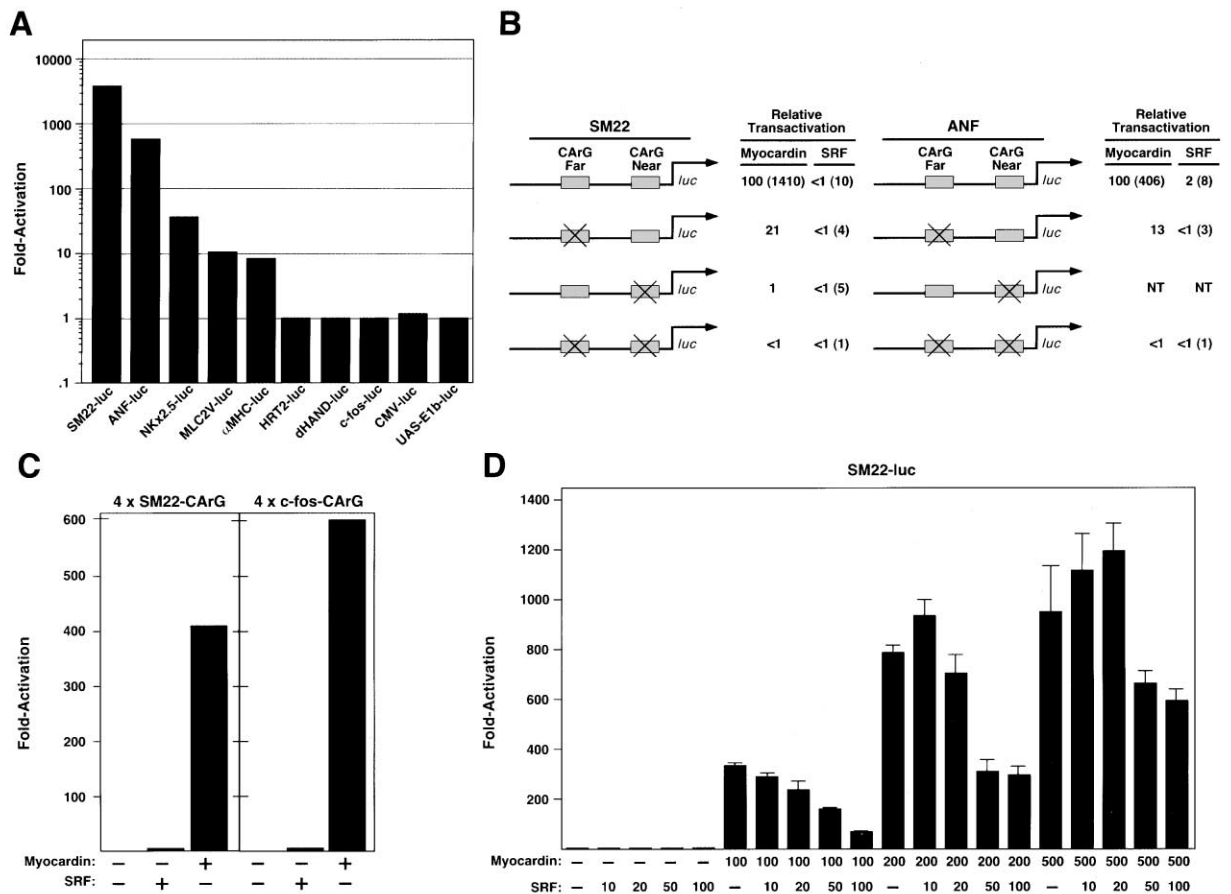


Figure 4. Activation of CArG Box-Dependent Promoters by Myocardin

(A) Activation of cardiac and smooth muscle reporter genes by myocardin in transfected COS cells. Values are expressed as the fold-increase in luciferase activity in the presence of myocardin expression plasmid compared to the level of activity with reporter plasmid alone.

(B) Analysis of the effects of CArG box mutations on activation of the *SM22* and *ANF* promoters by myocardin and SRF. Values are expressed as luciferase activity of each construct compared to the wild-type construct, which was assigned a value of 100. Values in parentheses indicate the fold-increase in activity in the presence of myocardin expression plasmid compared to the level of activity with reporter alone. NT, not tested.

(C) COS cells were transiently transfected with a luciferase reporter linked to the E1b basal promoter and four tandem copies of either CArG-near from the *SM22* promoter (left) or the c-*fos* SRE (right) and expression vectors for myocardin and SRF.

(D) COS cells were transiently transfected with the SM22-luciferase reporter and the indicated amounts (in ng) of myocardin and SRF expression plasmids. Results in (A), (B), and (C) are from representative experiments. Results in (D) are the average ± standard error of three independent experiments.

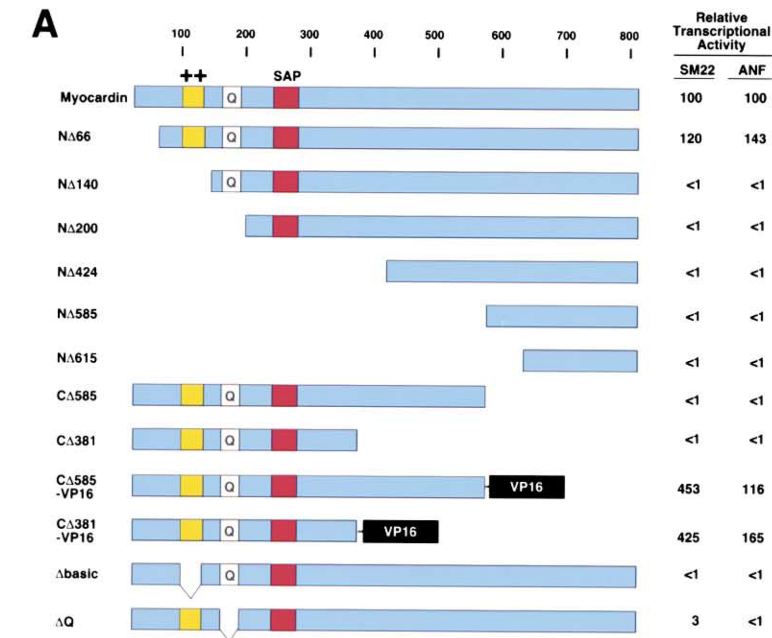
activation domain between residues 541 and the carboxyl terminus. Interestingly, when coexpressed with full-length myocardin, these carboxy-terminal deletion mutants acted in a dominant negative manner and severely impaired the ability of the wild-type protein to activate the *SM22* (Figure 5B) and *ANF* promoters (data not shown).

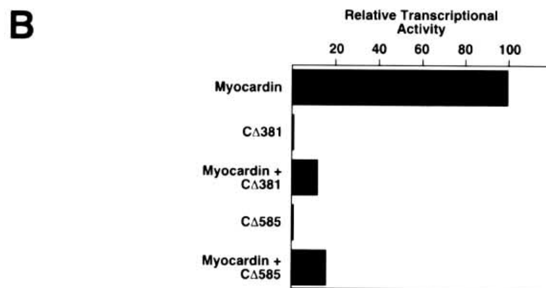
To determine whether the transactivation domain conferred specificity to myocardin, we fused the viral coactivator protein VP16 to the carboxy-terminal deletion mutants that were transcriptionally inactive. As shown in Figure 5A, myocardin-VP16 fusions containing residues 1–381 or 1–585 acted as potent activators of the *SM22* and *ANF* promoters. Together, these results suggest that the carboxyl terminus of myocardin functions as a general transcription activation domain, and that the basic and Q-rich domains near the amino terminus are required for directing the activation domain to CArG box-dependent promoters. The basic region also appears to be required for nuclear localization since deletion of this region resulted in relocalization of myocardin from the nucleus to the cytoplasm (data not shown).

We examined the functional importance of the SAP domain by introducing proline mutations into helix-1 or -2 (mutants PSF and PGH). These mutations had only a modest effect on the ability of myocardin to transactivate the *SM22* promoter, but they abolished activation of the *ANF* promoter. Similarly, deletion of the linker region between the two helices of the SAP domain, shown previously to be required for DNA binding by SAF-A (Kipp et al., 2000), had little effect on *SM22* activation, but eliminated *ANF* activation.

Myocardin Forms a Complex with SRF

To further determine the mechanism for CArG box-dependent transcriptional activation by myocardin, we





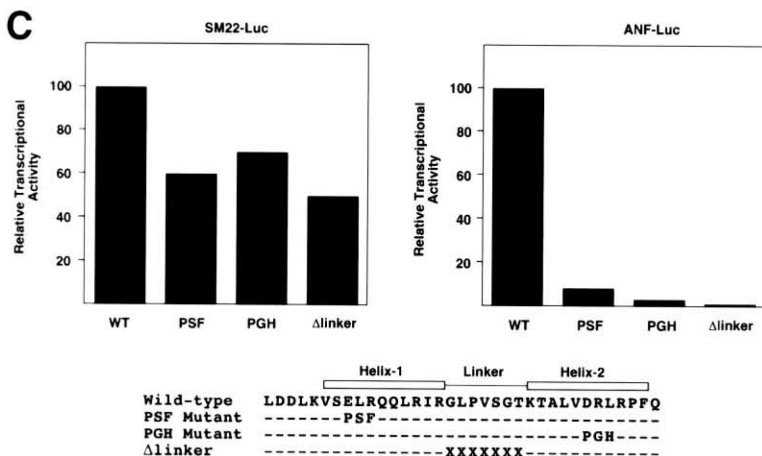


Figure 5. Mapping Domains of Myocardin Required for Activation of the *SM22* and *ANF* Promoters

(A) Deletion mapping of the domains of myocardin required for activation of the *SM22* and *ANF* promoters. All myocardin deletions contained a FLAG epitope at the amino terminus and their expression was confirmed by Western blot.

(B) Dominant negative effects of C-terminal deletion mutants in transfection assays. Equivalent amounts of expression plasmid for myocardin and myocardin mutants were transfected with the SM22-luciferase reporter plasmid and luciferase activity was determined.

(C) The indicated mutations were introduced into the SAP domain of myocardin and were used to test for transactivation of the SM22-luciferase and ANF-luciferase reporters. Values are expressed as luciferase activity of each construct compared to the wild-type construct, which was assigned a value of 100. Results of representative experiments are shown. Average values for assays varied by less than 5% of the mean.

tested whether myocardin translated in vitro could bind to the CArG boxes from the *SM22* promoter. SRF bound to both CArG boxes, but no binding of myocardin to either CArG box was detectable in gel mobility shift assays. However, myocardin in the presence of SRF gave rise to a prominent ternary complex with the CArG box sequence (Figure 6A). This ternary complex was supershifted by antibodies against SRF or FLAG-tagged myocardin. The total amount of SRF DNA binding was comparable in the presence and absence of myocardin, suggesting that association of SRF with myocardin does not alter the affinity of SRF for the CArG box. Myocardin and SRF also formed a ternary complex with the c-*fos* and *ANF* CArG boxes, the intensity of which correlated

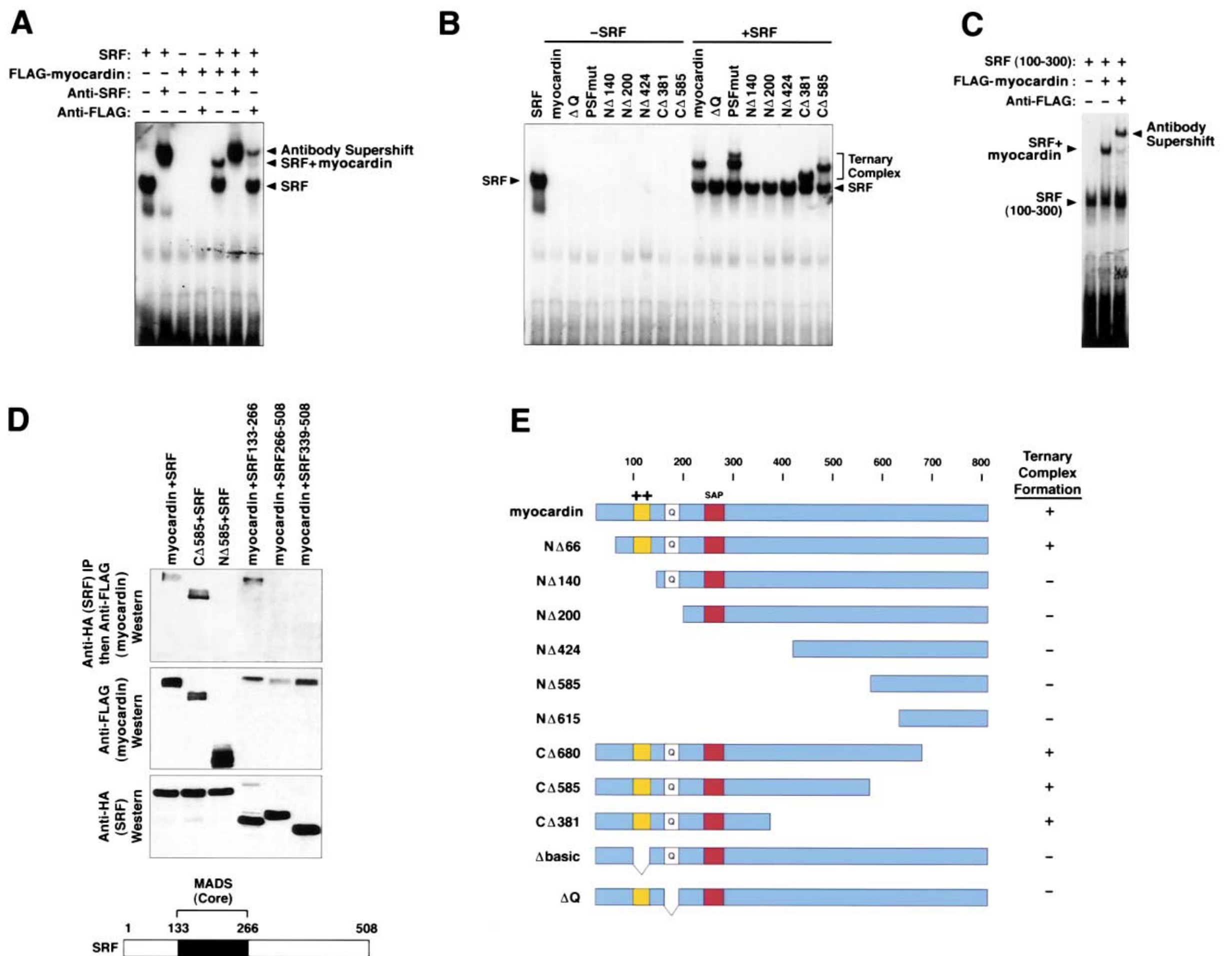


Figure 6. Ternary Complex Formation between Myocardin and SRF

(A) Gel mobility shift assays were performed with a ^{32}P-labeled oligonucleotide probe for SM22 CArG-near and in vitro translation products of FLAG-tagged myocardin in the presence and absence of SRF. Antibodies against SRF and the FLAG-epitope were included, as indicated.

(B) Gel mobility shift assays were performed as in (A) with SRF and wild-type and mutant forms of FLAG-tagged myocardin.

(C) Gel mobility shift assays were performed as in (A) with an SRF deletion mutant (SRF 100–300) lacking the amino and carboxyl termini.

(D) Coimmunoprecipitation of myocardin and SRF from transiently transfected COS cells. Immunoprecipitates were separated by SDS-PAGE and sequentially immunoblotted with anti-FLAG antibody (top panel). The same blot was then reprobed with anti-HA antibody to determine the presence of HA-tagged SRF (bottom panel). One-twentieth of the cell extract was directly immunoblotted with anti-FLAG antibody to detect the presence of FLAG-tagged myocardin (middle panel).

(E) Schematic diagrams of myocardin mutants showing the ability to form a ternary complex with SRF.

directly with the relative binding of SRF to the site (data not shown). The lack of obvious homology in the flanking sequences of these different CArG boxes suggests that myocardin associates directly with SRF and does not depend on specific DNA sequences for ternary complex formation (see below).

The region of myocardin required for ternary complex formation with SRF was determined using myocardin deletion mutants. Deletion of the amino-terminal 140 amino acids (NΔ140) abolished association with SRF, as did larger amino-terminal deletions (Figures 6B and 6D). In contrast, deletions from amino acid 381 to the carboxyl terminus did not affect SRF interaction (mutants CΔ381 and CΔ585). Deletion of the Q-rich domain or the basic region also abolished ternary complex formation, whereas mutation of the SAP domain (PSF mutant) did not. These findings are consistent with the interpretation that the amino terminus of myocardin confers transcriptional specificity by mediating association with SRF, whereas the carboxyl terminus activates transcription.

To determine whether myocardin interacted with the DNA binding or transcription activation domain of SRF, we performed gel mobility shift assays with an SRF deletion mutant encompassing the MADS domain but lacking the amino and carboxyl termini. This SRF mutant (SRF 100–300) bound the CArG box sequence and formed a ternary complex with myocardin (Figure 6C).

Association of myocardin and SRF was also readily detectable in coimmunoprecipitation assays of epitope-tagged proteins. Interaction was dependent on the amino-terminal region of myocardin, as demonstrated by the association of myocardin mutant CΔ585, but not NΔ585, with SRF (Figures 6D and 6E). The core MADS

domain of SRF (residues 133–266) was also necessary and sufficient to mediate association with myocardin in coimmunoprecipitation assays.

Together, these results demonstrate that myocardin interacts with SRF to form a stable ternary complex. The direct correlation between the ability of myocardin mutants to activate *SM22* and *ANF* transcription and to interact with SRF supports the conclusion that myocardin activates transcription via its association with SRF.

Inhibition of Cardiomyocyte Differentiation in *Xenopus* Embryos by Dominant Negative Myocardin

To investigate the functions of myocardin in vivo, we analyzed the effects of the dominant negative myocardin mutant CΔ585 on cardiac gene expression in *Xenopus* embryos, which express at least two myocardin-like genes (D. Wang, L. Sutherland, and E. Olson, unpublished data). Single dorsal-vegetal blastomeres of 8-cell *Xenopus* embryos were injected with synthetic mRNA encoding CΔ585. Due to the fate map of the 8-cell *Xenopus* embryo, injections targeted to the heart always result in coexpression in the developing somites. Injected embryos were assayed for expression of muscle markers by whole-mount in situ hybridization at the early tailbud stage. At this stage, the heart-forming region consists of two symmetrical domains of tissue located on either side of the ventral midline, allowing the uninjected side of the embryo to serve as an internal control for the injected, experimental side. The distribution of mutant myocardin transcripts was visualized by coinjection of GFP mRNA.

The side of the embryo injected with CΔ585 mRNA showed a dramatic reduction in expression of transcripts for cardiac α-actin and α-tropomyosin (Figures 7A and 7B), which are markers for cardiac and skeletal myocyte differentiation. The effects of dominant negative myocardin on cardiac differentiation were highly specific, as demonstrated first by the normal overall appearance of the embryo and second by the wild-type expression of these markers in the somite myotomes which also received the CΔ585 transcript. Expression of troponin I, a heart-specific differentiation marker, was also inhibited by CΔ585 (data not shown). In addition, expression of Nkx2.5 was severely reduced in CΔ585-injected embryos. The overall morphology of the embryos expressing CΔ585 was normal, indicating that the mutant did not affect general aspects of embryogenesis. We attempted to rescue CΔ585-injected embryos by injection of wild-type myocardin mRNA. However, overexpression of wild-type myocardin resulted in severe morphologic abnormalities and embryonic lethality.

Expression of the CΔ585 mutant resulted in a dose-dependent reduction in expression of cardiac markers, such that approximately 90% of injected embryos exhibited a reduction or complete elimination of cardiac gene expression on the injected side (Table 1). In contrast, defects were observed in 5% or less of embryos injected with the GFP marker alone. Together, these results demonstrate that dominant negative myocardin can specifically interfere with myocardial differentiation in vivo.

Discussion

Myocardin is a novel SRF cofactor with the following properties that implicate it in SRF-dependent activation of muscle gene expression: (1) during embryogenesis, myocardin is expressed in cardiac and a subset of smooth muscle cells, concomitant with the expression of SRF-dependent muscle genes. (2) Myocardin is an extraordinarily potent transcriptional activator that enhances transcriptional activation by SRF. (3) Transcriptional activation by myocardin is extremely sensitive to the level of SRF. If SRF levels exceed a narrow range, transcriptional activity of myocardin is diminished. (4) Transcriptional activation by myocardin is mediated by the CArG box sequence. (5) Myocardin associates with the CArG box sequence only in the presence of SRF, and mutants of myocardin that cannot interact with SRF cannot activate transcription. Conversely, mutants of myocardin that can associate with SRF, but which lack the transactivation domain, prevent CArG box-dependent transcription by wild-type myocardin. (6) The latter type of dominant negative myocardin mutant interferes with cardiomyocyte differentiation in vivo.

Myocardin Is Required for Heart Development

Myocardin expression is initiated in the cardiac crescent at the time of cardiogenic specification and is maintained throughout the atrial and ventricular chambers of the heart during later development. Myocardin is also expressed in embryonic vascular smooth muscle cells within the cardiac outflow tract and aortic arch arteries, as well as in developing visceral smooth muscle cells of the respiratory, gastrointestinal, and genitourinary tracts. However, myocardin is not expressed in the coronary vasculature or the dorsal aorta, nor in skeletal muscle cells. Since CArG boxes have been shown to be required for expression of muscle genes in all muscle cell types, there must be myogenic SRF cofactors in addition to myocardin. Whether other myocardin-related factors identified as EST or genomic sequences (Figure 1B) play this role remains to be determined.

Our results demonstrate that a dominant negative myocardin mutant that can associate with SRF, but cannot activate transcription, specifically interferes with cardiomyocyte differentiation when expressed in the dorsal-vegetal blastomere that gives rise to the cardiac lineage in *Xenopus*. Intriguingly, dominant negative myocardin did not inhibit skeletal muscle differentiation in the somite myotomes, as assayed by expression of cardiac α-actin and α-tropomyosin. These findings are consistent with the lack of myocardin expression in the skeletal muscle lineage and suggest that other factors in skeletal muscle interfere with the dominant negative effects of the myocardin mutant. The precise role of myocardin in vascular development remains to be determined.

The finding that dominant negative myocardin inhibits the expression of multiple cardiac structural genes, as well as *Nkx2.5*, which is normally expressed prior to cardiomyocyte differentiation, suggests that myocardin plays an essential early role in the cardiac developmental pathway. The effects of dominant negative myocardin in vivo suggest that this mutant sequesters SRF and prevents endogenous myocardin from forming a transcriptionally active complex. Accordingly, SRF is highly expressed in the early heart (Croissant et al., 1996). However, we cannot rule out the possibility that myocardin may have other partners in vivo that account for the inhibition of cardiac differentiation by this mutant.

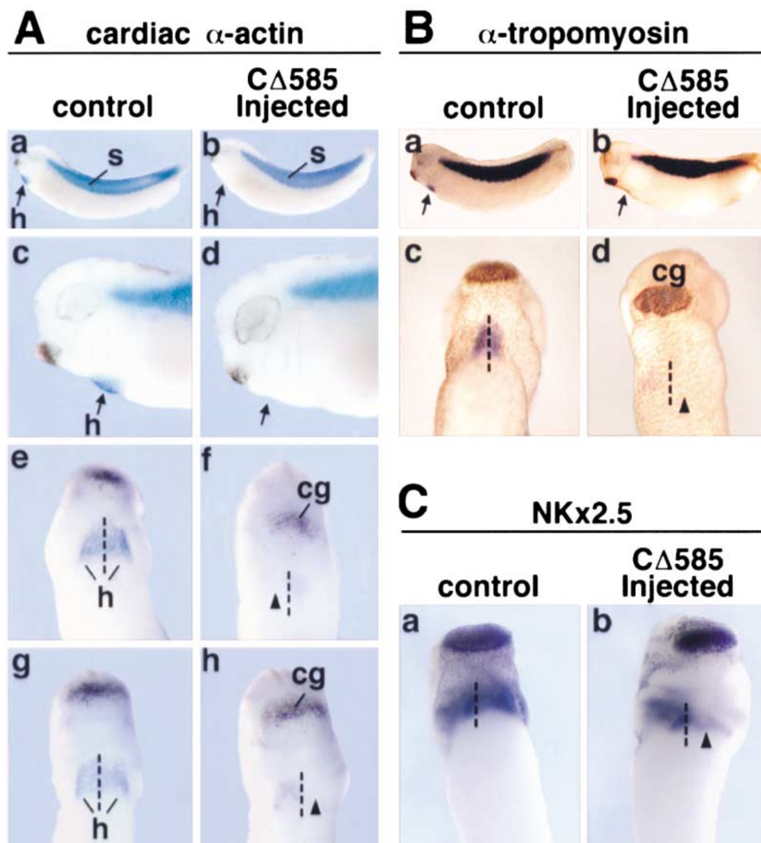


Figure 7. Inhibition of Myocardial Gene Expression by Expression of Dominant Negative Myocardin in *Xenopus* Embryos

Xenopus embryos at the 8-cell stage were injected with mRNA encoding the CΔ585 mutant of myocardin and GFP or GFP alone (control) and were assayed for expression of (A) cardiac α-actin, (B) α-tropomyosin, or (C) Nkx2.5 at stage 28 by whole-mount in situ hybridization. (A, e–h), (B, c–d), and (C) show ventral views; other panels show lateral views. The dotted line bisects the symmetrical heart-forming region. Arrowheads show the heart on the injected side, which displays a reduced level of expression of cardiac markers. cg, cement gland; h, heart; s, somites.

Previous studies showed that dominant negative Nkx2.5 mutants were able to prevent cardiac development in injected *Xenopus* embryos (Fu et al., 1998; Grow and Krieg, 1998). Thus, because the dominant negative myocardin mutant interferes with Nkx2.5 expression, its effects could be mediated by downregulation of Nkx2.5 or by interference with CArG box-dependent cardiac structural genes, or both.

Table 1. Summary of Dominant Negative Myocardin Phenotypes in Injected *Xenopus* Embryos

mRNA	Normal	Reduced/ Eliminated	# Embryos Assayed
α-Cardiac Actin			
Control	39 (95%)	2 (5%)	41
CΔ585 (200 pg)	8 (29%)	20 (71%)	28
CΔ585 (500 pg)	2 (9%)	21 (91%)	23
α-Tropomyosin			
Control	41 (95%)	2 (5%)	43
CΔ585 (150 pg)	9 (27%)	25 (73%)	34
CΔ585 (300 pg)	7 (20%)	28 (80%)	35
Nkx2.5			
Control	32 (97%)	1 (3%)	33
CΔ585 (200 pg)	7 (33%)	14 (67%)	21

Single dorsal-vegetal blastomeres of 8-cell *Xenopus* embryos were injected with the indicated amount of mRNA encoding the CΔ585 mutant of myocardin and 300 pg of GFP mRNA. Controls received only GFP mRNA. At stage 28, embryos were stained for expression of α-cardiac actin, α-tropomyosin, or Nkx2.5 by whole-mount in situ hybridization, and the extent of heart formation on the injected side was determined.

Coregulation of Muscle Gene Expression by SRF and Myocardin

In contrast to the association of SRF with p62TCF, which requires contact of TCF with a specific DNA sequence flanking the CArG box (Shaw et al., 1989), myocardin and SRF can form a complex in the absence of DNA. Association with myocardin does not change the DNA binding activity of SRF, suggesting that the enhanced transcriptional activity of SRF upon association with myocardin is due to the recruitment of the potent transcription activation domain of myocardin, rather than to an increase in DNA binding affinity of SRF. Myocardin interacts with the MADS domain region of SRF, which is structurally similar to that of MEF2 (Santelli and Richmond, 2000). However, we have detected no interaction between myocardin and MEF2 (data not shown).

Myocardin appears to require a very precise level of SRF for maximal transcriptional activity; elevation of SRF above a narrow range results in suppression of myocardin-dependent transcription. Since SRF is a much weaker activator than myocardin and can associate with myocardin in the absence of DNA binding, excess SRF would be expected to diminish transcriptional activation by myocardin through competition with SRF bound to DNA target sites, a phenomenon known as "squelching" (Ptashne, 1988). In support of this interpretation, previous studies have demonstrated that transcriptional activation by SRF is extremely sensitive to

SRF levels and that high amounts of SRF can inhibit activation by SRF, as well as other activators (Prywes and Zhu, 1992). These findings have been interpreted to indicate that excess SRF titrates out a common coactivator required for transcription.

The remarkable potency in which myocardin enhances SRF-dependent transcription raises interesting questions about the specificity of target gene activation. For example, different CArG box-dependent muscle genes exhibit different expression patterns; some are specific for a particular type of muscle cell (e.g., cardiac, skeletal, or smooth), and there is even specificity of expression within subsets of muscle cells of a given lineage. These unique expression patterns suggest that additional factors, either positive or negative, are likely to modulate the activity of myocardin and SRF.

Since growth-regulated genes controlled by CArG boxes are downregulated in post-mitotic differentiated muscle cells, there must be mechanisms that render them nonresponsive to myocardin, just as there must be mechanisms that prevent activation of SRF-dependent muscle genes by growth signals. Such differential responsiveness is likely to be dependent on the positions and numbers of CArG boxes within individual promoters and the other factors that bind nearby sites. Many muscle-specific genes are regulated by pairs of CArG boxes that act cooperatively (Chow and Schwartz, 1990; Lee et al., 1991; Mack and Owens, 1999), whereas many growth-regulated genes, such as *c-fos,* are controlled by single CArG boxes. Our findings demonstrate that mutation of a single CArG box in the *SM22* promoter results in a greater than 100-fold reduction in responsiveness to myocardin. Thus, a requirement for multiple CArG boxes to confer maximal sensitivity to myocardin could contribute to muscle specificity.

SRF has also been shown to activate cardiac genes in association with Nkx2.5 (Chen and Schwartz, 1996) and the zinc-finger protein GATA4 (Belaguli et al., 2000; Morin et al., 2001). However, there are several significant differences between the effects of myocardin and these other factors on SRF-dependent transcription: (1) myocardin potently activates transcription through the CArG box, whereas Nkx2.5 and GATA factors do not activate CArG box transcription when expressed alone in transfected cells. (2) Transcriptional activation by SRF with Nkx2.5 or GATA factors is relatively modest compared to activation by myocardin and SRF. (3) SRF does not appear to form stable DNA binding ternary complexes on the CArG box with Nkx2.5 or GATA4.

Regulation of Transcription and Chromosomal Organization by SAP Domain Proteins

SAP domains have been identified in a variety of proteins involved in RNA processing and transcriptional control (Aravind and Koonin, 2000). Recently, the SAP domain of SAF-A was shown to mediate binding to SARs/MARs (Kipp et al., 2000). These specialized AT-rich DNA sequences, usually between 300 and 3000 bp long, are thought to partition the genome into topologically independent loops. Binding of the SAP domain of SAF-A to SARs has been proposed to create chromatin loops that affect the expression of adjacent genes. While myocardin might have a role in chromatin remodeling, its primary function appears to be as a transcriptional activator because it contains a potent transcriptional activation domain and it activates transfected CArG box-dependent reporters that would not be expected to require complex alterations in chromatin conformation for expression.

The behavior of SAP domain mutants of myocardin suggests that transcriptional activation of the *SM22* and *ANF* promoters may involve different mechanisms. Whereas the SAP domain was dispensible for *SM22* activation, it was essential for *ANF* activation. Since the SAP domain is not required for association with SRF, the ability of SAP domain mutants to discriminate between the *SM22* and *ANF* promoters suggests that this domain may associate with other transcriptional regulators that differ between these two promoters.

Potential Roles for Myocardin in Signal-Dependent Gene Expression Programs

In addition to the role of SRF in regulation of muscle gene expression during development, SRF has also been implicated in reactivation of fetal cardiac genes in hypertrophic cardiomyocytes (Paradis et al., 1996). A variety of calcium-dependent signaling pathways have been shown to stimulate SRF activity in response to hypertrophic stimuli. SRF activity is also enhanced in response to changes in actin dynamics, which is likely to have important effects on muscle gene expression (Sotiropoulos et al., 1999). Whether such signals regulate the expression or activity of myocardin is an interesting issue for the future.

Experimental Procedures

Cloning and Bioinformatics

We screened for novel cardiac-specific genes in silico by performing a BLAST search with ESTs from mouse embryonic heart cDNA libraries in the database. Two criteria were used to identify novel cardiac genes: (1) sequences had to have been found only in cardiac cDNA libraries, and (2) sequences had to be novel, without any known conserved domains. From this search, we identified 20 candidate cardiac-specific genes that were unknown. The corresponding cDNA fragments were cloned by polymerase chain reaction (PCR) and used as probes to perform whole-mount in situ hybridization on E9.5 mouse embryos. One of the cDNA sequences identified in this screen (accession number AI607474) corresponded to the 3′ untranslated region of the myocardin transcript.

In Situ Hybridization and Northern Analysis

Whole-mount and section in situ hybridization and Northern analyses were performed as described (Wang et al., 1999).

Generation of Myocardin Mutants

Myocardin expression plasmids were generated through conventional or PCR-based cloning. Site-directed mutagenesis was performed using the QuickChange kit (Stratagene). For mutant ΔQ, cDNA sequences encoding amino acids 139–192 were removed. For mutant Δbasic, cDNA sequences encoding amino acids 115–134 were removed.

Transfection Assays

All myocardin expression vectors were cloned in pcDNA3.1 (Invitrogen), which was driven by the CMV promoter. A CMV-driven human SRF eukaryotic expression vector (Chen and Schwartz, 1996) was used. The SM22-luciferase reporter contained the 1434 bp promoter and the CArG box mutations within it have been described (Li et al., 1997). The ANF-luciferase reporter contains the 638 bp promoter (Sprenkle et al., 1995). The *ANF* CArG box mutations were introduced by changing the consensus sequences as follows: CArG-

far from CCATAAAAGG to CAATAAAAGC, and CArG-near from CCA AATATGG to CGAATTCTGG. The α-*MHC* (Lu et al., 1999), *MLC-2V* (Zhu et al., 1991), *HRT2* (Nakagawa et al., 2000), *dHAND* (McFadden et al., 2000), and *Nkx2.5* (Lien et al., 1999) luciferase reporters have been described.

Transfections were performed with FuGENE6 (Roche) according to manufacturer's instructions. Unless otherwise indicated, 100 ng of reporter and 100 ng of each activator plasmid were used. The total amount of DNA per well was kept constant by adding the corresponding amount of expression vector without a cDNA insert. CMV-lacZ was used as an internal control to normalize for variations in transfection efficiency. All the proteins were expressed at a very similar level as confirmed by Western blot.

Immunostaining and Immunoprecipitation

Immunostaining and coimmunoprecipitation experiments were performed as described previously (Lu et al., 1999).

Gel Mobility Shift Assays

SRF and FLAG-tagged myocardin were translated in vitro with a TNT T7-coupled reticulocyte lysate system (Promega). The sequence of the top strand oligonucleotide for SM22 CArG-far was: CTAGGTTT CAGGGTCCTG<u>CCATAAAAGG</u>TTTTTCCCGGCCGCC. Gel mobility shift assays were performed as described (Chang et al., 2001).

Xenopus Injections and Analysis

Xenopus laevis embryos were collected using standard techniques and were staged according to Nieuwkoop and Faber (1994). Synthetic mRNAs encoding a truncated myocardin protein CΔ585 were microinjected into the left dorsal-vegetal blastomere of 8-cell embryos using a variable automatic injector, as described (Cleaver et al., 1996). Injected embryos were cultured at 18°C in 3% Ficoll/100% Steinberg's buffer for 12 hr and then in 20% Steinberg's buffer until heart differentiation had occurred. In the standard protocol, between 150 and 500 pg of myocardin mRNA was coinjected with 300 pg of GFP mRNA. Injection results were assayed by whole-mount in situ hybridization (Harland, 1991), using *Xenopus* α-tropomyosin, cardiac α-actin, or Nkx2.5 probes.

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References

Aravind, L., and Koonin, E.V. (2000). SAP-a putative DNA-binding motif involved in chromosomal organization. Trends Biochem. Sci. *25*, 112–114.

Belaguli, N.S., Sepulveda, J.L., Nigam, V., Charron, F., Nemer, M., and Schwartz, R.J. (2000). Cardiac tissue enriched factors serum response factor and GATA-4 are mutual coregulators. Mol. Cell. Biol. *20*, 7550–7558.

Black, B.L., and Olson, E.N. (1998). Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. Annu. Rev. Cell. Dev. Biol. *14*, 167–196.

Chang, P.S., Li, L., McAnally, J., and Olson, E.N. (2001). Muscle-specificity encoded by specific serum response factor binding sites. J. Biol. Chem. *276*, 17206–17212.

Chen, C.Y., and Schwartz, R.J. (1996). Recruitment of the Tinman homolog Nkx-2.5 by serum response factor activates cardiac alpha-actin gene transcription. Mol. Biol. Cell *16*, 6372–6384.

Chow, K.L., and Schwartz, R.J. (1990). A combinatorial of closely associated positive and negative cis-acting promoter elements regulates transcription of the skeletal alpha-actin gene. Mol. Cell. Biol. *10*, 528–538.

Cleaver, O.B., Patterson, K.D., and Krieg, P.A. (1996). Overexpression of the tinman-related genes XNkx-2.5 and XNkx-2.3 in Xenopus embryos results in myocardial hyperplasia. Development *122*, 3549–3556.

Croissant, J.D., Kim, J.H., Eichele, G., Goering, I., Lough, J., Prywes, R., and Schwartz, R.J. (1996). Avian serum response factor expression restricted primarily to muscle cell lineages is required for α-actin gene transcription. Dev. Biol. *177*, 250–264.

Fu, Y., Yan, W., Mohun, T.J., and Evans, S.M. (1998). Vertebrate tinman homologues XNkx2–3 and XNkx2–5 are required for heart formation in a functionally redundant manner. Development *125*, 4439–4449.

Gille, H., Sharrocks, A.D., and Shaw, P.E. (1992). Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at the c-fos promoter. Nature *358*, 414–417.

Gilman, M.Z., Wilson, R.N., and Weinberg, R.A. (1986). Multiple protein-binding sites in the 5′-flanking region regulate c-fos expression. Mol. Cell. Biol. *6*, 4305–4316.

Grow, M.W., and Krieg, P.A. (1998). Tinman function is essential for vertebrate heart development: elimination of cardiac differentiation by dominant inhibitory mutants of the tinman-related genes, XNkx2–3 and XNkx2–5. Dev. Biol. *204*, 187–196.

Harland, R.M. (1991). In situ hybridization, an improved whole mount method for Xenopus embryos. In Methods in Cell Biology, B.K. Kay and H.G. Peng, eds. (San Diego, CA: Academic Press), pp. 685–695.

Hauschka, S.D. (1994). The embryonic origin of muscle. In Myology, 2nd edition, A.G. Engel and C. Franzini-Armstrong, eds. (New York: McGraw Hill, Inc.), pp.3–73.

Hines, W.A., Thorburn, J., and Thorburn, A. (1999). A low-affinity serum response element allows other transcription factors to activate inducible gene expression in cardiac myocytes. Mol. Cell. Biol. *19*, 1841–1852.

Kim, S., Ip, H.S., Lu, M.M., Clendenin, C., and Parmacek, M.S. (1997). A serum response factor-dependent transcriptional regulatory program identifies distinct smooth muscle cell sublineages. Mol. Cell. Biol. *17*, 2266–2278.

Kipp, M., Gohring, F., Ostendorp, T., Van Drunen, C.M., Van Driel, R., Przybylski, M., and Fackelmayer, F.O. (2000). SAF-box, a conserved protein domain that specifically recognizes scaffold attachment region DNA. Mol. Cell. Biol. *20*, 7480–7489.

Lee, T.C., Chow, K.L., Fang, P., and Schwartz, R.J. (1991). Activation of skeletal alpha-actin gene transcription: the cooperative formation of serum response factor-binding complexes over positive cis-acting promoter serum response elements displaces a negative-acting nuclear factor enriched in replicating myoblasts and nonmyogenic cells. Mol. Cell. Biol. *11*, 5090–5100.

Li, L., Miano, J.M., Cserjesi, P., and Olson, E.N. (1996). SM22 alpha, a marker of adult smooth muscle, is expressed in multiple myogenic lineages during embryogenesis. Circ. Res. *78*, 188–195.

Li, L., Liu, Z., Mercer, B., Overbeek, P., and Olson, E.N. (1997). Evidence for serum response factor-mediated regulatory networks governing SM22alpha transcription in smooth, skeletal, and cardiac muscle cells. Dev. Biol. *187*, 311–321.

Lien, C.L., Wu, C., Mercer, B., Webb, R., Richardson, J.A., and Olson, E.N. (1999). Control of early cardiac-specific transcription of Nkx2–5 by a GATA-dependent enhancer. Development *126*, 75–84.

Lints, T.J., Parsons, L.M., Hartley, L., Lyons, I., and Harvey, R.P. (1993). Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. Development *119*, 419–431.

Lu, J.R., McKinsey, T.A., Xu, H., Wang, D.Z., Richardson, J.A., and Olson, E.N. (1999). FOG-2, a heart- and brain-enriched cofactor for GATA transcription factors. Mol. Cell. Biol. *19*, 4495–4502.

Mack, C.P., and Owens, G.K. (1999). Regulation of smooth muscle alpha-actin expression in vivo is dependent on CArG elements within the 5′ and first intron promoter regions. Circ. Res. *84*, 852–861.

Marais, R., Wynne, J., and Treisman, R. (1993). The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cell *73*, 381–393.

McFadden, D.G., Charite, J., Richardson, J.A., Srivastava, D., Firulli, A.B., and Olson, E.N. (2000). A GATA-dependent right ventricular enhancer controls dHAND transcription in the developing heart. Development *127*, 5331–5341.

Morin, S., Paradis, P., Aries, A., and Nemer, M. (2001). Serum response factor-GATA ternary complex required for nuclear signaling by a G-protein coupled receptor. Mol. Cell. Biol. *21*, 1036–1044.

Nakagawa, O., McFadden, D.G., Nakagawa, M., Yanagisawa, H., Hu, T., Srivastava, D., and Olson, E.N. (2000). Members of the HRT family of basic helix-loop-helix proteins act as transcriptional repressors downstream of Notch signaling. Proc. Natl. Acad. Sci. USA *97*, 13655–13660.

Nieuwkoop, P.D., and Faber, J. (1994). Normal Table of Xenopus laevis (New York: Garland Publishing).

Norman, C., Runswick, M., Pollock, R., and Treisman, R. (1988). Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-fos serum response element. Cell *55*, 989–1003.

Paradis, P., MacLellan, W.R., Belaguli, N.S., Schwartz, R.J., and Schneider, M.D. (1996). Serum response factor mediates AP-1-dependent induction of the skeletal alpha-actin promoter in ventricular myocytes. J. Biol. Chem. *271*, 10827–10833.

Prywes, R., and Zhu, H. (1992). In vitro squelching of activated transcription by serum response factor: evidence for a common coactivator used by multiple transcriptional activators. Nucleic Acids Res. *20*, 513–520.

Ptashne, M. (1988). How eukaryotic transcriptional activators work. Nature *335*, 683–689.

Reecy, J., Belaguli, N.S., and Schwartz, R.J. (1998). SRF/homeobox protein interactions. In Heart Development, R. Harvey and N. Rosenthal, eds. (San Diego, CA: Academic Press), pp. 273–290.

Santelli, E., and Richmond, T.J. (2000). Crystal structure of MEF2A core bound to DNA at 1.5 Å resolution. J. Mol. Biol. *297*, 437–449.

Shaw, P.E., Schroter, H., and Nordheim, A. (1989). The ability of a ternary complex to form over the serum response element correlates with serum inducibility of the human c-fos promoter. Cell *56*, 563–572.

Shore, P., and Sharrocks, A.D. (1995). The MADS-box family of transcription factors. Eur. J. Biochem. *229*, 1–13.

Sotiropoulos, A., Gineitis, D., Copeland, J., and Treisman, R. (1999). Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. Cell *98*, 159–169.

Sprenkle, A.B., Murray, S.F., and Glembotski, C.C. (1995). Involvement of multiple Cis-elements in α-adrenergic agonist-inducible ANF transcription. Roles for SREs and an Sp-1-like element. Circ. Res. *77*, 1060–1069.

Treisman, R. (1994). Ternary complex factors: growth factor regulated transcriptional activators. Curr. Opin. Genet. Dev. *4*, 96–101.

Wang, D.Z., Reiter, R.S., Lin, J.L., Wang, Q., Williams, H.S., Krob, S.L., Schultheiss, T.M., Evans, S., and Lin, J.J. (1999). Requirement of a novel gene, Xin, in cardiac morphogenesis. Development *126*, 1281–1294.

Zhu, H., Garcia, A.V., Ross, R.S., Evans, S.M., and Chien, K.R. (1991). A conserved 28-base-pair element (HF-1) in the rat cardiac myosin light-chain-2 gene confers cardiac-specific and alpha-adrenergic-inducible expression in cultured neonatal rat myocardial cells. Mol. Cell. Biol. *11*, 2273–2281.

Accession Numbers

The deduced amino acid sequence of mouse myocardin was deposited in GenBank under accession number AF384055.