MUSCLE-SPECIFIC REGULATION OF SERUM RESPONSE FACTOR BY

DIFFERENTIAL DNA BINDING AFFINITY AND

COFACTOR INTERACTIONS

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To my family: Mom, Dad, Derek, and Ivan.

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by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

May, 2001

ACKNOWLEDGEMENTS

So many people have helped me during my years in graduate school. My mentor, Eric Olson, is my model of the ideal scientist. He taught me how to ask the key questions, and just as importantly, how to ask the next questions. He was always around whenever I needed guidance, but he also provided enough space to let me learn how to work and think independently. Without the help of Da-Zhi Wang, I would not have a thesis. He cloned Myocardin and performed the initial characterization experiments, and graciously let me work on the SM22 aspect of the project, which grew into the second half of my dissertation. Our collaboration was both productive and exciting, and I greatly appreciate his extensive scientific knowledge and his upbeat attitude. Zhigao Wang and Lilian Sutherland also work on Myocardin and have shared ideas and data. The transgenic technician for our lab is John McAnally, and his skill at injections greatly advanced my thesis work. Li Li made the 1343-SM22lacZ reporter mice and also provided the SM22 CArG box mutant luciferase reporters. Jim Richardson and his histology core group did the sectioning of the SM22 and c-fos lacZ embryos and the Myocardin *in situ* experiments. Alisha Tizenor helped make many of the figures in this dissertation and in my papers. She was always eager to share her expertise in graphics, and everything I know about Adobe Photoshop I learned from her. Robin Downing and Carolyn Paulk deserve special thanks for taking care of all the administrative red tape for the MSTP students and also for listening whenever I needed to talk. I would like to thank my thesis committee, Ray MacDonald, Jane Johnson, and Tom Sato for their helpful suggestions and critiques, and their neverending patience. Renee Valdez is my benchmate, and we have

had innumerable discussions about every topic imaginable during the three years we have been working next to each other. She is a great friend, and I am thrilled that we are going back to medical school together. My husband, Ivan Liang, has been a constant source of love and encouragement. He makes me happy and keeps me sane. Finally, my mom and dad are wonderful parents. They always had confidence in me, even during the times when I was convinced there was no light at the end of the tunnel. Words cannot express my gratitude.

Priscilla Chang

May, 2001

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Publication No.

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The University of Texas Southwestern Medical Center at Dallas, 2001

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Serum response factor (SRF) is a MADS-box transcription factor that regulates muscle-specific and growth factor-inducible genes by binding the CArG box consensus sequence $CC(A/T)_6GG$. Because SRF expression is not muscle-restricted, its expression alone cannot account for the muscle-specificity of some of its target genes. To further understand the role of SRF in muscle-specific transcription, two distinct approaches were taken.

First, tandem multimers of different CArG boxes with flanking sequences were analyzed in transgenic mice. CArG elements from the *SM22* and *skeletal α-actin* promoters directed highly restricted expression in developing smooth, cardiac, and skeletal muscle cells during early embryogenesis. In contrast, the CArG box and flanking sequences from the *c-fos* promoter directed expression throughout the embryo, with no preference for muscle cells. Systematic swapping of the core and flanking sequences of the *SM22* and *c-fos* CArG boxes revealed that cell type-specificity was dictated in large part by sequences immediately flanking the CArG box core. Sequences that directed widespread expression bound SRF more strongly than those that directed muscle-restricted expression. Therefore, sequence variations among CArG boxes influence cell type-specificity of expression and account, at least in part, for the ability of SRF to distinguish between growth factor-inducible and muscle-specific genes *in vivo*.

Second, a novel transcriptional cofactor for SRF called Myocardin was characterized. Myocardin belongs to the SAP domain family of nuclear proteins, is expressed specifically in cardiac and smooth muscle cells, and is a potent activator of cardiac and smooth muscle genes, including SM22. Myocardin activates through CArG boxes, and its activation is dependent on its interaction with the MADS box domain of SRF. Myocardin is the founding member of a new class of muscle-specific transcription factors and provides another mechanism whereby SRF can convey myogenic activity to muscle-specific genes.

These results describe two mechanisms for muscle-specific activation of target genes by SRF. Muscle-specific genes contain CArG boxes with relatively low affinities for SRF, and thus are only able to respond to the high levels of SRF found in muscle. Also,

Myocardin, a muscle-specific transcription factor, is able to associate with SRF and cooperatively activate transcription of muscle genes.

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ANF	atrial natriuretic factor
bHLH	basic helix-loop-helix
CArG	CC (A+T rich)$_6$ GG
CBP	CREB binding protein
cDNA	complimentary deoxyribonucleic acid
CREB	cyclic AMP response element binding protein
dHAND	deciduum, heart, autonomic nervous system, neural crest derivatives
DNA	deoxyribonucleic acid
E	embryonic day
eHAND	extraembryonic tissues, heart, autonomic nervous system, neural crest derivatives
FBS	fetal bovine serum
HDAC4, 5	histone deacetylase 4, 5
HMG	high-mobility-group
hsp68	heat shock protein 68
MADS	MCM1, Agamous, Deficiens, SRF
MAP	mitogen activated protein
MAR	matrix attachment region
MCK	muscle creatine kinase
MEF	myocyte enhancer factor
MHC	myosin heavy chain

MLC	myosin light chain
MRF4	muscle regulatory factor 4
NF-AT	nuclear factor of activated T cells
PIAS	protein inhibitor of activated STAT
PBS	phosphate buffered saline
PCR	polymerase chain reaction
SAP	SAF A/B, Acinus, PIAS
SAR	scaffold attachment region
SRF	serum response factor
STAT	signal transducer and activator of transcription
TCF	ternary complex factor
TnT	transcription and translation
VP16	viral protein 16

CHAPTER I

INTRODUCTION

The three major muscle cell types, skeletal, cardiac, and smooth, express overlapping but distinct sets of muscle-specific genes, and exhibit unique contractile and physiologic properties. How these different types of muscle cells arise during development, and what genes are important in these developmental pathways, are questions that are just beginning to be answered. Many human diseases are due to perturbations in normal muscle development or regulation, including congenital abnormalities such as muscular dystrophy or valvular and septal cardiac defects, and problems acquired later in life such as atherosclerosis or heart failure. Understanding muscle development is directly relevant to understanding the pathogenesis of these diseases and developing possible treatments.

Overview of Embryonic Muscle Development

The overall events that occur in the development of all mammalian embryos are very similar. Thus, the mouse is a good model system to study as a means to eventually understanding the processes that govern muscle development in humans. Mouse embryogenesis lasts 19 days from fertilization to birth (Kaufman, 1995; Theiler, 1989). Implantation of the embryo in the uterine epithelium occurs at E4.5, four and a half days after fertilization. At E7.0, the primitive streak appears and the first mesodermal cells begin to develop. Somites first start forming at E8.0, appearing in pairs on either side of the embryo and in a rostral to caudal direction. The heart also starts forming at this stage, condensing in

a curved band of cells across the front of the embryo. The paired dorsal aorta and the aortic arches of the embryonic vasculature also begin forming at this time. The heart begins to loop at E8.5, and different regions within the heart begin to develop into distinct structures, from which the four chambers of the heart and the great vessels will eventually arise. At E9.0, the heart is capable of beating regularly and continuously and the paired dorsal aorta begin to fuse, eventually forming a single aorta running down the midline of the embryo. The heart is still a looped tube at E10.5, although the septum is beginning to develop. By E11.0, the cervical somites are no longer visible, though the somites in the lower portion of the embryo are still clearly visible. At E13.0, the somites are visible only in the distal portion of the tail, and all the valves of the heart have now formed. At E15.0, the vasculature of the embryo is now set, and will not change substantially in conformation until after birth. The heart is also fully differentiated at this time, with all four chambers clearly recognizable. From this stage until birth at E19.0, the embryo continues to grow in size but no substantial changes occur in the cardiovascular system.

Skeletal Muscle Development

Skeletal muscle development is relatively well understood compared to what is known about the development of the heart or of smooth muscle. Most skeletal muscle cells originate from multipotent mesodermal precursor cells in the somites (reviewed in Lassar and Munsterberg, 1994; Yun and Wold, 1996). Somites are round epithelial structures that arise from paraxial mesoderm. Initally, somites form two distinct compartments, the dermomyotome and the sclerotome. The sclerotome is not involved in myogenesis; instead,

cells from the sclerotome eventually form the vertebrae and ribs. A certain population of cells from the dermomyotome exit the cell cycle and give rise to a third somitic compartment, the myotome. Cells from both the dermomyotomal and myotomal regions of the somite become muscle. Muscles of the trunk come from the myotome, while the tongue, diaphragm, and limb muscles develop from migrating cells originating from the dermomyotome. Head musculature is largely formed from migratory prechordial and cranial paraxial mesodermal cells. Later during embryogenesis, the dermomyotome matures into the dermotome, which gives rise to dermal structures.

Members of the MyoD basic helix-loop-helix (bHLH) family play key roles in the determination and differentiation of skeletal muscle. There are four myogenic bHLH proteins- myogenin, Myf5, MyoD, and MRF4. As with all the other members of the bHLH family, these proteins recognize DNA sites called E-boxes (CANNTG) (reviewed in Olson, 1990). To bind these sites, they form heterodimers with ubiquitous bHLH cofactors termed E proteins. All of these muscle regulatory factors have the ability to convert nonmuscle cells into skeletal muscle cells that express proper skeletal markers. Myf5 is the first of these muscle regulatory factors to be expressed in muscle precursor cells during embryonic mouse development, at approximately E8.0, and the other factors all come on within the next few days (reviewed in Rudnicki and Jaenisch, 1995). All four of the myogenic regulatory factors have been deleted in mice, individually and in combination. *Myogenin* null embryos have the most severe skeletal muscle phenotype, with significantly less skeletal muscle tissue than wild-type embryos (Hasty et al., 1993; Nabeshima et al., 1993). Myoblasts, however, are present where the muscle fibers should be, indicating that myogenin functions at the level of

differentiation of specified muscle precursor cells. Mice lacking either Myf5 or MyoD have no overt muscle defects, but *Myf5*/*MyoD* double knockout mice have a complete lack of both differentiated skeletal muscle and myoblasts (Braun et al., 1992; Rudnicki et al., 1992; Rudnicki et al., 1993). Also, no expression of myogenin, MRF4, or other skeletal muscle markers can be detected in these mice. Myf5 and MyoD therefore act as determination factors that specify muscle precursor cells. MRF4 null mice, like the Myf5 and MyoD single knockout mice, have overtly normal muscle development (Braun and Arnold, 1995; Patapoutian et al., 1995; Zhang et al., 1995). Interestingly, the *MRF4/MyoD* double mutant mice have a muscle differentiation phenotype similar to the *myogenin* null mice, suggesting that MRF4 and MyoD also function in muscle differentiation (Rawls et al., 1998). As evidenced by the different knockout phenotypes, the four vertebrate muscle regulatory factors have overlapping and partially redundant functions in determination and differentiation of skeletal muscle. The sole myogenic bHLH in *Drosophila*, *nautilus*, is expressed in both somatic precursor cells and differentiated muscle fibers (Michelson et al., 1990; Paterson et al., 1991). *Nautilus* mutants have disrupted muscle fiber formation, demonstrating that *nautilus* is required for the differentiation of muscle precursor cells (Keller et al., 1998; Misquitta and Paterson, 1999).

The myocyte enhancer factor 2 (MEF2) family is also important in skeletal muscle development. The four vertebrate MEF2 proteins, MEF2A, MEF2B, MEF2C, and MEF2D, belong to the MADS box family of transcription factors, and are highly expressed in all three muscle lineages during embryogenesis, including skeletal muscle (reviewed in Black and Olson, 1998). MEF2C is expressed at E8.0, and is the first family member expressed in

skeletal muscle. The other MEF2 factors are expressed later in development, and all four factors continue to be expressed in adult skeletal muscle. Currently, only the *mef2c* null mutant has been published (Lin et al., 1997). These mice have severe cardiac defects, which precludes analysis of any potential skeletal muscle defects, and will be discussed later. Further studies need to be done to determine the *in vivo* roles of the MEF2 factors in vertebrate myogenesis. There is only a single MEF2 gene, *D-mef2*, in *Drosophila* (Lilly et al., 1994). Null embryos show a complete loss of differentiated muscle in all three lineages-somatic (skeletal), cardiac, and visceral (smooth) (Bour et al., 1995; Lilly et al., 1995). Although there is no muscle in these embryos, the myoblasts are normal, indicating that *D-mef2* functions at a later stage of muscle differentiation.

The MEF2 proteins interact with numerous other proteins, including the class II histone deacetylases HDAC4 and HDAC5, which are transcriptional repressors (Lu et al., 2000). HDAC4 and HDAC5 are expressed in skeletal and cardiac muscle and have overlapping expression patterns with the MEF2 factors. The HDACs are recruited to muscle promoters by MEF2, and inhibit MEF2-dependent transcriptional activation. HDAC4 and HDAC5 are also able to block the myogenic conversion ability of MyoD, and thus are negative regulators of muscle differentiation. Another set of factors that interact with the MEF2 proteins are the myogenic bHLHs. The MEF2 factors and the myogenic bHLHs synergistically activate transcription of muscle promoters, and MEF2 binding sites are frequently located near E-boxes (Wright et al., 1991). Synergy between the MEF2 and myogenic bHLH factors is mediated by direct interaction between the MADS and bHLH

regions (Molkentin et al., 1995). Clearly, combinatorial regulation between the MEF2 and myogenic bHLH proteins are central in the control of skeletal muscle development.

Cardiac Muscle Development

Although cardiac development is not as well understood as skeletal muscle development, recent discoveries have led to a better understanding of the mechanisms of cardiac muscle differentiation. Cardiac development begins when precursor cells located on either side of the embryo in the lateral plate mesoderm are specified to become cardioblasts. These specified precursor cells form a crescent shaped cardiogenic field across the ventral midline of the embryo. These cells then migrate towards the midline and form a linear heart tube. At this point the heart tube is already patterned into different compartments- the aortic sac, the conotruncus (outflow tract), right ventricle, left ventricle, and atria, though they are not morphologically identifiable. The linear heart tube then undergoes rightward looping, so that the chambers of the heart are roughly in the proper positions and the inflow and outflow tracts are aligned. Endothelial cells then migrate into cardiac cushions, which eventually fuse into the valves and septa which divide the heart into its familiar four chambers (reviewed in Olson and Srivastava, 1996; Srivastava and Olson, 2000).

The *Nkx2.5* homeobox gene is the earliest known marker of heart development in vertebrates (Komuro and Izumo, 1993; Lints et al., 1993). It is expressed in cardiac progenitor cells in the cardiac crescent at E7.75 and its expression in the heart continues throughout embryonic development. *Nkx2.5* null mice have defects at the looping heart tube stage, indicating that *Nkx2.5* plays a key role in cardiac development (Lyons et al., 1995). In fact, it

is surprising that these knockout mice survive to even this stage, because studies in flies indicate that this gene is absolutely required for cardiogenesis. Loss of function mutations of the *Drosophila* ortholog of *Nkx2.5*, *tinman*, result in the complete absence of the dorsal vessel, the fly equivalent of the heart (Bodmer, 1993). This difference suggests that other Nkx family members may play redundant or complementary roles in vertebrate heart development.

Some members of the zinc finger GATA transcription factors are also essential in cardiac development. GATA4, GATA5, and GATA6 are all expressed in cardiac progenitor cells during embryogenesis (reviewed in Charron and Nemer, 1999). Deletion of *GATA4* in mice results in severely deformed embryos that die at approximately E9.0, and do not form a linear heart tube (Kuo et al., 1997; Molkentin et al., 1997). In normal cardiac development, the cardiogenic cells migrate ventrally and fuse at the midline to form a heart tube, but in the *GATA*4 null mutant embryos, the cardiac precursor cells fail to migrate properly, although cardiac precursor cells are properly specified, and are even able to differentiate into cardiac structures. Studies in zebrafish have shown that GATA5 is also required for normal heart tube development and mutant embryos often have cardiac bifida (Reiter et al., 1999). In mice, *GATA5* null embryos are viable, and no heart abnormalities have been described (Molkentin et al., 2000), suggesting that functional redundancy between the different GATA factors may be present.

Two other factors that are important in cardiac development are the bHLH factors dHAND and eHAND. Both of these genes are expressed in cardiac progenitor cells and become restricted to specific segments of the linear heart tube. *dHAND* is expressed in the

region of the future right ventricle, while *eHAND* is expressed in the future left ventricle (Srivastava et al., 1997). As expected from its expression pattern, deletion of *dHAND* results in a hypoplastic right ventricle. *eHAND* null mice die before linear heart tube formation due to placental defects, though these mice would be predicted to also have left ventricular defects (Firulli et al., 1998). Zebrafish hearts have only two chambers, one atrium and one ventricle, and mutation of the single zebrafish HAND gene causes ablation of the ventricular chamber (Yelon et al., 2000).

As mentioned previously, *Mef2c* null mice have malformed hearts (Lin et al., 1997). Null *Mef2c* mice die at E9.5 from cardiac insufficiency, with unlooped heart tubes, hypoplastic ventricular precursor regions and pericardial effusion. MEF2C therefore is required in cardiac development as well as in skeletal muscle development.

NF-ATc/NF-ATc1/NF-AT2 (nuclear factor of activated T cells) is important in cardiac valve formation. NF-ATc is a member of a family of proteins which, in response to calcium signaling, are dephosphorylated by calcineurin, translocate to the nucleus, and activate target genes (reviewed in Rao et al., 1997). NF-ATc is expressed in numerous tissues, including skeletal and cardiac muscle. Deletion of NF-ATc in mice results in defects in valve formation, leading to embryonic death (de la Pompa et al., 1998; Ranger et al., 1998). Recently, the calcineurin/NFAT pathway has also been implicated in the induction of cardiac hypertrophy, suggesting another role for this pathway in cardiac development (Molkentin et al., 1998). Although knockout experiments have identified these factors as being essential for cardiac development, little is understood about the mechanisms of action of these factors, how these factors are regulated, or how these factors interact in the developing heart.

Smooth Muscle Development

In comparison to skeletal and cardiac muscle, very little is understood about smooth muscle development. This is at least partially due to the fact that smooth muscle cells, unlike skeletal and cardiac cells, display remarkable phenotypic plasticity. Smooth muscle serves extremely diverse functions in different tissues throughout the body, including the vasculature, the digestive tract, the urinary tract, and the respiratory system. Smooth muscle cells are able to reversibly modulate their phenotype in response to various stimuli, switching between a resting state expressing contractile proteins, to a synthetic proliferative state (Frid et al., 1992; Glukhova et al., 1991). In addition, smooth muscle cells in different tissues develop from different precursor populations found in multiple locations throughout the embryo. For example, smooth muscle cells of the aorta arise from mesenchymal neural crest cells, while smooth muscle cells of the coronary arteries are of mesodermal origin (reviewed in Schwartz et al., 1990). Visceral smooth muscle cells arise from distinct populations of local mesenchyme (Cunha et al., 1992).

Mef2c null mice, in addition to their cardiac malformations, also exhibit defects in smooth muscle development (Lin et al., 1998). The *Mef2c* mutant embryos have a disorganized nonfunctional vasculature, with a complete lack of differentiated smooth muscle cells. MEF2C therefore plays multiple roles during the development of all three muscle lineages.

Even though some factors required for skeletal, cardiac, and smooth muscle development have been found, much remains unknown. The molecular mechanisms of

muscle specification, development, and differentiation are still not well understood. Downstream transcription factors for all three muscle lineages are still being found and characterized, and the regulatory interactions between all of these factors have yet to be uncovered.

Serum Response Factor

Serum Response Factor (SRF) is a 67 kD protein that has been shown to play a central role in the regulation of numerous muscle genes. SRF is a member of the MADS box protein superfamily, an ancient and well-conserved family of transcription factors (reviewed in Treisman, 1995). The MADS box is named after the proteins MCM1, a yeast protein important in mating type determination, Agamous and Deficiens, plant proteins important in homeotic flower development, and SRF. The MADS box comprises of approximately 60 amino acids, and mediates DNA binding, homodimerization, and interaction with accessory proteins. The MADS box of SRF is located in the N-terminal region of the protein, and the activation domain is located in the C-terminal region. SRF is highly conserved among various vertebrate species including human, mouse, chick, zebrafish, and Xenopus, and is even similar to Drosophila SRF within the MADS box (Affolter et al., 1994; Belaguli et al., 1997; Croissant et al., 1996; Mohun et al., 1991; Norman et al., 1988; Vogel and Gerster, 1999).

SRF binds as a dimer to the DNA consensus sequence $CC(A/T)_6GG$, known as a CArG box (Norman et al., 1988). CArG boxes are found in the promoters of numerous muscle genes, including *cardiac α-actin*, *skeletal α-actin*, *muscle creatine kinase*, *smooth muscle myosin heavy chain*, *smooth muscle α-actin*, *smooth muscle calponin*, *telokin*, and *SM22*

(Blank et al., 1992; Chow and Schwartz, 1990; Herring and Smith, 1997; Katoh et al., 1994; Kim et al., 1997; Li et al., 1997; Miano et al., 2000; Mohun et al., 1989; Sternberg et al., 1988; Walsh, 1989). The CArG boxes are essential in the regulation of all of these muscle genes, as is the ability of SRF to bind to these CArG boxes. If the CArG boxes are mutated such that SRF is no longer able to bind, then these genes are no longer active.

However, how SRF specifically activates muscle genes is not clearly understood. In fact, SRF was first identified as a factor that conferred serum-inducibility to the *c-fos* proto-oncogene, hence its name (Norman et al., 1988). SRF activates immediate early response genes such as *c-fos* and *egr-1* in response to growth factor signals, by binding to serum response elements that contain CArG boxes. Full serum responsiveness is mediated by the association of SRF with ternary complex factor (TCF), later identified as the three proteins Elk-1, SAP-1, and SAP-2/ERP/NET (Dalton and Treisman, 1992; Giovane et al., 1994; Hipskind et al., 1991; Lopez et al., 1994). The TCF proteins are members of the Ets domain protein family, and share three regions of high homology (Price et al., 1995). TCF binds the Ets motif CAGGAT, located directly 5' of the *c-fos* CArG box, but only in the presence of SRF (Shaw et al., 1989). Ternary complex formation is dependent on the MADS box of SRF and the N-terminal conserved region of TCF. SRF-dependent transcription of *c-fos* in response to growth factor stimulation can be activated by two different signaling pathways. First, the TCF factors are direct targets of MAP kinase signaling pathways (reviewed in Treisman, 1994). Activation of the Ras/Raf/MAP kinase cascade phosphorylates TCF, allowing it to bind SRF and DNA, and to activate *c-fos* transcription (Marais et al., 1993). In the second pathway, the Rho family of GTPases activate SRF-dependent transcription in a TCF-

independent manner (Hill et al., 1995). The Rho GTPases promote the polymerization of actin filaments and the depletion of free actin monomers, which induce SRF activation (Sotiropoulos et al., 1999). Therefore, multiple signal transduction pathways converge on SRF to regulate the induction of immediate early genes.

SRF plays a central role in the activation of both growth factor genes and muscle-specific genes. The expression pattern of SRF is consistent with its dual role in cellular growth and muscle differentiation. SRF is expressed ubiquitously, at low levels, starting early during embryogenesis at E6.5 and continuing throughout adulthood (Norman et al., 1988). However, its expression is also upregulated and enriched in mesodermal and neuroectodermal lineages during embryogenesis, subsequent to the onset of organogenesis (Belaguli et al., 1997). In situ hybridizations of E11.5 mouse embryos show high levels of SRF transcripts in the myocardial region of the heart, the myotomal portion of the somites, and the smooth muscle layers of arteries and veins. SRF transcripts were also seen in the neuroectoderm of the brain and in the neural tube, but were virtually undetectable in endodermal tissues such as the liver, the lungs, or the spleen. Similar results were described for zebrafish and chick embryos (Croissant et al., 1996; Vogel and Gerster, 1999).

To further study the role of SRF *in vivo*, the *Srf* allele was mutated, and mice homozygous for the mutated allele were studied. The knockout embryos die at approximately E9.5 (Arsenian et al., 1998). These embryos are gastrulation defective and completely lacking in mesodermal cells. Mesodermal markers such as *Brachyury (T)*, which encodes a transcription factor required for posterior mesoderm formation, were absent. The SRF target genes *skeletal*, *cardiac*, and *smooth muscle α-actin* were also undetectable,

highlighting the essential role of SRF in the activation of CArG box-containing muscle genes. As expected, expression levels of the immediate-early genes *c-fos* and *egr-1* were also dramatically decreased, though still present at low basal levels.

Mechanisms of SRF regulation

Interestingly, the requirement of SRF for the expression of both growth factor-inducible and muscle-specific genes seems mutually exclusive, because growth factor genes are downregulated in differentiated muscle cells. Although SRF activity has been shown to be regulated by many different mechanisms, it is not yet fully understood how SRF specifically activates muscle-specific CArG box genes in muscle.

Alternative RNA splicing is a common strategy used to create multiple different gene products from a single locus. Several distinct isoforms of SRF have been identified, which are expressed relatively abundantly in skeletal, cardiac, and smooth muscle (Belaguli et al., 1999; Kemp and Metcalfe, 2000). These splice variants lack regions of the C-terminal transactivation domain and consequently have little to no activation ability, but can still bind DNA and interact with full-length SRF. However, the functional significance of these alternative splicing dominant negative isoforms of SRF is still unknown.

Another mechanism that modulates SRF activity is phosphorylation. SRF contains numerous serine residues which can be phosphorylated by casein kinase II or DNA-activated protein kinase in response to growth-factor stimulation (Janknecht et al., 1992; Liu et al., 1993; Marais et al., 1992). Phosphorylation of serines in the MADS box increases the affinity of SRF for its binding site, and thus increases transcriptional activation of the immediate early

genes. Phosphorylation of the sites located in the activation domain is also required for the full activity of SRF.

A recent study suggests that changes in the subcellular distribution of SRF may be another pathway for controlling SRF activity. SRF is normally expressed in the nucleus in cultured cells. Under conditions of serum deprivation, both SRF transcriptional activation and SRF binding to CArG boxes are reduced due to extranuclear redistribution of SRF (Camoretti-Mercado et al., 2000). Upon the addition of serum, SRF relocalizes to the nucleus. It has yet to be determined what factor(s) in serum are responsible for the reversible translocation of SRF between the nucleus and cytoplasm.

Numerous positive and negative cofactors for SRF have also been identified. The best characterized cofactor is TCF, whose interaction with SRF on the *c-fos* promoter has already been discussed. However, CArG boxes from muscle promoters are not located adjacent to Ets-binding motifs, as is the case for the *c-fos* promoter, so TCF would not be expected to regulate these muscle genes. Another factor which interacts with SRF is CREB-binding protein (CBP)/p300, a ubiquitous protein which was originally characterized as a coactivator of CREB, a cAMP-responsive transcription factor (Chrivia et al., 1993; Kwok et al., 1994). CBP has two transactivation domains and histone acetyltransferase activity, and may regulate SRF activity through multiple mechanisms, either by recruitment of the transcriptional machinery or by remodelling nucleosomes (Bannister and Kouzarides, 1996; Ogryzko et al., 1996). Neither of these models of action though, would seem to be able to direct muscle-specific transcription. Another ubiquitously expressed protein, HMG-I(Y),

potentiates SRF activation of CArG box promoters by enhancing SRF binding to DNA (Chin et al., 1998).

Several muscle cofactors for SRF have also been described. The homeodomain protein Phox/Mhox/prx1 interacts with SRF to increase its affinity for CArG boxes (Grueneberg et al., 1992). Phox is specifically expressed in mesodermal cell types, including skeletal, cardiac, and smooth muscle, but also in mesenchymal cells within the branchial arches and in the limb buds (Cserjesi et al., 1992). The myogenic bHLH factors have also been reported to interact with SRF to regulate skeletal muscle genes (Groisman et al., 1996). Finally, Nkx2.5 and GATA4 interact with SRF to activate certain cardiac-specific genes (Chen and Schwartz, 1996; Sepulveda et al., 1998). These cofactors definitely contribute to the ability of SRF to distinguish between different CArG box-regulated genes, but almost certainly other mechanisms of SRF regulation and other SRF cofactors remain undiscovered.

Objectives of Dissertation

There have been numerous mechanisms already identified that regulate SRF activity. This thesis describes studies on two novel aspects of SRF regulation as possible mechanisms for muscle-specific transcription by SRF: A) Differential affinities of SRF for different CArG boxes as a mechanism for distinguishing between growth factor-inducible and muscle-specific genes. B) Characterization of Myocardin, a muscle-specific cofactor for SRF.

CHAPTER II

MATERIALS AND METHODS

Construction of CArG box multimer constructs

Oligonucleotides containing the appropriate CArG box core (10 bp) and flanking sequences (15 bp each side) with HindIII sites on both ends were phosphorylated, phenol extracted, and annealed. Correct multimers were subcloned into the hsp68-lacZ vector (Kothary et al., 1989) for generation of transgenic mice. The number and orientations of the CArG repeats were determined by DNA sequencing. Sequences of top strand oligonucleotides (not including the HindIII sites) with CArG boxes underlined were as follows:

SM22: ACTTGGTGTCTTTCCCCAAATATGGAGCCTGTGTGGAGTG

Skeletal α-actin: TCTAGTGCCCGACACCCAAATATGGCTTGGGAAGGGCAGC

c-fos: CTTTACACAGGATGTCCATATTAGGACATCTGCGTCAGCA

SFS: ACTTGGTGTCTTTCCCCATATTAGGAGCCTGTGTGGAGTG

FSF: CTTTACACAGGATGTCCAAATATGGACATCTGCGTCAGCA

FFS: CTTTACACAGGATGTCCATATTAGGAGCCTGTGTGGAGTG

SFF: ACTTGGTGTCTTTCCCCATATTAGGACATCTGCGTCAGCA

FSS: CTTTACACAGGATGTCCAAATATGGAGCCTGTGTGGAGTG

SSF: ACTTGGTGTCTTTCCCCAAATATGGACATCTGCGTCAGCA

Generation of transgenic mice

DNA was gel purified and eluted using a Nucleospin DNA purification kit (Clontech). Transgenic mice were created by pronuclear injection of DNA into fertilized oocytes and LacZ expression was assayed in F0 embryos as described (Cheng et al., 1992; Hogan, 1994).

β-galactosidase staining and histology

Embryos were dissected in PBS, fixed in 2% formaldehyde/0.2% glutaraldehyde in PBS on ice for 1 hour, washed twice with PBS, and stained overnight at room temperature in 5 mM ferricyanide/5 mM ferrocyanide/2 mM $MgCl_2$/1 mg/ml X-Gal in dimethylformamide in PBS. Embryos were postfixed overnight in 4% formaldehyde after 2 washes in PBS. Embryos were then successively dehydrated (30%, 50%, 70%, 90%, and 100% methanol solutions) for 1 hour each, and left in 100% methanol overnight. Embryos were cleared for 2 hours in 2:1 benzyl benzoate/benzyl alcohol, embedded in paraffin, sectioned at 5 μm, rehydrated and stained with Nuclear Fast Red (Moller and Moller, 1994).

Gel mobility shift assays

SRF and Flag-Myocardin were translated in vitro with a TnT T7-coupled reticulocyte lysate system (Promega). The same *SM22 CArG-near*, *c-fos*, and *skeletal α-actin* CArG box oligonucleotides used for the construction of the CArG box-dependent transgenes were used as probes in gel mobility shift assays. Sequences of the top strand oligonucleotides (with CArG boxes underlined) for *SM22* CArG-far, *egr*-1 (Tsai-Morris et al., 1988) and *muscle creatine kinase (MCK)* were as follows:

CArG-far: GTTTCAGGGTCCTGCCCATAAAAGGTTTTTCCCGGCCGCC

egr-1: GCCGACCCGGAAACGCCATATAAGGAGCAGGAAGGATCCC

MCK: ACGGGTCTAGGCTGCCCATGTAAGGAGGCAAGGCCTGGGG

Complementary oligonucleotides were annealed and labeled with Klenow polymerase and α^{32}P-dCTP. $5x10^4$ cpm of labeled probe was incubated for 20 minutes at room temperature with the appropriate in vitro translated protein(s) and poly (dI-dC) in gel shift buffer, as described (Brennan and Olson, 1990). Antibody supershift experiments were performed with rabbit anti-SRF antiserum (Santa Cruz) or mouse monoclonal anti-FLAG antibody (Sigma). Unlabeled competitor DNA was added at 25-, 50- and 100-fold excess over labeled probe. DNA-protein complexes were separated by gel electrophoresis on a 5% nondenaturing polyacrylamide gel. Relative DNA binding was determined by visualizing the shifted probe with a Phosphorimager and quantified using ImageQuant Program (Molecular Dynamics).

Construction of SRF and Myocardin Deletion Mutants

All SRF and 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 deleted. For mutant Δbasic, cDNA sequences encoding amino acids 115-134 were removed. Mutations within the SAP domain and the amino- and carboxy-terminal deletions are indicated in the text.

Reporter Mutagenesis

The 1.3 kb *SM22* luciferase reporter construct and the two CArG box mutation constructs have been described previously (Li et al., 1997). The ANF CArG box mutations were generated within the context of the 638 bp promoter (Sprenkle et al., 1995) using a PCR-based mutagenesis strategy, and are as follows:

CArG1: from CCTTATTTGG to ATCGATCTGG

CArG2: from CTTTAAAAGG to GGATCCCAGG

Transfection assays

COS and F9 cells were grown in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Cellgro). Transfections were performed with FuGENE6 (Roche) according to the manufacturer's instructions. Briefly, 100ng of reporter and 100 ng of each activator plasmid were incubated with 3ul of FuGENE6 diluted in 100ul of DMEM and added to cells in six-well plates. The total amount of DNA in each transfection was kept constant by the addition of corresponding amounts of parent vector DNA. Cells were harvested 48 hours after transfection for cell extracts. To prepare the extracts, cells in each well were scraped into 400ul lysis buffer (Promega), vortexed, and centrifuged. Luciferase activities were assyed with a luminometer, and CMV-lacZ was used as an internal control to normalize for variations in transfection efficiency. Transfections were performed in duplicate, and all transfections were repeated multiple times.

CHAPTER III

MUSCLE SPECIFICITY ENCODED BY SRF-BINDING SITES

SM22 encodes a structural troponin-related protein which is expressed specifically in developing smooth, skeletal, and cardiac muscle during early embryogenesis, before becoming restricted to smooth muscle during late embryonic and postnatal development. (Li et al., 1996a; Solway et al., 1995; Takahashi and Nadal-Ginard, 1991). SM22 expression is first detected in the developing heart at E8.0. SM22 is expressed throughout the entire heart, before becoming restricted to the right ventricle at E12.5. This heart expression becomes undetectable by E14.5. Somitic expression of SM22 is first seen at E9.5, and continues until approximately E13.5. Expression of SM22 is seen only in the myotomal compartment of developing somites, and never in the differentiated skeletal muscle of the embryo. SM22 transcripts can also be detected in both visceral and vascular smooth muscle. SM22 is expressed in the smooth muscle layer of the bronchi and gut at E13.5, and this expression continues into adulthood. SM22 expression is also observed in the smooth muscle layer of the vasculature, in both arteries and veins. Because of its muscle restricted expression pattern, SM22 has been studied extensively as a model for muscle regulatory pathways.

Previous studies have shown that 1.3 kb of the *SM22* promoter is sufficient to direct expression of a lacZ reporter in transgenic mice in developing smooth, cardiac, and skeletal muscle cells during mouse embryogenesis (Li et al., 1996b). LacZ expression is first seen in the heart and dorsal aorta at E9.0. Somite expression of the transgene can be detected a half a day later at E9.5, and lacZ expression can clearly be seen in all three muscle lineages at

E11.5. By E13.5, expression in the heart and somites is markedly reduced, while expression in the vasculature is still present at high levels. The expression of the SM22-lacZ transgene overlaps completely with the expression of endogenous *SM22* transcripts in the cardiac, skeletal, and vascular smooth muscle lineages. Also, there is no ectopic expression of lacZ anywhere else in the embryo. However, the SM22-lacZ transgene does not fully recapitulate endogenous *SM22* expression. Unlike endogenous *SM22*, the transgene is not expressed in either visceral smooth muscle or in the smooth muscle layer of veins, though it is expressed in arterial vasculature. Most likely, additional regulatory sequences control expression in those tissues, but the 1343 nucleotides of the *SM22* promoter contained in this transgene are sufficient to confer muscle-restricted expression.

There are two CArG boxes located within these 1343 nucleotides. They are referred to as CArG-near (at -150/-141) and CArG-far (at -273/-264), with CArG-near being the proximal CArG box located closer to the start site of transcription. Within the context of the 1.3 kb promoter, both of these CArG boxes were individually mutated such that they no longer could bind SRF. CArG-near, but not CArG-far, was found to be essential for the muscle-specific expression of SM22 (Kim et al., 1997; Li et al., 1997). The mutated CArG-near construct was transcriptionally silent in transgenic embryos, with virtually no lacZ expression. The mutated CArG-far construct showed some perturbation of expression, but there was still significant lacZ expression in the muscle lineages. These experiments also demonstrate the essential role of SRF in the proper regulation of SM22 expression.

Muscle-specificity of multimerized *SM22* CArG elements

To determine whether CArG-near might be sufficient to confer muscle-specificity, I created a transgene (4xSM22-lacZ) containing four tandem copies of CArG-near with 15 nucleotides of flanking sequence on each side (Fig. 1). The CArG boxes were linked in a head-to-tail orientation upstream of a *lacZ* reporter under control of the *heat shock protein (hsp)-68* basal promoter, which is transcriptionally silent in mouse embryos (Kothary et al., 1989). I chose to analyze this transgene in E11.5 embryos because SM22 expression is prominent in all three muscle lineages at this time point.

The multimerized *SM22* CArG-near element directed lacZ expression in a highly restricted pattern in F0 transgenic mouse embryos at E11.5 (Fig. 2B-D). Similar expression patterns were observed in 9 transgenic F0 embryos harboring this transgene. Three representative embryos are shown. As observed with the 1.3kb *SM22* promoter (Fig. 2A), lacZ expression directed by the multimerized *SM22* CArG box was observed throughout the dorsal aorta and cranial vasculature, as well as in the heart and somite myotomes. Expression in the vasculature and somites appeared to mimic that of the 1343-bp *SM22* promoter. The multimerized *SM22* CArG box directed lacZ expression at very high levels throughout the atrial and ventricular chambers of the heart at E11.5. This is in contrast to the native *SM22* promoter, which is active specifically in the future right ventricle following looping morphogenesis. The 4xSM22-lacZ transgene was also expressed in the ventral region of the neural tube where the endogenous 1343-bp SM22 promoter is not expressed, though this expression may be due to the *hsp68* promoter. There was virtually no expression of the transgene outside of these cell types. The expression pattern of the multimerized CArG-near transgene is similar to that of SRF, which is enriched in muscle cells and the ventral neural tube during embryogenesis (Belaguli et al., 1997; Croissant et al., 1996; Vogel and Gerster, 1999).

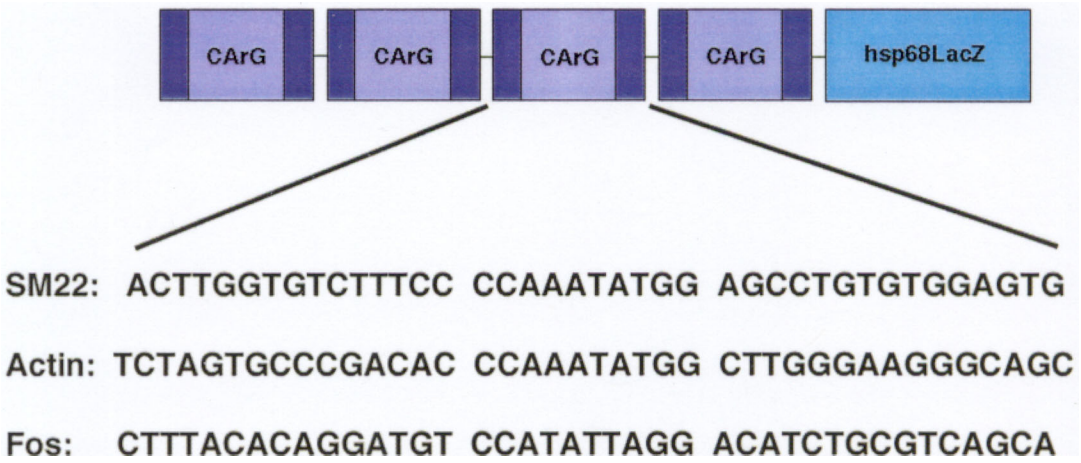


Fig. 1. Diagram of multimerized CArG box LacZ reporters.

All three LacZ constructs contain four CArG elements linked in a head-to-tail orientation. Each CArG element has a 10 bp core CArG box and 15 bp of flanking sequence on each side of the CArG box. The multimerized CArG boxes are fused to the *hsp68* basal promoter driving a LacZ reporter. The sequences of the CArG box elements from *SM22*, *skeletal α-actin*, and *c-fos* are shown.

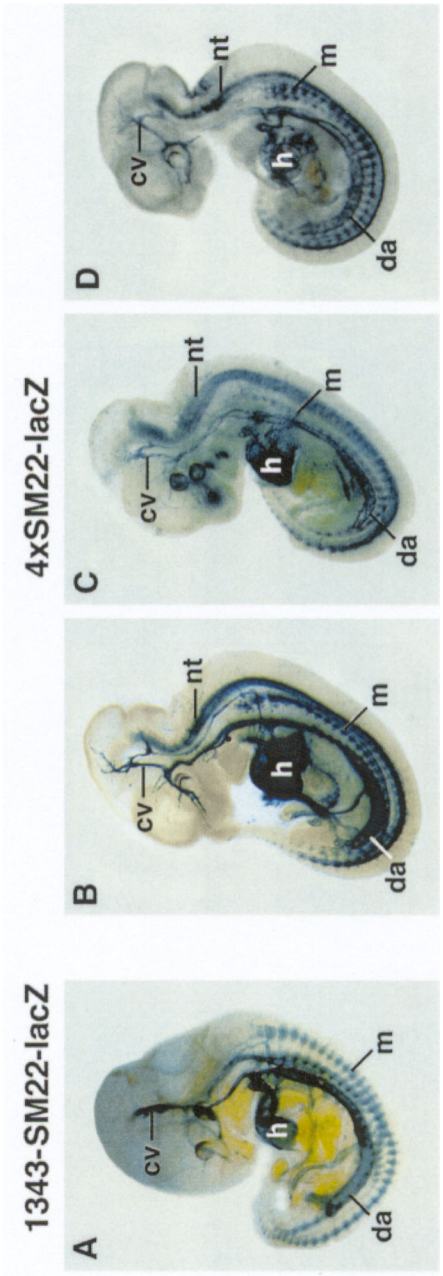

1343-SM22-lacZ
4xSM22-lacZ
A
B
C
D
cv
nt
h
m
da

Fig. 2. LacZ expression patterns directed by the 1343-bp *SM22* reporter and by a multimerized *SM22* CArG box reporter.

E11.5 transgenic mouse embryos harboring *lacZ* transgenes controlled by (A) the 1343-bp *SM22* promoter or (B,C,D) synthetic multimers of *SM22* CArG-near upstream of the *hsp68* basal promoter were stained for lacZ expression. Strong expression of lacZ can be seen specifically in the heart (h) and somite myotomes (m), as well as the dorsal aorta (da), cranial vasculature (cv), and other vascular structures in all embryos. The multimerized *SM22* CArG box also directed expression in the neural tube (nt). Three representative F0 embryos harboring 4xSM22-lacZ are shown. The lacZ expression pattern was similar for all the embryos, but the intensity of lacZ staining varied between the embryos.

Lack of muscle-specificity of the *c-fos* CArG box

To determine if the muscle-restricted activity of the *SM22* CArG box reflected a general property of CArG boxes or was unique to CArG-near, I examined the expression pattern of the *hsp68-lacZ* transgene linked to four copies of the CArG boxes from the *skeletal α-actin* and *c-fos* promoters. Like the 4xSM22-lacZ transgene, the *skeletal α-actin* CArG-box transgene (4x-Actin-lacZ) directed lacZ expression in cardiac, skeletal, and smooth muscle cell lineages, as well as in the ventral neural tube, at E11.5 (Fig. 3A-C). In fact, the expression patterns for the 4x-Actin-lacZ transgenic embyos were virtually identical to that of the 4x-SM22-lacZ transgenic embryos, differing only in the intensity of lacZ staining.

In contrast to the highly specific expression patterns of the *SM22* and *skeletal α-actin* CArG boxes, the *c-fos* CArG box directed widespread embryonic expression (Fig. 3D-F). This transgene was expressed in the heart, somites, and aorta, but there was also extensive staining throughout the embryo, suggesting that the *c-fos* CArG element was active in a wider range of cell types than the *SM22* and *skeletal α-actin* CArG boxes. The broad expression pattern of the 4xfos-lacZ transgene suggests that the *c-fos* CArG box is active in cells that express SRF at relatively low levels.

Transverse sections of transgenic embryos harboring the multimerized *SM22* CArG and *c-fos* CArG box transgenes confirmed the differences in their expression patterns. LacZ was expressed specifically in the heart, somites, dorsal aorta, and ventral neural tube in embryos harboring the 4xSM22-lacZ transgene, whereas lacZ expression was observed throughout the entire embryo with the 4xfos-lacZ transgene (Fig. 4A,B). These experiments show that not all CArG boxes are able to direct muscle-restricted expression, suggesting that

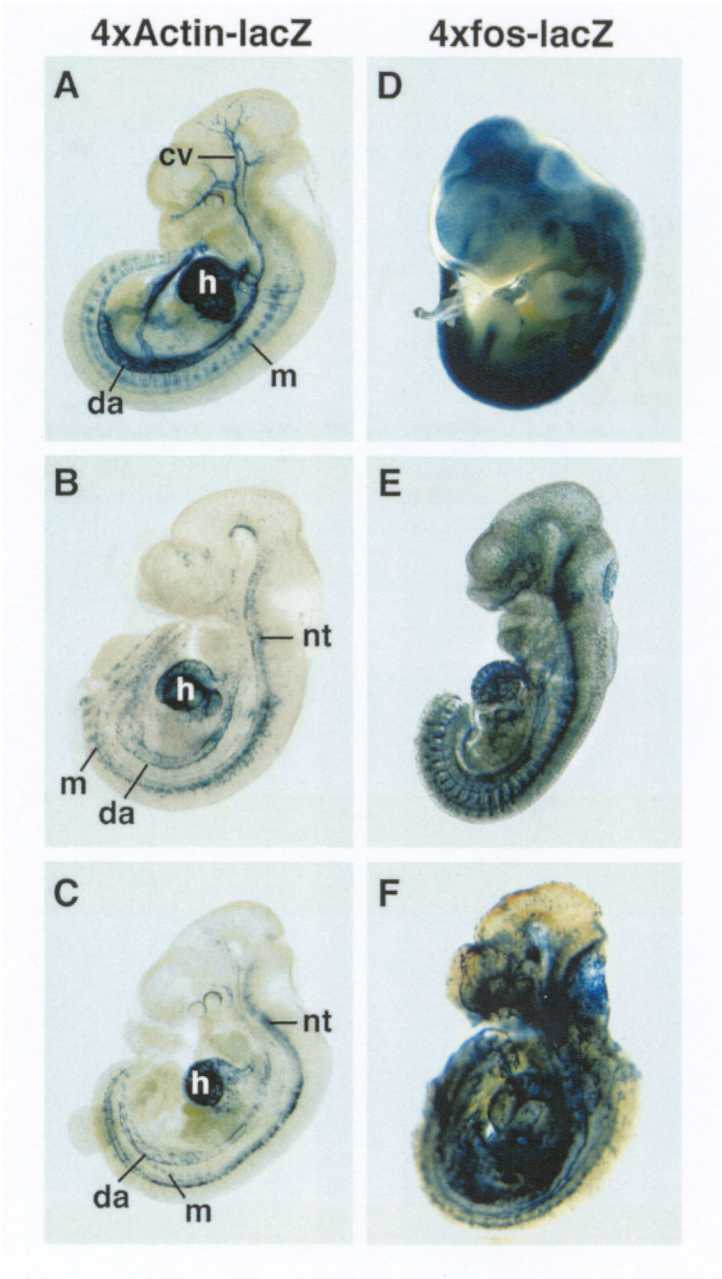

4xActin-lacZ
4xfos-lacZ
A
cv
h
m
da
D
B
nt
h
m
da
E
C
nt
h
da
m
F

Fig. 3. LacZ expression patterns directed by multimerized *skeletal α- actin* and *c-fos* CArG box reporters.

E11.5 transgenic mouse embryos harboring (A-C) 4xActin-lacZ and (D-F) 4xfos-lacZ transgenes were stained for lacZ expression. In the embryos harboring 4xActin-lacZ, strong expression can be seen specifically in the heart (h), somite myotomes (m), the dorsal aorta (da), cranial vasculature (cv) and other vascular structures, as well as in the neural tube (nt). In the embryo harboring 4xfos-lacZ, lacZ expression was widespread and not specific for myogenic cell types. Three representative F0 embryos harboring each transgene are shown. The expression pattern for 4xActin-lacZ was similar for all the embryos, but the intensity of lacZ expression varied between the embryos. The 4xfos-lacZ transgene showed much more widespread and variable expression of lacZ.

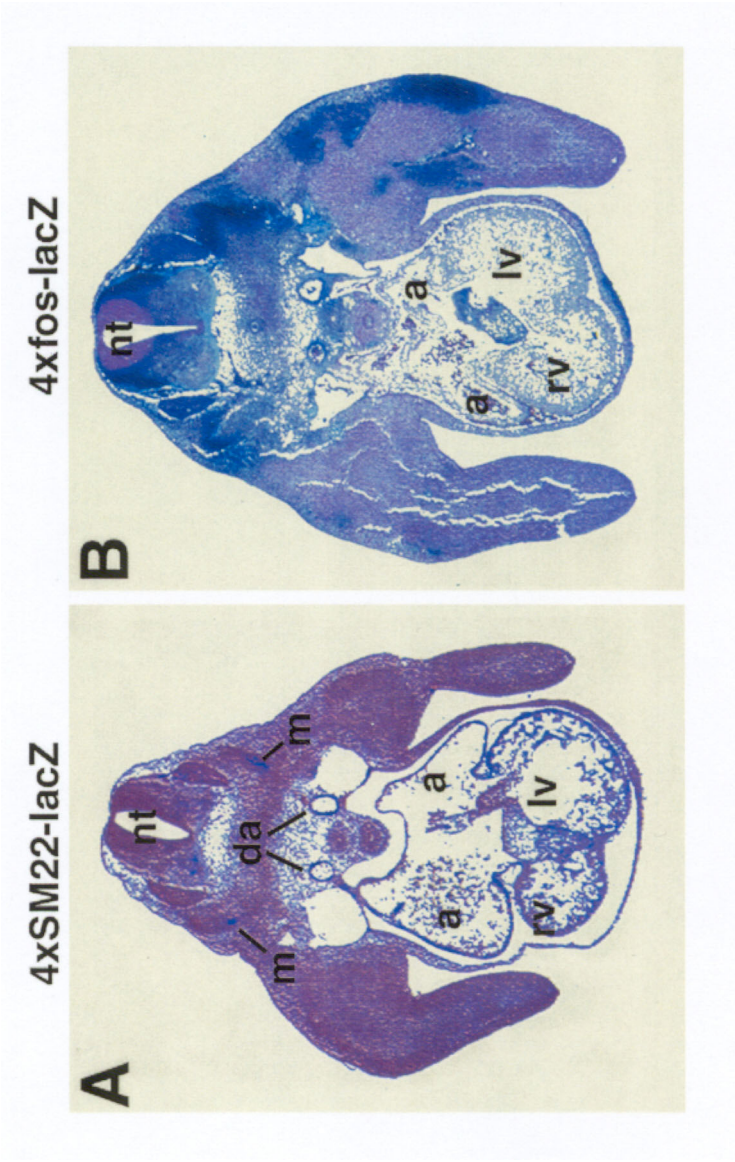

A
4xSM22-lacZ
nt
da
m
m
a
a
lv
rv
B
4xfos-lacZ
nt
a
a
lv
rv

Fig. 4. Sections of transgenic embryos harboring lacZ transgenes containing multimerized *SM22* and *c-fos* CArG boxes.

E11.5 transgenic mouse embryos harboring the (A) *4xSM22-lacZ* or (B) *4xfos-lacZ* transgenes were stained for lacZ expression, sectioned at the level of the heart and stained with Nuclear Fast Red. In (A), lacZ expression can be seen in the atria (a) and right and left ventricles (rv and lv) of the heart, the myotome portion of the somites (m), the paired dorsal aorta (da), and in a subset of cells in the ventral neural tube (nt). In (B), staining can be seen throughout the embryo.

the muscle-specific expression patterns of the *SM22* and *skeletal α-actin* CArG boxes are encoded within the CArG boxes themselves.

Analysis of chimeric CArG elements

I next sought to identify the DNA sequences responsible for the distinctly different patterns of transgene expression directed by the *SM22* and *c-fos* CArG boxes. I therefore created chimeric CArG elements by systematically swapping the core sequences and surrounding nucleotides of the two CArG boxes. Tandem copies of these chimeric CArG elements were linked to the *hsp68-lacZ* reporter and tested in F0 transgenic embryos at E11.5. As with the multimerized *SM22*, *c-fos*, and *skeletal α-actin* CArG constructs, all of the CArG elements were organized in a head-to-tail orientation. Each chimeric CArG box was named according to the identity of the 5'- flanking, core ($CC(A/T)_6GG$), and 3'-flanking nucleotides, with S referring to *SM22* and F referring to *c-fos* (Fig. 5). The number of CArG boxes contained in each transgene is shown in Table 1. At least three independent transgenic embryos were examined with each construct. As seen with previous constructs, the overall expression pattern was similar in different embryos harboring a given construct, but the intensity of expression varied, presumably because of differences in transgene integration sites. I also examined the expression pattern of various transgenes containing between 3 and 6 tandem copies of one particular CArG box, but saw no significant differences in expression pattern for a given transgene, indicating that the number of CArG boxes in the transgenes did not influence their patterns of expression. Representative embryos with each transgene are shown in Fig. 6.

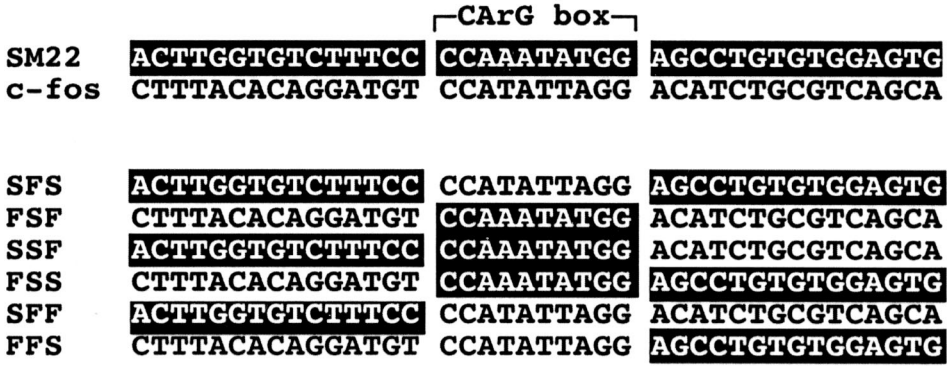


Fig. 5. Diagram of chimeric CArG elements.

Sequences of the CArG boxes and flanking regions of the *SM22* CArG, *c-fos* CArG, and chimeric CArG elements. Each CArG element contains the 10 nucleotide CArG box and 15 nucleotides of flanking sequence both 5' and 3' of the core CArG box. *SM22* sequences are shaded in black.

Table 1. Summary of CArG-box containing transgenes.

Reporter	CArG copies	Transgenic Embryos
SM22 (SSS)	4	9
SFS	3	5
FSS	3	3
SSF	3	7
SFF	3	6
FFS	3	4
FSF	6	4
c-fos (FFF)	4	4

Embryos harboring the indicated chimeric CArG boxes upstream of hsp68-lacZ were stained for lacZ expression at E11.5. Forty-two total transgenic embryos were analyzed. The number of CArG boxes in each of the transgene constructs and the number of transgenic embryos examined are indicated.

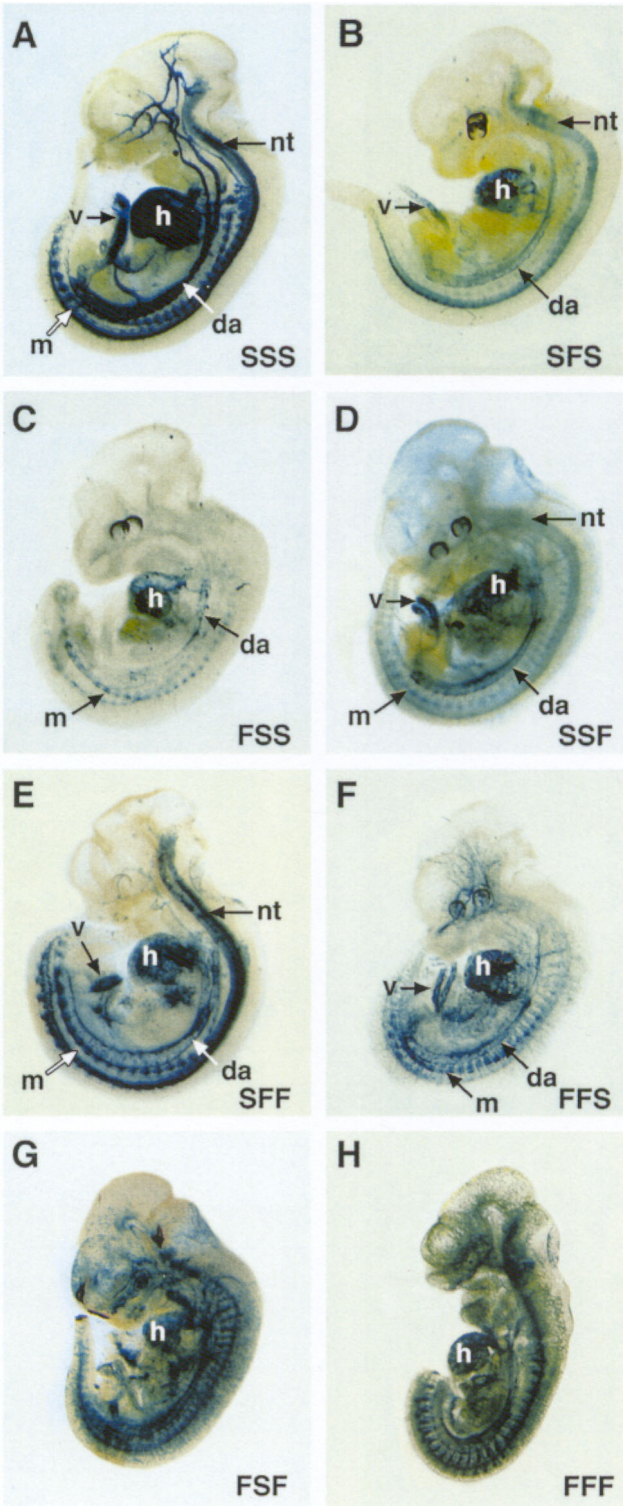

A
nt
v
h
da
m
SSS
B
nt
h
v
da
SFS
C
h
da
m
FSS
D
nt
h
v
da
m
SSF
E
nt
v
h
da
m
SFF
F
h
v
da
m
FFS
G
h
FSF
H
h
FFF

Fig. 6. Expression patterns of lacZ transgenes linked to multimerized chimeric CArG element reporters.

E11.5 transgenic mouse embryos harboring *hsp68-lacZ* transgenes linked to the indicated multimerized CArG boxes were stained for lacZ expression. Sequences of the CArG boxes are shown in Fig. 5. The number of tandem copies of the each CArG box are shown in Table 1, along with the number of transgenic embryos analyzed. (da) dorsal aorta; (h) heart; (m) myotome; (nt) neural tube; (v) umbilical vessel. The SSS and SFS CArG elements direct lacZ expression specifically in muscle lineages and in the neural tube. The FFF and FSF embryos show ubiquitous expression of lacZ, and the FSS, SSF, SFF, and FFS elements direct intermediate expression patterns, with lacZ present in muscle but also in other tissues.

The CArG box element SFS, containing the *SM22* flanking sequences and the *c-fos* core sequence, directed expression in a pattern similar to that of the *SM22* CArG box (SSS) (Fig. 6, compare A and B), except that the level of expression was weaker. This result suggested that the core CArG sequence was not responsible for the specificity of the *SM22* CArG box expression pattern. Conversely, the CArG box FSF, containing the *c-fos* flanking sequences and the *SM22* core sequence, showed a widespread expression pattern, reminiscent of the *c-fos* CArG box (FFF) (Fig. 6, compare G and H). High background staining with FSF and FFF was especially pronounced in the head. The other four chimeric CArGs (FSS, SSF, SFF, and FFS) directed expression in patterns that appeared to be intermediate between the highly specific pattern seen with the *SM22* CArG and the widespread pattern seen with the *c-fos* CArG (Fig. 6C-F). Together, these data suggest that the differences in expression pattern of different CArG element multimers are determined primarily by the 15 flanking nucleotides on both sides of the core CArG boxes.

High-affinity binding of SRF to CArG boxes correlates with widespread transgene expression

To determine whether there might be a correlation between DNA binding affinity and expression pattern, gel mobility shift assays were performed with *in vitro*-translated SRF and the different chimeric CArG elements. As shown in Fig. 7, the *c-fos* CArG element bound SRF more avidly than the *SM22* CArG element (compare lanes 1 and 2). This difference in SRF binding appeared to be attributable to the flanking sequences of these CArG boxes, because the chimeric CArG SFS (lane 3) bound SRF with a reduced affinity similar to that of

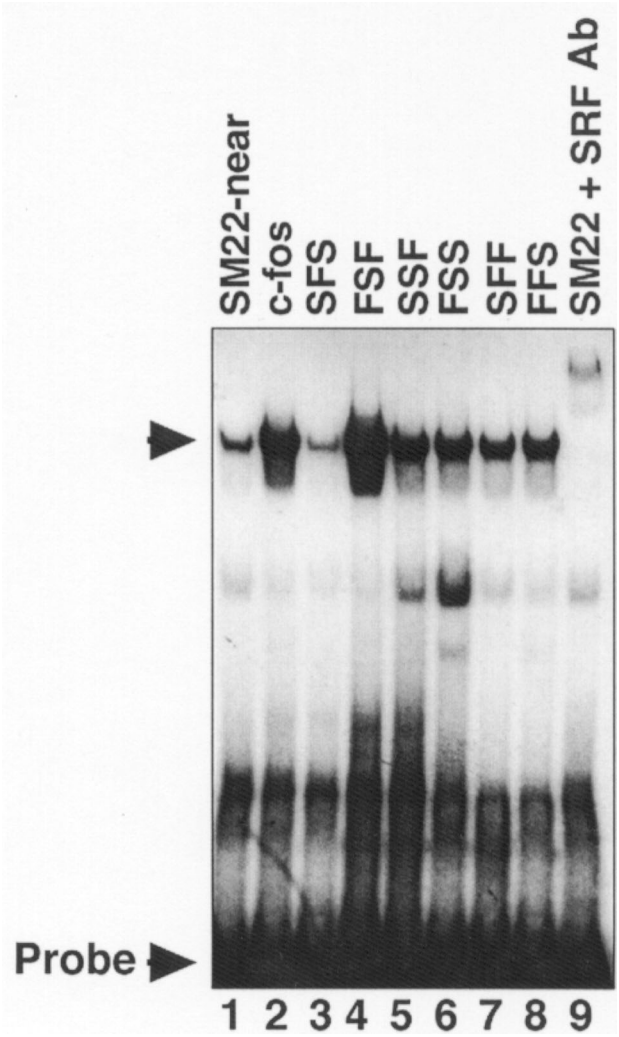

SM22-near
c-fos
SFS
FSF
SSF
FSS
SFF
FFS
SM22 + SRF Ab
Probe
1 2 3 4 5 6 7 8 9

Fig. 7. Gel mobility shift assays of SRF binding to chimeric CArG elements.

Oligonucleotide probes corresponding to the indicated CArG elements (see Fig. 5) were used as probes in gel mobility shift assays with *in vitro*-translated SRF. The *SM22* CArG and SFS (lanes 1 and 3) bound SRF relatively weakly, whereas the *c-fos* CArG and FSF (lanes 2 and 4) bound SRF most strongly. SSF, FSS, SFF, and FFS (lanes 5-8) had intermediate affinities for SRF. Note that the SRF-containing complex was specifically supershifted with SRF antibody (lane 9). The position of the SRF-DNA complex is shown with an arrowhead to the left of each panel. All assays contained equal amounts of labeled probe, as described in Materials and Methods.

the *SM22* CArG, whereas FSF (lane 4) bound SRF very strongly, like the *c-fos* CArG box. The CArGs with mixed flanking sequences (FSS, SSF, FFS, and SFF, lanes 4-7) showed SRF binding intermediate between that of the other CArG elements. The single major complex observed in gel mobility shift assays was confirmed to contain SRF by supershift with SRF antibody (lane 9).

To further compare the relative affinities of SRF for the *SM22* and *c-fos* CArG boxes, I performed competition experiments with each of the chimeric CArG sequences and ^{32}P-labeled probes for the *SM22* and *c-fos* CArG elements (Fig. 8). Results from competition experiments are plotted in Fig. 9. Binding of SRF to the *SM22* CArG probe was competed most effectively by the FSF and FFF sequences. The SFS and SSS sequences were the least effective competitors and other chimeric CArG sequences showed intermediate abilities to compete for SRF binding. A similar order of effectiveness in competition for SRF DNA binding by the different CArG sequences was observed with the *c-fos* CArG probe. Thus, those CArG sequences that contained the *c-fos* flanking regions and directed widespread expression in vivo showed the strongest binding of SRF. Conversely, those CArG sequences that contained the *SM22* flanking regions and directed muscle-restricted expression in vivo showed relatively weak binding of SRF.

Comparison of SRF binding to different CArG boxes

The above findings revealed a correlation between strength of SRF binding and specificity of expression. As a further test of this correlation, I examined the binding of SRF to CArG

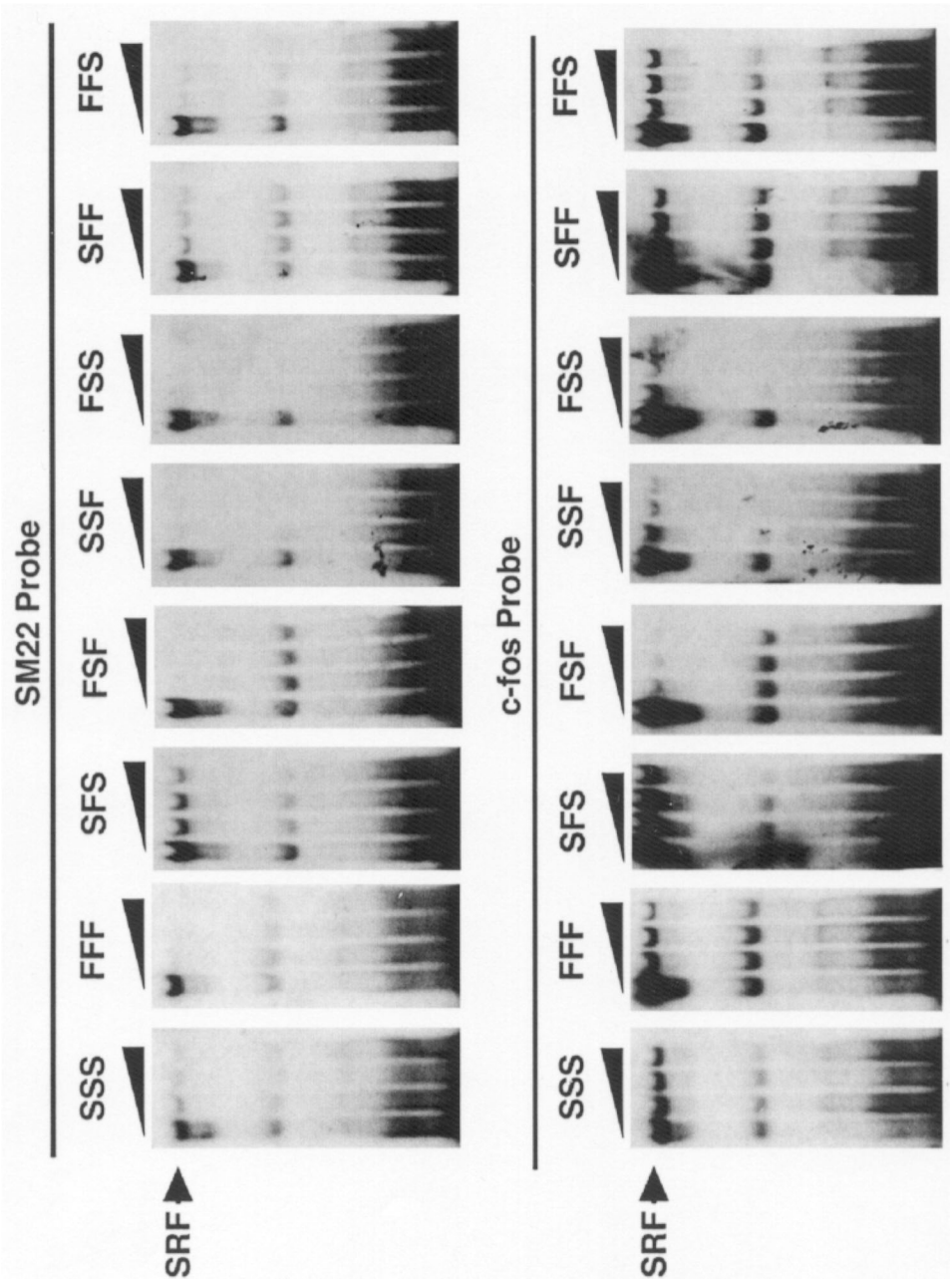
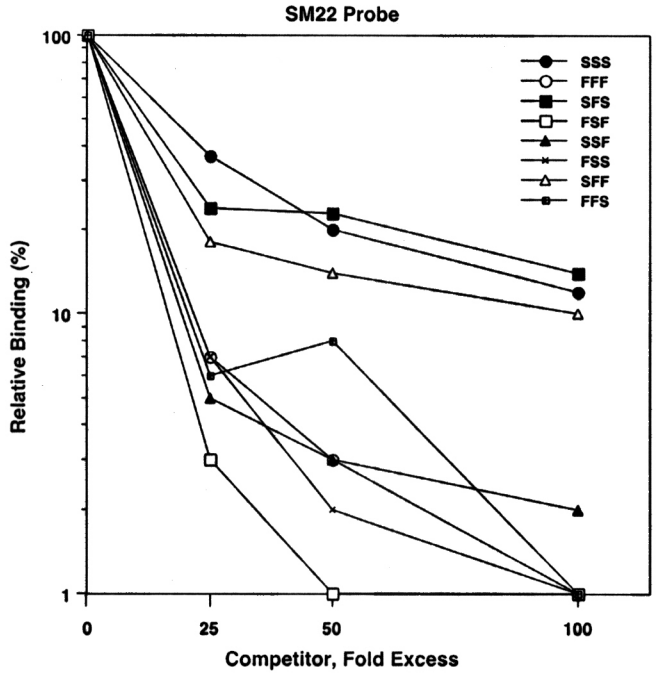

SM22 Probe
SSS
FFF
SFS
FSF
SSF
FSS
SFF
FFS
SRF
c-fos Probe
SSS
FFF
SFS
FSF
SSF
FSS
SFF
FFS
SRF

Fig. 8. Competition assays for SRF binding to the *SM22* and *c-fos* CArG boxes.

Oligonucleotide probes corresponding to the *SM22* (top panel) and *c-fos* (bottom panel) CArG boxes were used as probes in gel mobility shift assays with *in vitro*-translated SRF. Each of the CArG sequences shown in Figure 5 was used as unlabeled competitor at 25-, 50-, and 100-fold excess over labeled probe. The increasing concentrations are indicated by the black triangles. CArG boxes containing the *c-fos* flanking sequences competed for SRF binding more effectively than those containing the *SM22* flanking sequences.


SM22 Probe
100
10
1
Relative Binding (%)
0
25
50
100
Competitor, Fold Excess
SSS
FFF
SFS
FSF
SSF
FSS
SFF
FFS

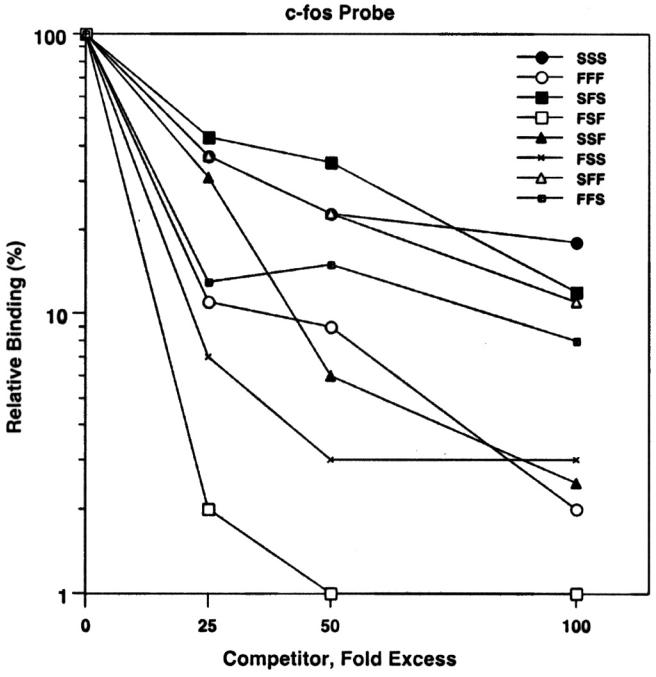

c-fos Probe
100
10
1
Relative Binding (%)
0
25
50
100
Competitor, Fold Excess
SSS
FFF
SFS
FSF
SSF
FSS
SFF
FFS

Fig. 9. Quantitation of competition assays from Figure 8.

Relative binding of SRF from to the *SM22* (top panel) and *c-fos* (bottom panel) probes was quantitated by phosphorimager and plotted as percent of maximal SRF binding in the absence of competitor.

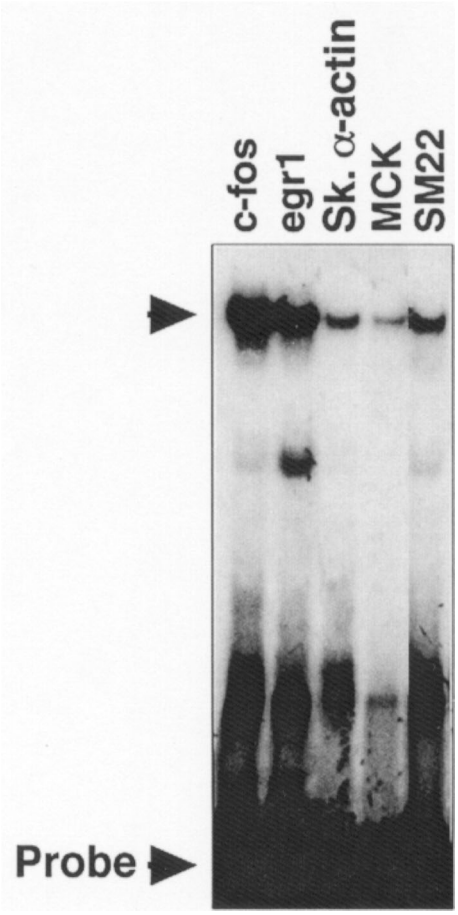


Fig. 10. Gel mobility shift of SRF binding to CArG boxes from various promoters.

Oligonucleotide probes corresponding to the indicated CArG boxes were used as probes in gel mobility shift assays with *in vitro*-translated SRF. SRF bound strongly to the CArG elements from *c-fos* and *egr1* and weakly to CArG boxes from *skeletal α-actin*, *MCK*, and *SM22*.

boxes from other muscle-specific and ubiquitously expressed genes (Fig. 10). The *skeletal α-actin* CArG box, which directed a muscle expression pattern similar to that of *SM22* CArG, bound SRF relatively weakly. Similarly, the CArG box in the skeletal muscle-specific enhancer of the *muscle creatine kinase (MCK)* gene, also bound SRF weakly. In contrast, SRF bound strongly to the CArG box from *egr-1*, which, like *c-fos,* is widely expressed.

Summary

Four multimers of *SM22* CArG-near are sufficient to direct muscle expression of a lacZ reporter in transgenic mice. Another CArG box from a muscle gene, that of *α-skeletal actin*, can also direct muscle-specific expression. However, a multimerized *c-fos* CArG box construct gives ubiquitous expression, indicating that not all CArG boxes are able to direct muscle-specific expression. Chimeric CArG elements were created by swapping core and flanking CArG box sequences from the SM22 CArG-near and c-fos CArG boxes. Analysis of transgenic mice containing these chimeric CArG elements demonstrate that the flanking sequences surrounding the CArG boxes, and not the sequences within the CArG boxes themselves, determine the specificity of expression. Gel mobility shift assays show that the flanking sequences also determine the binding affinity of SRF for the CArG elements. SRF binds much more avidly to CArG elements that contain *c-fos* flanking sequences than to those that contain flanking sequences from *SM22* CArG-near. The relative affinities of the CArG boxes for SRF correlate well with the expression patterns of the multimerized transgenes. The CArG boxes that direct ubiquitous expression, such as the *c-fos* CArG box,

bind SRF more strongly than CArG boxes that direct muscle-specific expression, such as those from *SM22 ορ α-skeletal actin*. These results suggest that SRF is able to differentiate between different CArG box-containing genes based on its affinity for the CArG box, and that this differential affinity contributes to the specificity of SRF activation of muscle genes in muscle.

CHAPTER IV

CHARACTERIZATION OF MYOCARDIN, A NOVEL SRF COFACTOR

In an effort to identify novel cardiac genes, Da-Zhi Wang searched EST databases for sequences found only in cardiac cDNA libraries that did not correspond to known genes. These sequences were then used as probes for in situ hybridization in E9.5 mouse embryos and for Northern blot analysis of adult mouse tissues, in order to further confirm their cardiac-specific expression. One of the cDNAs identified in this screen corresponded to a 3' untranslated region, which was used to isolate full length cDNAs from an embryonic mouse heart cDNA library. The extended cDNA sequence encoded a novel open reading frame of 807 amino acids. This protein was named Myocardin because of its specific expression in the adult myocardium and its essential role in myocardial gene expression *in vivo*. The amino acid sequence of Myocardin contains several notable features. Myocardin has a basic region located between residues 115-132, an extended stretch of glutamine (Q) residues between amino acids 159-192, a SAP domain at residues 252-286, and an extended amphipathic alpha-helix resembling a leucine zipper between residues 396-424 (Fig. 11A).

SAP domains (Fig. 11B) are found in a variety of proteins that influence nuclear architecture and transcription (reviewed in Aravind and Koonin, 2000). The SAP domain is named for Scaffold Attachment Factors A and B (SAF-A and SAF-B), which recognize chromosomal regions known as matrix attachment or scaffold attachment regions (MARs/SARs) (Romig et al., 1992), Acinus, a caspase-3-activated protein required for apoptotic chromatin condensation (Sahara et al., 1999), and PIAS1, an inhibitor of STAT-

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
PGFSNLTSSGPSIFNIDFLDVTDLNLNSPMDLHLQQW 807
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B

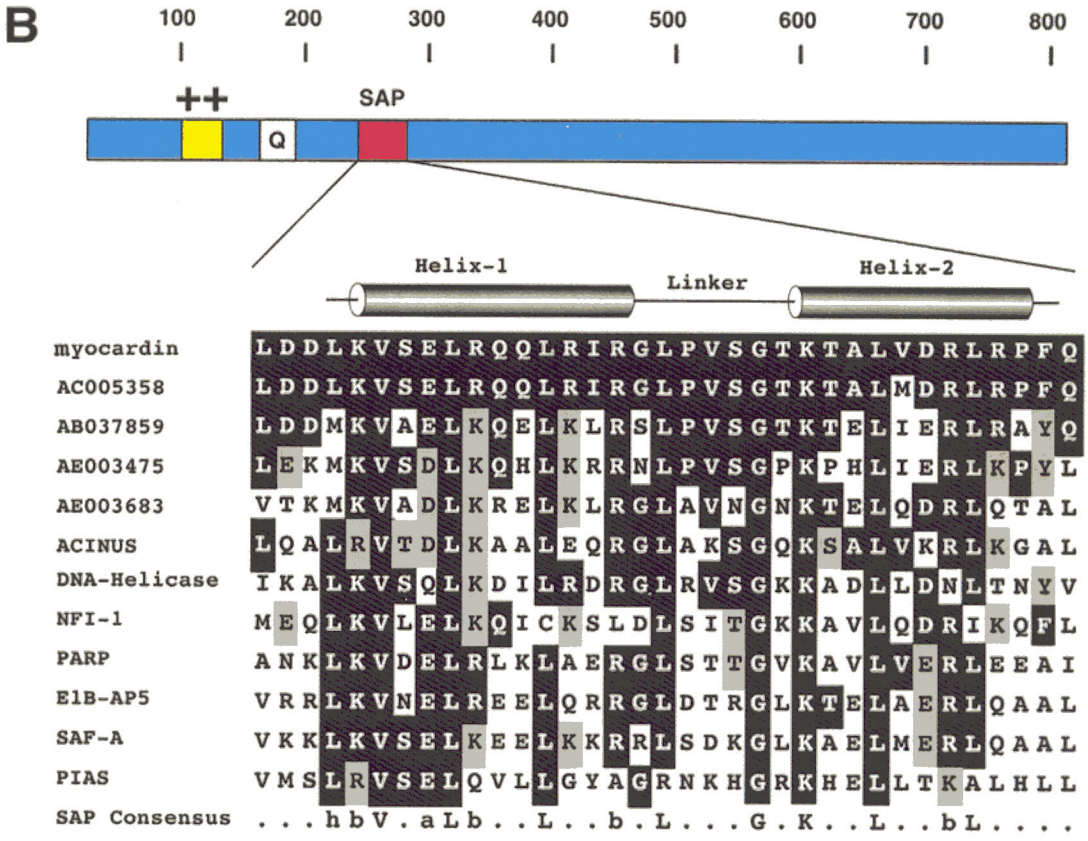


Fig. 11. Deduced amino acid sequence and homology of Myocardin with other SAP domain proteins.

(A) Deduced amino acid sequence of mouse Myocardin, with structural domains noted.

(B) Homology of Myocardin with other 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. Consensus sequence for the SAP domain is shown at the bottom. a, acidic; b, basic; h, hydrophobic. The experiments described in this figure were performed by Da-Zhi Wang.

mediated gene activation (Liu et al., 1998). The SAP domain is a 35-amino acid motif containing two amphipathic helices separated by an intervening region. Myocardin does not show homology any other known SAP proteins outside of the SAP domain, but additional mouse, human and *Drosophila* ESTs encoding novel proteins with homology to the SAP and basic domains of Myocardin have been identified (Fig.11B).

Northern blot analysis of adult mouse tissues performed by Da-Zhi Wang revealed multiple Myocardin transcripts ranging in size from 3.5 to 7.5 kb specifically in adult heart, with prominent species of 3.5 and 5 kb (Wang et al., 2001). There was no detectable expression of Myocardin in any of the other tissues examined (Fig. 12A). In situ hybridization of mouse embryos showed that Myocardin transcripts were first detected in the cardiac crescent at E7.75 (Fig. 12B, panel 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 (panel b), and throughout the developing atrial and ventricular chambers until birth (panels a-f). Myocardin was also expressed in a subset of embryonic vascular and visceral smooth muscle cells. At E13.5, Myocardin expression was evident in the smooth muscle cells lining the walls of the esophagus, the aortic arch arteries, and the pulmonary outflow tract (panel g). Expression in these smooth muscle cell types was still apparent, but was diminished, by E15.5. Myocardin expression was also detected in developing smooth muscle cells within the developing lung and gut (panels h,i). Myocardin was not expressed at detectable levels in skeletal muscle cells during embryogenesis or postnatally.

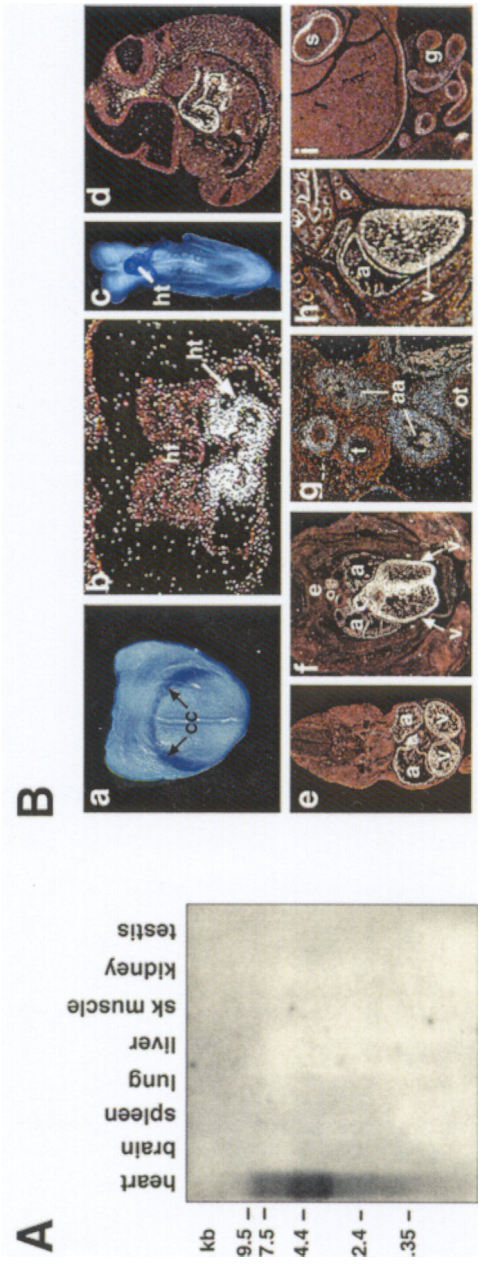

A
heart
brain
spleen
lung
liver
sk muscle
kidney
testis
kb
9.5
7.5
4.4
2.4
.35
B
a
b
c
d
e
f
g
h
i
cc
ht
ot
aa
v
s
g

Fig. 12. Expression of Myocardin transcripts in embryonic and adult mouse tissues.

(A) A cDNA probe encompassing the 3' untranslated region of Myocardin was used as a probe for an adult mouse tissue Northern blot (Clontech). Myocardin transcripts are detected only in the heart and migrate as a heterogeneous series of bands, with predominant species of 3.5 and 5 kb.

(B) Myocardin transcripts were detected by whole-mount (a,c) or section (b, 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 show Myocardin expression in the smooth muscle cell layers of the outflow tract (ot), trachea (t), and aortic arch arteries (aa). (h) Myocardin is expressed in the smooth muscle of the stomach (s) and gut (g). (i) E15.5. Transverse section shows Myocardin expression in the heart and esophagus (e). The experiments described in this figure were performed by Da-Zhi Wang.

As a first step toward determining its function, the subcellular distribution of Myocardin in transfected COS cells was examined. Myocardin was localized predominantly to the nucleus and showed a punctate intranuclear staining pattern with exclusion from nucleoli (Fig. 13A). The nuclear localization of Myocardin suggested that it might function as a transcription factor. In an effort to identify potential target genes for Myocardin, various muscle promoters linked to luciferase reporters were tested for their responsiveness to Myocardin. 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 the *Nkx2.5* enhancer (Fig. 13B). In contrast, Myocardin failed to activate the *HRT1* promoter or the *dHAND* enhancer. The *cytomegalovirus* (CMV) and *E1b* promoters, which show no tissue-specificity, were also not activated by Myocardin, indicating that it does not act as a general transcriptional activator. All of the initial characterization of Myocardin was done by Da-Zhi Wang.

Myocardin activates transcription through binding sites for SRF

Of all of the muscle promoters tested, the *SM22* promoter was the most responsive to Myocardin, being up-regulated by several thousand-fold over basal transcription. As described previously, SM22 is expressed in all three muscle lineages during early embryogenesis before becoming restricted specifically to the smooth muscle lineage. Transcription of *SM22* in cardiac and smooth muscle cells is dependent on two CArG boxes in the promoter that bind SRF. These sites, CArG-near and CArG-far, bind SRF and are essential for *SM22* transcription, though SRF transactivates the *SM22* promoter only about

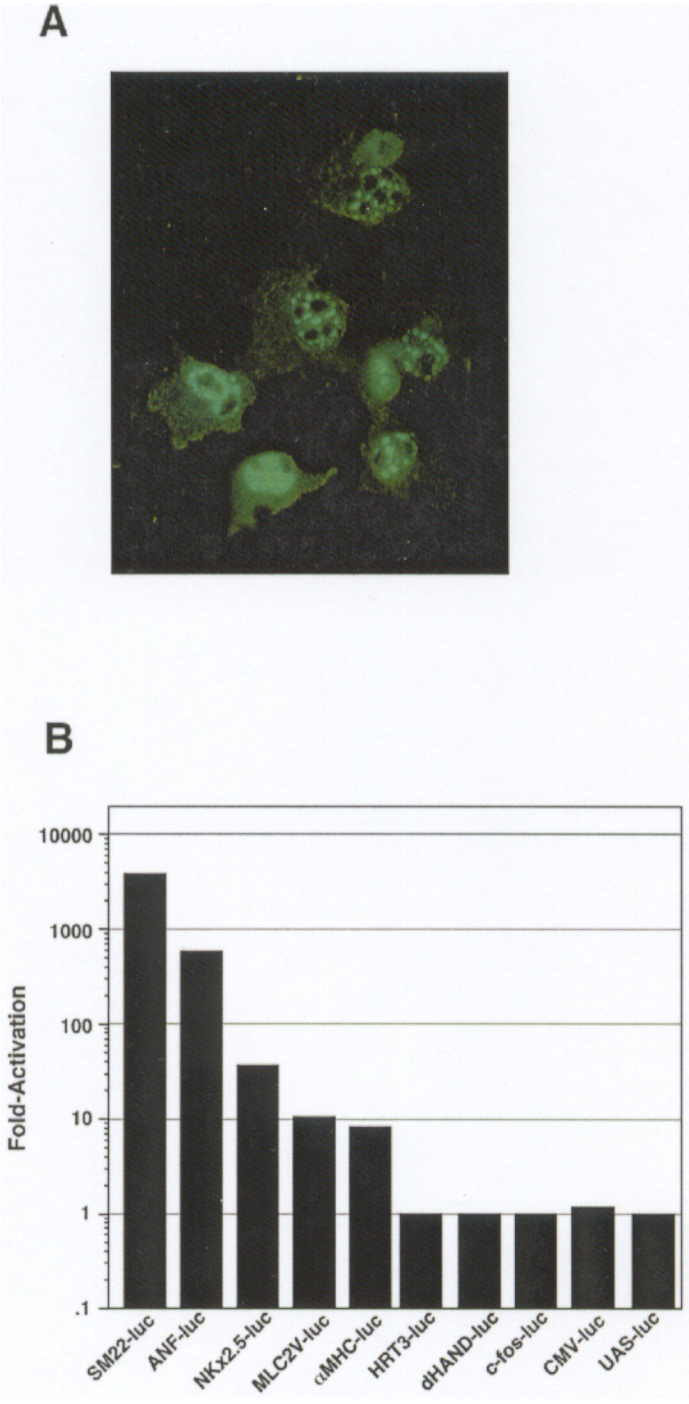

A
B
10000
1000
100
10
1
.1
Fold-Activation
SM22-luc
ANF-luc
NKx2.5-luc
MLC2V-luc
αMHC-luc
HRT3-luc
dHAND-luc
c-fos-luc
CMV-luc
UAS-luc

Fig. 13. Nuclear localization and transcriptional activity of Myocardin.

(A) The subcellular location of Myocardin protein was determined by immunofluorescence of COS cells transiently transfected with FLAG-tagged Myocardin.

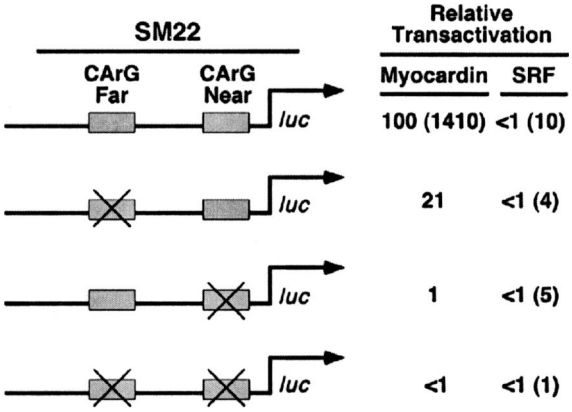
(B) Activation of cardiac and smooth muscle reporter genes by Myocardin. The indicated reporter genes were transfected into COS cells with a Myocardin expression plasmid and luciferase activity was determined on cell extracts. Fold-activation values are expressed as the fold-increase in luciferase activity in the presence of Myocardin expression plasmid compared to the level of activity with vector alone. The experiments described in this figure were performed by Da-Zhi Wang.

ten-fold, compared to the several thousand-fold activation of the same reporter by Myocardin. This suggests that an additional SRF cofactor may be required for full activity of the *SM22* promoter in muscle cells, and that Myocardin could be this SRF cofactor.

The next step was to determine what sequences in the SM22 promoter were required for this high transactivation ability of Myocardin. As the CArG boxes are the best characterized regulatory sites in the *SM22* promoter, they were the logical candidates to study first. To investigate the potential requirement of the CArG boxes for responsiveness to Myocardin, I tested whether mutations in either CArG box in the context of the 1343 bp SM22 promoter impaired responsiveness to Myocardin (Fig. 14). Mutation of CArG-far reduced Myocardin activation by 5-fold, but this mutant promoter construct was still activated several hundred-fold. However, mutation of CArG-near almost completely eliminated the ability of the promoter to respond to Myocardin, and mutation of both CArG boxes completely abolished all activation by Myocardin. Therefore, the CArG boxes, and especially CArG-near, were required for Myocardin transactivation of *SM22*.

The *ANF* promoter also contains two CArG boxes that have been shown to be required for transcriptional activity in cardiomyocytes (Hines et al., 1999). As with the *SM22* promoter, mutation of the distal CArG box severely reduced *ANF* activation by Myocardin, and a reporter containing mutations of both CArG boxes was completely unable to respond to Myocardin (Fig. 14). Interestingly, the c-fos promoter, which contains a single essential CArG box, was activated less than 2-fold by Myocardin (Fig. 13B).

CArG boxes are therefore necessary for the activation of *SM22* and *ANF* by Myocardin. To determine if CArG boxes were sufficient to confer transcriptional


SM22
CArG Far
CArG Near
luc
Relative Transactivation
Myocardin
SRF
100 (1410)
<1 (10)
21
<1 (4)
1
<1 (5)
<1
<1 (1)

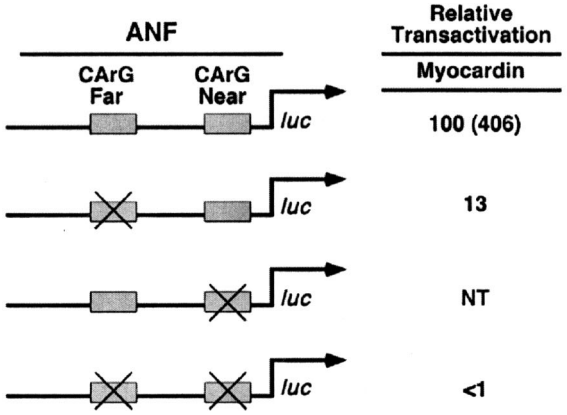

ANF
CArG Far
CArG Near
luc
Relative Transactivation
Myocardin
100 (406)
13
NT
<1

Fig. 14. Activation of SM22 and ANF by Myocardin is CArG box-dependent.

Analysis of the effects of CArG box mutations on activation of the *SM22* (top) and *ANF* (bottom) promoters by Myocardin. Each CArG box from the *SM22* and *ANF* promoter was mutated to a sequence that could not bind SRF and the effects on activation by Myocardin of the reporters were determined in transfected COS cells. Relative transactivation values are expressed as the percentage of luciferase activity of each construct compared to the wild-type reporter, which was assigned a value of 100. Values in parentheses indicated the fold-increase in activation by Myocardin compared to the level of activity of vector alone. NT, not tested.

responsiveness to Myocardin, luciferase reporter genes containing the *E1b* basal promoter linked to four tandem copies of either *SM22* CArG-near or the *c-fos* CArG box were constructed. As shown in Fig. 15, Myocardin was able to transactivate these reporters several hundred-fold. In contrast, SRF was only able to activate expression of the multimerized CArG box reporters 8-fold. Together, these results demonstrate that the CArG box is a target for transcriptional activation by Myocardin, which is a much more potent transactivator than SRF.

Myocardin is highly sensitive to the level of SRF

The ability of Myocardin and SRF to coordinately synergize transcription was also examined by Da-Zhi Wang. Myocardin was extremely sensitive to the level of SRF, such that at low concentrations of SRF, Myocardin and SRF synergistically activated *SM22* transcription, whereas at higher concentrations of SRF, transcriptional activation by Myocardin was reduced (Fig. 16). Inhibition of Myocardin-dependent transcription by excess SRF could be relieved by increasing the amount of Myocardin.

Thus, the ratio of SRF to Myocardin is extremely important for transcriptional activation by Myocardin, and exceeding an optimal ratio with an excess of SRF results in attenuation of Myocardin activity. Such a model suggests that transcriptional activation by Myocardin in the absence of exogenous SRF is mediated by endogenous SRF.

Domain mapping of Myocardin

To further define the mechanism of Myocardin-dependent transcriptional activation,

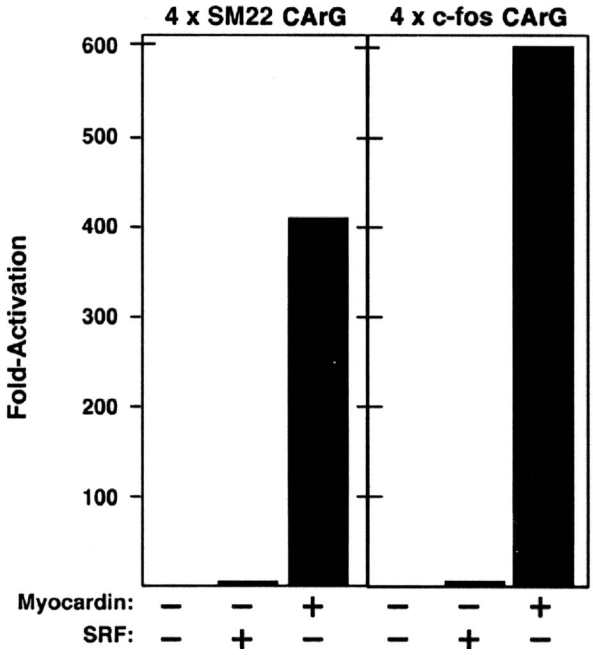


Fig. 15. CArG boxes are sufficient for Myocardin activation.

Activation of luciferase reporters containing the E1b basal promoter and four tandem copies of either *SM22* CArG-near or the *c-fos* CArG box. Myocardin was able to activate these minimal CArG box reporters.

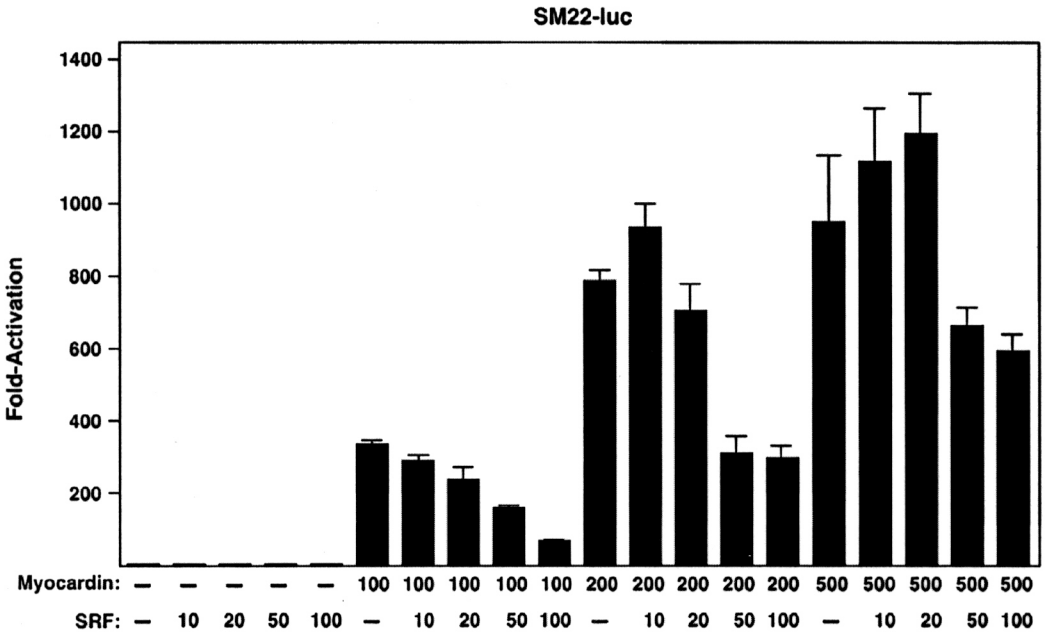


Fig. 16. Myocardin and SRF cooperatively activate transcription.

COS cells were transiently trasfected with 100ng of the SM22-luciferase reporter and the indicated amounts (in ng) of Myocardin and SRF. Myocardin activation is exquisitely sensitive to the levels of SRF. This experiment was performed by Da-Zhi Wang.

the transcriptional activities of a series of amino- and carboxy-terminal deletion mutants were assessed. Deletion of the first 66 residues of the amino-terminus (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 (Fig. 17). The loss in activity of this mutant appears to be due to deletion of the basic region, because an internal deletion mutant (mutant Δbasic) lacking only the basic region was also unable to activate transcription. All further N-terminal deletions up to amino acid 615 were also transcriptionally inactive. An internal deletion of the Q-rich domain within the context of the full length protein (mutant ΔQ) also eliminated transcriptional activity.

Carboxy-terminal deletion of residues 585-807 (mutant CΔ585) or 381-807 (mutant CΔ381) also eliminated all transcriptional activity (Fig. 18). These results are consistent with the results of GAL4 fusion experiments, which indicated the existence of a transcription activation domain between residues 541 and 807(Z. Wang, data not shown). To determine whether this carboxy-terminal region also serves some additional function required for Myocardin transactivation, constructs were created containing the transcriptionally inactive carboxy-terminal deletion mutants fused to the VP16 transcription activation domain. As shown in Fig. 18, the Myocardin-VP16 fusion construct that contained residues 1-381 (CΔ381-VP16) was a potent activator of the *SM22* and *ANF* promoters. This result indicates that the carboxy-terminal residues from 382 to 807 can be completely substituted for by the activation domain of VP16, and thus function solely as an activation domain. Note that

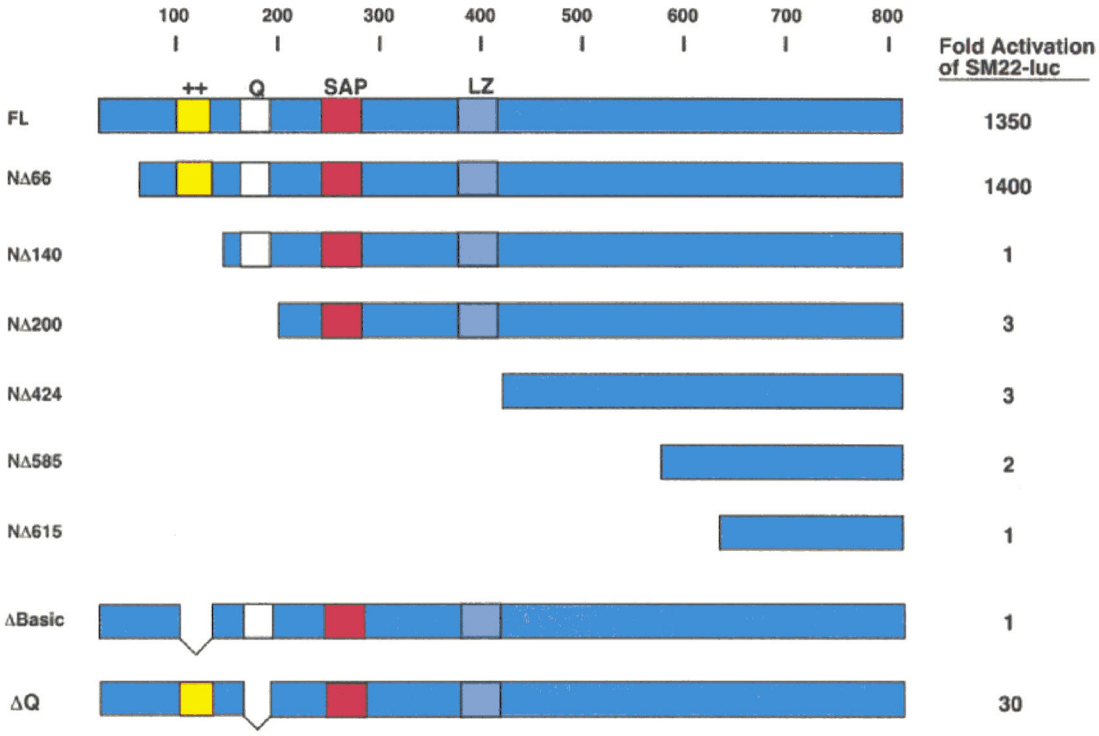


Fig. 17. Myocardin domain mapping.

Deletion mapping of the domains of Myocardin required for activation of the *SM22* and *ANF* promoters. The amino-terminus contains two domains required for Myocardin activation. All Myocardin deletions contained a FLAG epitope at the N-terminus and their expression was confirmed by Western blot.

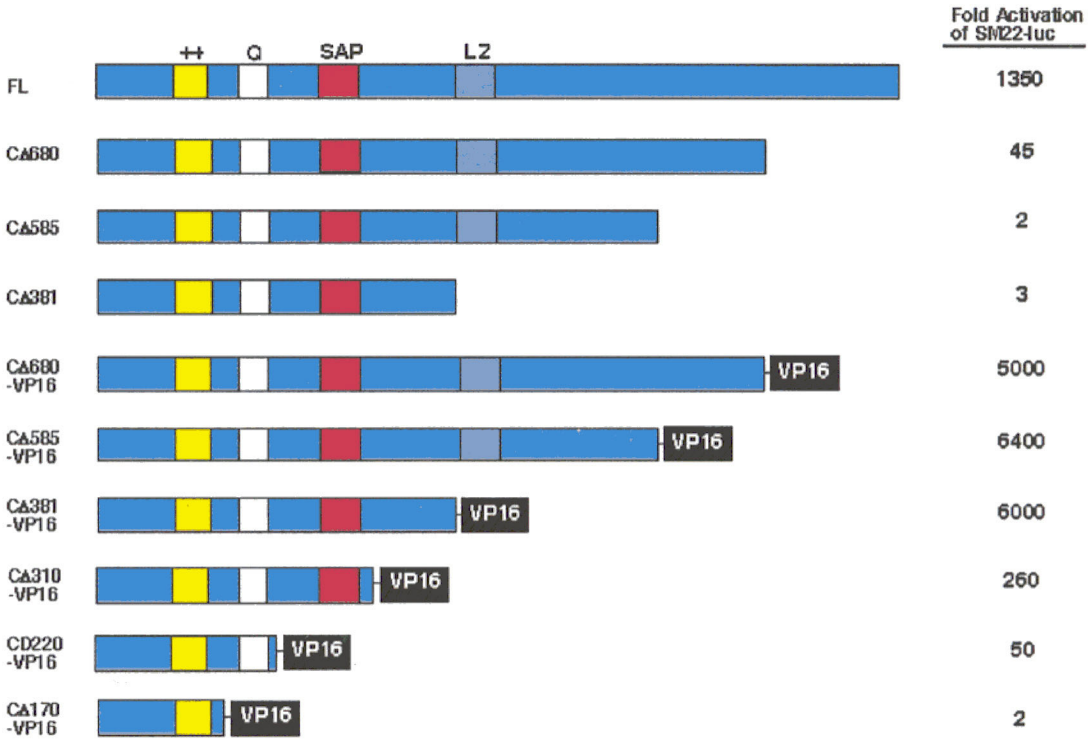


Fig. 18. More Myocardin domain mapping.

Further deletion mapping of Myocardin domains required for activation. The carboxy-terminus is required for Myocardin activation, but it can be substituted for by the VP16 activation domain. Again, all Myocardin deletions contained a FLAG epitope at the N-terminus and their expression was confirmed by Western blot.

Myocardin-VP16 fusion constructs with more carboxy-terminal residues deleted, such as CΔ170-VP16, were unable to activate either the *SM22* or *ANF* reporters.

The transcriptionally inactive Myocardin deletion mutants were further studied to determine if they might interfere with transcriptional activation by the wild-type protein. Indeed, when coexpressed in transfected COS cells, the carboxy-terminal deletion mutants severely impaired the ability of wild-type Myocardin to activate the *SM22* promoter (Fig. 19).

To assess the functional importance of the SAP domain, three different mutations were introduced into this region (Fig. 20). Introduction of proline mutations into helix 1 or 2 and mutations of conserved leucine residues (mutants PSF and PGH), had only a modest effect on the ability of Myocardin to transactivate the *SM22* promoter. However, these mutations abolished activation of the *ANF* promoter by Myocardin. Similarly, deletion of the linker region between the two helices of the SAP domain (Δlinker), a region shown previously to be required for DNA binding by SAF-A (Kipp et al., 2000), had little effect on *SM22* activation, but eliminated activation of *ANF*.

Together, these results suggest that the residues located at the carboxy-terminus of Myocardin function 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 requirement of the SAP domain for Myocardin activation seems to be promoter dependent.

Myocardin forms a complex with SRF

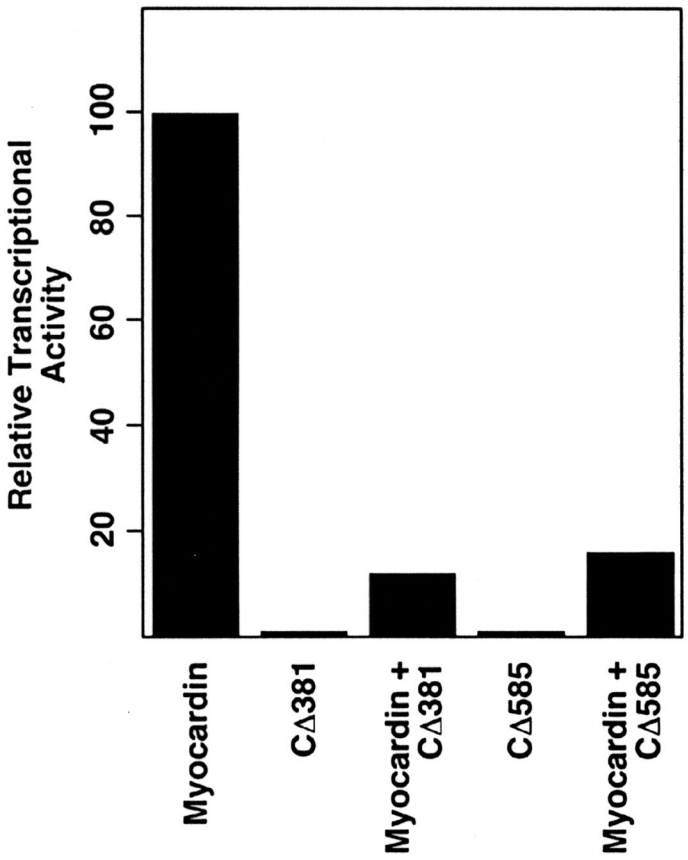


Fig. 19. Myocardin mutants block activation of wild-type Myocardin.

Dominant negative effects of carboxy-terminal deletion mutants in transfection assays. Carboxy-terminal Myocardin deletion mutants inhibit transcriptional activation by wild-type Myocardin. Values are expressed as luciferase activity of each construct compared to the wild-type construct, which was assigned a value of 100.

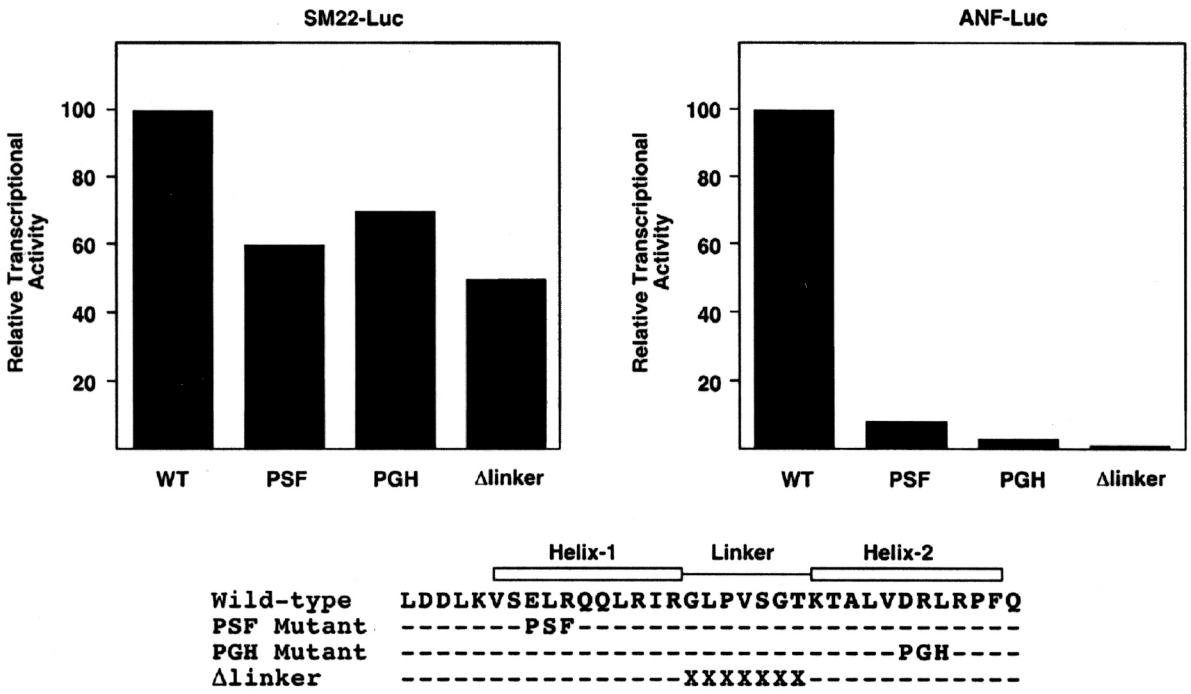


Fig. 20. The SAP domain is not required for activation of SM22 but is required for ANF activation.

Three different mutations were made in the SAP domain of Myocardin.

To further determine the mechanism of CArG-box-dependent transcriptional activation by Myocardin, the ability of *in vitro*-translated Myocardin to bind to CArG boxes was tested. SRF was able to bind to both *SM22* CArG boxes, but no binding of Myocardin alone 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 (Fig. 21). This complex could be super-shifted by antibodies directed against either 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 either CArG-near or CArG-far. I also examined ternary complex formation on the *c-fos* CArG box and both *ANF* CArG boxes. All of these sites gave rise to a ternary complex, the intensities of which correlated directly with the relative affinities of SRF for the sites (data not shown). The fact that these different CArG boxes do not share any obvious homology in their flanking sequences suggests that Myocardin associates directly with SRF and does not depend on specific DNA sequences for ternary complex formation.

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 of Myocardin (NΔ140) abolished association with SRF, as did larger N-terminal deletions (Fig. 22). In contrast, carboxy-terminal deletions from amino acid 381 or 585 to the end of the protein (mutants CΔ381 and CΔ585) did not affect the ability of Myocardin to interact with SRF. 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

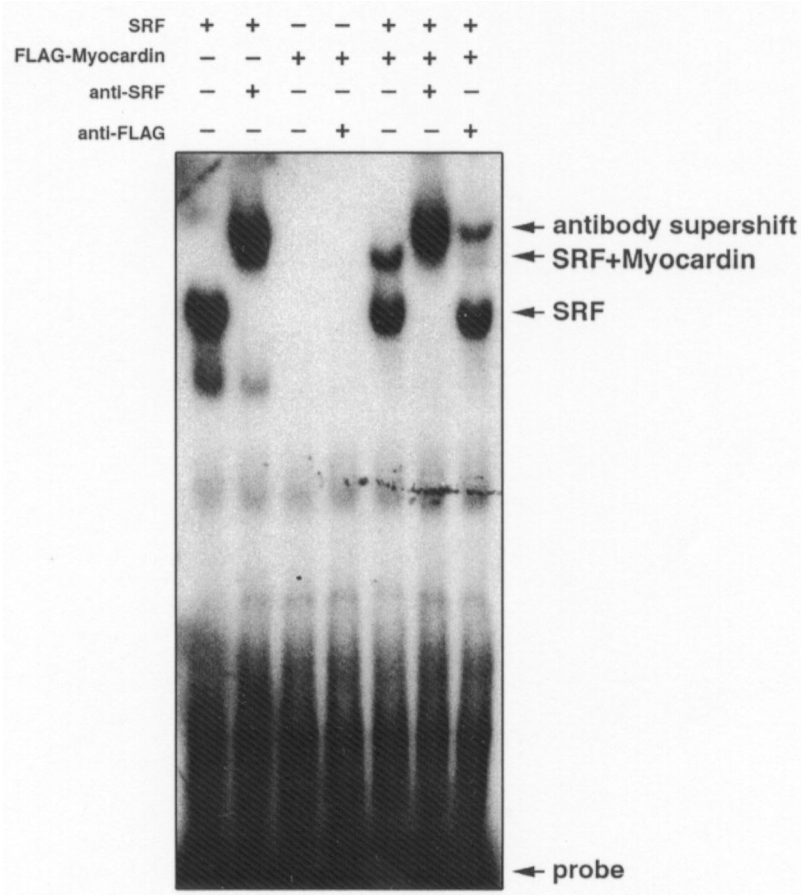


Fig. 21. Ternary complex formation between Myocardin and SRF.

Gel mobility shift assays were performed with a ^{32}P-labeled oligonucleotide probe for *SM22* CArG-far and in vitro translation products of wild-type and mutant forms of FLAG-tagged Myocardin in the presence and absence of SRF. Similar results were seen for *SM22* CArG-near, and the *c-fos*, and *ANF* CArG boxes.

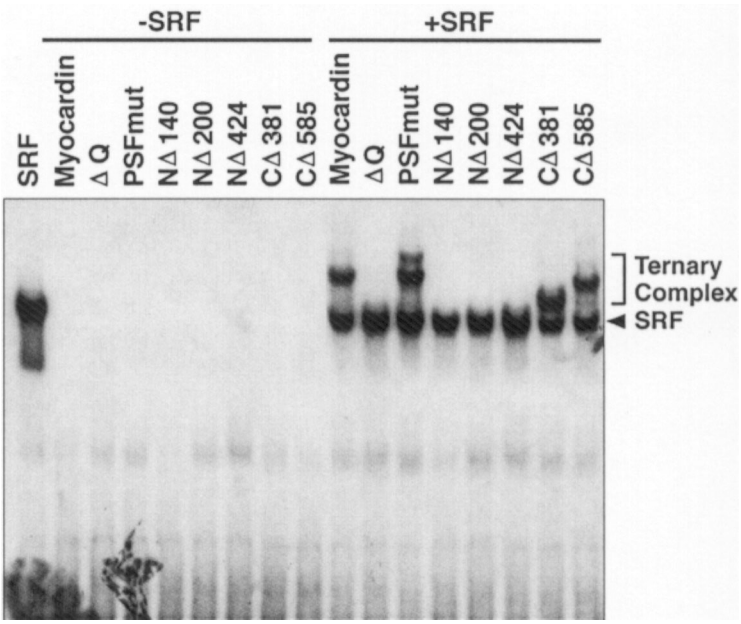


Fig. 22. Basic and Q domains of Myocardin are required for interaction with SRF.

(A) Gel mobility shift assays were performed as in Figure 19 with SRF and FLAG-tagged Myocardin. Antibodies against SRF and the FLAG-epitope were included, as indicated. The C-terminal residues of Myocardin are not required for ternary complex formation.

(B) Summary of ternary complex formation ability of the different Myocardin mutants.

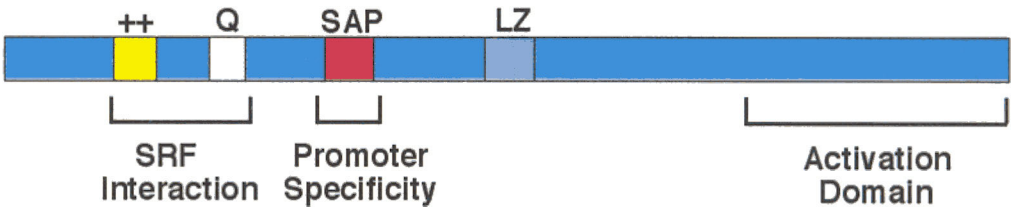


Fig. 23. Functional domains of Myocardin.

Myocardin activation of CArG box genes is dependent on three functional domains. The basic and Q-rich regions are required for interaction with SRF. The SAP domain mediates promoter-specific interactions, and the carboxy-terminal region contains a potent activation domain.

transcriptional specificity by mediating association with SRF, while the carboxyl-terminus activates transcription, but does not confer specificity for SRF (Fig. 23).

To determine the region of SRF that interacts with Myocardin, gel mobility shift assays were performed with an SRF deletion mutant containing the MADS domain but lacking both the amino- and carboxyl-terminal residues. This SRF mutant (SRF 100-300) was still able to bind the CArG box and form a ternary complex with Myocardin (Fig. 24). Therefore, Myocardin interacts with the DNA binding and dimerization domain of SRF. The interaction of the MADS box of SRF with the N-terminal region of Myocardin is corroborated by coimmunoprecipitation assays using epitope-tagged proteins in transfected COS cells (D. Wang, data not shown). This experiment also shows that the Myocardin-SRF interaction does not require the presence of DNA.

Together, these results demonstrate that Myocardin interacts with SRF and forms a stable ternary complex on CArG boxes. 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.

Summary

Myocardin is a novel protein that is highly expressed in muscle. It is able to potently activate several muscle reporters in transfected cells, including the smooth muscle gene SM22. The CArG boxes in the SM22 promoter are both necessary and sufficient for this activation by Myocardin. Mutation of the CArG boxes results in loss of Myocardin activation of the SM22 reporter, while a multimerized reporter containing four CArG boxes

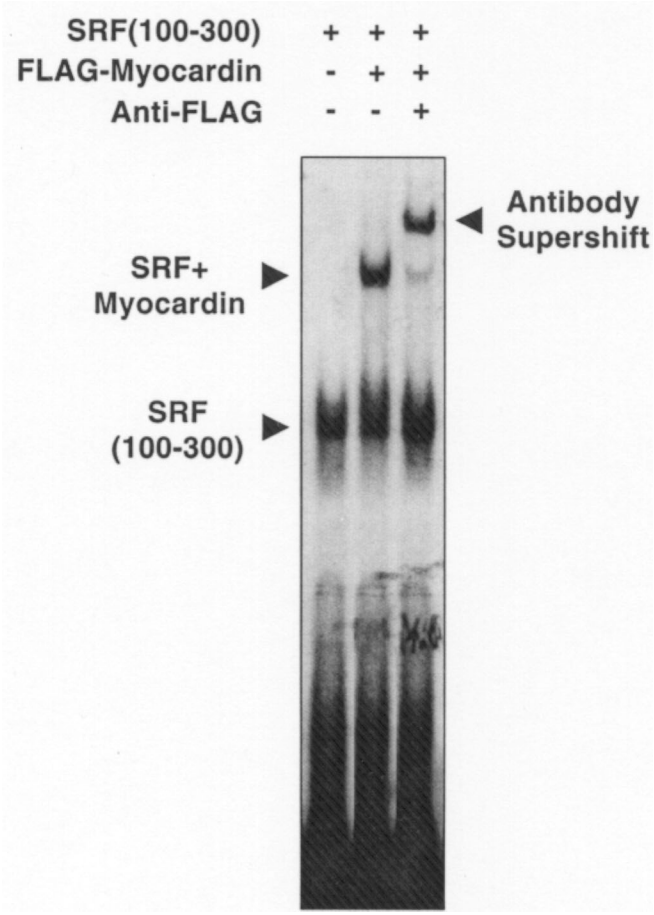


Fig. 24. MADS box of SRF is required for interaction with Myocardin.

Gel mobility shift assays were performed with an SRF deletion mutant (SRF 100-300), lacking the amino- and carboxyl-termini. This deletion construct was still able to form a ternary complex with Myocardin.

is transactivated by Myocardin. Myocardin and SRF can synergistically activate transcription of SM22. Myocardin contains several domains that are important in its transactivation ability. The basic and Q-rich regions in the amino-terminus of the protein are required, as is the acidic region in the carboxy-terminus. Myocardin and SRF form a ternary complex on CArG boxes, which requires the basic and Q-rich regions of Myocardin and the MADS box of SRF. These results demonstrate that Myocardin acts as a cofactor for SRF and contributes to the muscle-specific expression of target genes.

CHAPTER V

DISCUSSION

SRF is an important regulator of both growth factor-inducible and muscle-specific genes, but how SRF distinguishes between these two sets of genes, which have entirely different expression patterns, is not fully resolved. The results described in this dissertation demonstrate two mechanisms whereby SRF can direct muscle-specific gene expression of CArG box-containing genes.

First, CArG boxes with different flanking sequences can direct distinct temporospatial expression patterns during mouse embryogenesis. Whereas CArG boxes from the *SM22* and *skeletal α-actin* promoters can direct muscle-restricted transcription, the *c-fos* CArG box directs widespread embryonic expression. The different activities of these CArG boxes correlate with their relative affinities for SRF; CArG boxes that direct muscle-restricted expression bind SRF relatively weakly compared to CArG boxes that direct widespread expression.

Second, Myocardin is a novel protein with the following properties that implicate it as a cofactor for the SRF-dependent activation of muscle genes. 1) During embryogenesis, Myocardin is expressed specifically in developing 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.

Differential cellular responsiveness of CArG boxes

How might different CArG boxes encode differential information for cell type-specificity? A possible mechanism is that CArG boxes with relatively high affinities for SRF are able to detect low levels of SRF in a wide range of cell types, whereas muscle-specific CArG boxes, which exhibit reduced affinities for SRF, are only able to detect the higher levels of SRF that exist in muscle cells (as well as in certain neural cell types). According to this model, the multimerized CArG boxes transgenes respond to endogenous SRF levels with different sensitivities, resulting in different cellular expression patterns (Fig. 25). Consistent with this interpretation, SRF expression is highly enriched in developing muscle cell lineages and in a subset of neuroectodermal derivatives during embryogenesis, resembling the expression patterns of the *SM22* and *skeletal α-actin* CArG-containing transgenes.

The possibility that the multimerized CArG boxes contained in our transgenes "read" SRF levels *in vivo* is also suggested by the similarity in expression pattern seen with the *SM22* and *skeletal α-actin* CArG multimers. During embryogenesis, the endogenous *SM22* and *skeletal α-actin* genes show different temporospatial expression patterns in developing

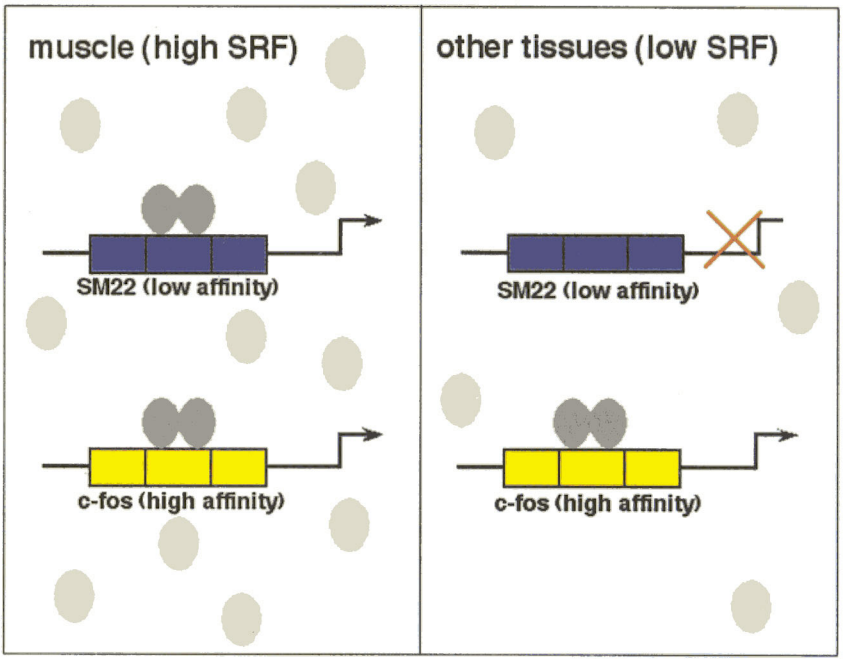


Fig. 25. Affinity model of SRF activation of target genes.

A model for muscle-specific activation of muscle genes by SRF. SRF is expressed at high levels in muscle and low levels elsewhere. Ubiquitously expressed genes such as c-fos or egr-1 contain CArG boxes with high affinities for SRF, and thus are able to bind even low levels of SRF. Correspondingly, muscle-specific genes such as SM22 or skeletal a-actin contain CArG boxes with lower affinities for SRF, and thus are only able to bind SRF at the high concentrations of SRF found in muscle.

muscle cell lineages (Li et al., 1996a; Lyons et al., 1991; Ruzicka and Schwartz, 1988; Solway et al., 1995). The fact that the multimerized *SM22* and *skeletal α-actin* CArG boxes direct the same expression patterns in skeletal, cardiac and smooth muscle cells in vivo also indicates that SRF is not solely responsible for the normal expression patterns of those genes.

Multimerized SRF and MEF2 Binding Site Reporters

A similar approach studying transgenic mice harboring multimerized binding sites for the MADS-box transcription factor MEF2 has previously been described (Naya et al., 1999). During embryogenesis, these MEF2 "sensor" mice showed specific expression of lacZ in developing muscle and neural cell lineages, the same cell types in which MEF2 is expressed at highest levels. Multimerized MEF2 binding sites from the *desmin* enhancer, which is muscle-specific, and the *c-jun* promoter, which is growth factor-inducible, both directed similar lacZ expression patterns *in vivo*. After birth, the MEF2-lacZ transgene was downregulated in skeletal and cardiac muscle despite high levels of MEF2 protein in these tissues, but could be activated in response to various calcium-dependent signal transduction pathways (Passier et al., 2000; Wu et al., 2000). These findings suggest that SRF and MEF2 may use different mechanisms to confer cell type-specificity through their target sequences.

Comparisons with other CArG box studies

This is the first analysis of the potential interchangeability of CArG boxes in transgenic mice described in the literature. However, previous studies of other CArG boxes in transfection assays have also suggested a correlation between low-affinity DNA binding of

SRF and muscle-restricted activity. Replacement of the most proximal CArG box in the *skeletal α-actin* promoter with the CArG box from the *c-fos* promoter resulted in constitutive expression of the muscle promoter in transfected nonmuscle cells (Santoro and Walsh, 1991). Similarly, replacement of two CArG boxes from the s*mooth muscle α-actin* promoter with the *c-fos* CArG box caused a relaxation in cell-specific expression in transfected cells (Hautmann et al., 1998).

Simplified regulation of CArG multimers

The approach described in the first half of this thesis was to investigate the activity of isolated CArG boxes and adjacent sequences outside the context of their native promoters. This is, of course, an over-simplification of the actual regulatory events that govern the activity of these sequences *in vivo*, and does not discount the potential importance of other regulatory factors that bind sites surrounding the CArG boxes in their native promoters. These results demonstrate that SRF can discriminate between different target genes based on differential affinity for CArG boxes. Such differential binding is likely to contribute to the specificity of expression of SRF-dependent genes in vivo and is likely to be profoundly influenced by cofactor interactions and intracellular signals.

Regulation of transcription and chromosomal organization by SAP domain proteins

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 to SARs has been proposed to create chromatin loops that affect the expression of adjacent genes. While Myocardin might have a role in binding to SARs, its primary function appears to be as a transcriptional activator, because it contains a potent transcriptional activation domain, and because it activates CArG box-dependent reporters in transfection assays that would not be expected to require complex alterations in chromatin conformation.

The behavior of SAP domain mutants of Myocardin suggests that transcriptional activation of the *SM22* and *ANF* promoters may involve different mechanisms. The SAP domain was dispensible for *SM22* activation, but it was essential for *ANF* activation. Because 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. Another possibility is that the SAP domain may interact with DNA when tethered to a promoter by SRF, thus providing promoter specificity, but this interaction is not strong enough to detect by gel mobility shift assays in the absence of SRF.

Members of the GATA family of zinc-finger transcription factors bind a site between the two CArG boxes in the *ANF* promoter (Morin et al., 2001). Notably, SRF has been shown to associate with GATA transcription factors (Belaguli et al., 2000; Morin et al., 2001). Studies are underway to determine if GATA factors interact with Myocardin, and if the SAP domain mediates transcriptional synergy between SRF and GATA factors.

Myocardin is expressed in cardiac and smooth muscle lineages

Myocardin expression is initiated in the cardiac crescent at the time of cardiogenic specification and is maintained throughout the atrial and ventricular chambers during pre- and postnatal 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 esophagus and 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 other myogenic SRF cofactors in addition to Myocardin. Whether the other Myocardin-related factors identified from ESTs might play this role remains to be determined.

Coregulation of muscle gene expression by SRF and Myocardin

Myocardin appears to require a very precise level of SRF for maximal transcriptional activity. Transfection of more than 10 ng of SRF results in suppression of Myocardin-dependent transcription. Previous studies have also 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). A possible explanation for this observation is that excess SRF competes with SRF-Myocardin complexes for binding to CArG-boxes. 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-Myocardin complexes bound to DNA target sites. Another interpretation of these

results is that excess SRF titrates out a common factor required for transcription. Competition for association with TFIIF has been shown to account for the ability of SRF to squelch itself and other activators (Joliot et al., 1995). Thus, it is also possible that Myocardin and excess SRF compete for association with TFIIF or other basal transcription factors.

Myocardin does not bind the CArG box alone, but forms a stable complex with SRF on DNA. In contrast to the association of SRF with TCF, which requires binding of both SRF and TCF to DNA, Myocardin and SRF can interact in the absence of DNA. Association of Myocardin with SRF is dependent on the basic and Q-rich regions near the amino-terminus. 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, as has been shown to occur in response to association of SRF with Phox and HMG-I(Y). However, gel shift assays using suboptimal amounts of Myocardin and SRF were not performed, so any possible effects by Myocardin on SRF binding affinity cannot be ruled out.

As mentioned previously, SRF has been shown to associate with Nkx2.5 and GATA4. However, there are several significant differences between the effects of Myocardin and these other factors on SRF-dependent transcription. 1) Myocardin activates transcription by several thousand-fold through the CArG box, whereas Nkx.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) It has not been possible to demonstrate the existence of a DNA-binding ternary complex between SRF and Nkx.5 or GATA4.

Regulation of Myocardin activation of CArG box genes

The remarkable potency by 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 genes are specific for a particular type of muscle cell (e.g. cardiac, skeletal, or smooth), or even for a subset of cells within 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 such as *c-fos*, which are also regulated by CArG boxes, are downregulated in post-mitotic differentiated muscle cells, there must also be mechanisms that render them nonresponsive to Myocardin. Such differential responsiveness is likely to be dependent on the context of CArG boxes within individual promoters and the other factors that bind nearby sites. This type of specificity may be achieved, at least in part, through combinations of CArG boxes. Many muscle-specific genes are regulated by two or more CArG boxes that act cooperatively, whereas many growth-regulated genes, such as *c-fos*, are controlled by single CArG boxes. These 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 provide another mechanism for muscle-specificity.

Other Myocardin-interacting proteins

Myocardin interacts with the MADS domain of SRF. MEF2, another MADS box protein, forms a similar DNA-binding dimer to SRF and recognizes an extended AT-rich binding site that resembles a CArG box (Santelli and Richmond, 2000). However, despite the homology between the MADS domains of SRF and MEF2, I have not been able to demonstrate an interaction between Myocardin and MEF2 (data not shown).

Myocardin also activated transcription from the *MLC-2V*, *α-MHC* and *Nkx2.5* regulatory regions, although not as potently as from the *SM22* and *ANF* promoters (Fig. 13). These genes have not been shown to be regulated by SRF. Whether their activation by Myocardin reflects a direct role for SRF in their regulation or is mediated by an SRF-independent mechanism remains to be determined. It is conceivable that Myocardin may directly bind DNA outside CArG boxes. However, I have performed DNA binding assays with probes spanning the entire *SM22* promoter and have not detected Myocardin binding in the absence of SRF (data not shown). More likely, Myocardin has partners other than SRF that mediate activation of these other genes.

Role of Myocardin in muscle developmental pathways

Preliminary studies suggest that Myocardin may play an important role in early cardiac development. A dominant negative Myocardin mutant construct (CΔ585) was injected into Xenopus embryos, and the embryos were assayed for expression of cardiac markers by *in situ* hybridization. Injection of dominant-negative Myocardin results in a

dramatic reduction of expression of cardiac *a*-actin, *a*-tropomyosin, troponin I, and Nkx2.5 transcripts (Wang et al., 2001). Currently, mouse transgenic and knockout experiments are underway to determine the *in vivo* function of Myocardin in mammalian muscle development.

Conclusion

SRF regulation is complex, and many pathways that regulate its activity have already been identified. The data presented in this thesis describe two novel mechanisms of SRF regulation. The differential DNA binding affinities of SRF for different CArG boxes and the interaction of SRF with Myocardin are important elements in the specific activation of muscle genes by SRF. Future studies of these aspects of SRF regulation will contribute to the elucidation of the regulatory networks that control SRF activation and function, and will eventually provide a fuller understanding of the molecular mechanisms of muscle development.

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