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Transcriptional Regulation During Heart Development

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Transcriptional Regulation During Heart Development

by

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Dedication

I am eternally grateful to my parents Tom and Mary Ellen Small, for their inexhaustible encouragement and support, and dedicate this doctoral dissertation to them.

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I extend the deepest gratitude to all of my mentors and colleagues during my graduate school career. In particular, I am indebted to my advisor Paul Krieg who has been an unequaled source of suggestions, expertise and encouragement. I credit Peter Vize for my introduction to the rapidly changing field of developmental biology, and impressing upon me the power and excitement of this field. I am grateful for the interactions, discussions, and friendships with a number of talented co-workers, including Ondine Cleaver, Tom Carroll, John Wallingford, Craig Newman, Wendy Gerber, Steve Vokes, Rob Garriock and Andy Warkman. I have also benefited from the faculty and students of the Cell Biology and Anatomy Department at the University of Arizona, especially Ray Runyan, Parker Antin, Carol Gregorio and Mark Bales. I am particularly indebted to Tim Mohun and Branko Latinkic for technical assistance with transgenesis methods, which were the foundation of much of my research, and valuable discussions on interpreting experimental data. I thank Benoit Bruneau and Eric Olson for critical comments on this research and for providing information prior to publication. I would also like to thank David Hillis for obtaining the *Rana catesbiana* that was used in my earliest experiments.

Transcriptional Regulation During Heart Development

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Cardiac gene expression is commonly thought to be the result of combinatorial interactions between heart-specific and globally expressed transcription factors. Myocardin has recently been identified as a cardiac and smooth muscle specific cofactor for the globally expressed serum response factor (SRF) protein. Using a combination of gain-of-function and loss-of-function approaches, we show that myocardin is both necessary and sufficient for the transcriptional activation of a wide range of cardiac differentiation products. We also demonstrate that myocardin cooperates with additional factors to induce gene expression, fitting with previous models of cardiac gene regulation. Myocardin is able to induce ectopic expression of *cTnI*, *cardiac α-actin*, *MHCα*, and *MLC2*, in addition to smooth muscle markers in whole embryos or animal pole explants. Conversely, suppression of myocardin gene activity by morpholino knockdown results in the inhibition of cardiac differentiation products. The expression of the early cardiac specification gene *Nkx2-5* however, is unaffected by either myocardin

misexpression or loss-of-function. From this data, we conclude that myocardin is the key intermediate between cardiac specification and differentiation.

The process of cardiac differentiation includes the formation of the atrial and ventricular chambers which display distinct contractile, physiological, conductive, and genetic properties. The *atrial natriuretic factor* (*ANF*) gene is initially expressed throughout the myocardial layer of the heart, but during subsequent development, expression becomes limited to the atrial chambers. We have isolated the *Xenopus ANF* promoter in order to examine the temporal and spatial regulation of the *ANF* gene in vivo using transgenic embryos. The mammalian and *Xenopus ANF* promoters show remarkable sequence similarity, including an Nkx2-5 binding site (NKE), two GATA sites, two SRF binding sites (SREs) and a Tbx binding site (TBE). Our transgenic studies show that mutations in either the NKE or proximal GATA elements result in ectopic *ANF* promoter activity in the kidneys, facial muscles, and aortic arch artery associated muscles, and causes persistent expression in the ventricle and outflow tract of the heart. We propose that the NKE and proximal GATA elements serve as crucial binding sites for assembly of a repressor complex, that is required for atrial-specific expression of the *ANF* gene.

Table of Contents

List of Figures

Chapter 1: General Cardiac Development

1.1. INTRODUCTION

The heart is the first organ to form during vertebrate development and is arguably the most important step in creating a living organism. The initiation of cardiogenesis occurs during the third week of human development (E7.25 in mouse or day 2 in *Xenopus laevis*). Rhythmic contractions begin shortly thereafter, at the beginning of the fourth week of development in humans (Sissman, 1970; Niewkoop and Faber, 1994), meaning that the developmental events, from the onset of cardiac gene expression to the establishment of a fully functional circulatory system occurs in less than one week. A functioning cardiovascular system is an absolute requirement at these early stages of development; rapid growth results in an embryo that is too large to be supplied with oxygen and nutrients by simple diffusion. In order for this exquisitely intricate process to occur, multiple steps must take place flawlessly, including heart specification, differentiation, and morphogenesis. These morphogenetic processes rapidly transform a simple cardiogenic tube into a mature four chambered heart that is comprised of multiple tissue types that exhibit distinct genetic, contractile, conductive and physiological properties (Lyons et al., 1990; Dagnino et al., 1991; Small and Krieg, 2000). Errors in

this process result in congenital heart disease and occur in about 1 percent of live human births. The frequency of fatal congenital heart defects resulting in spontaneous abortion is thought to be as high as 5-10% (Hoffman, 1995). This chapter will briefly outline what is known of the basic processes involved in the formation of a normal mature heart.

1.2. Cardiac morphogenesis

In the amphibian, the specification of pre-cardiac mesoderm occurs early during gastrulation, as the involuting cells destined to become the anterior mesoderm migrate through the blastopore and come into close proximity to Spemann's organizer (Jacobson and Sater, 1988). Signals emanating from the Spemann organizer and the underlying endoderm are necessary and sufficient to initiate the process of cardiogenesis in amphibians (Sater and Jacobson, 1990). After passing through the blastopore, the pre-cardiac cells migrate to dorso-lateral positions at the anterior end of the embryo resulting in bilateral heart patches. These heart patches then migrate to the ventral midline and fuse, creating the cardiac "tube" that initiates rhythmic contractions and acts as a simple pump, circulating blood throughout the circulatory system by peristaltic movements (Fishman and Chien, 1997).

The heart tube then undergoes a series of tissue rearrangements and morphogenetic movements that result in formation of the atrial and ventricular chambers, and placement of the chambers in the precise locations to facilitate the coordinated interconnections of the cardiovascular and pulmonary systems. These tissue movements result in the heart tube undertaking a counterclockwise looping motion. The pre atrial and ventricular chamber myocardium "balloons" out from the outer curvature of the looping tube and takes on distinct morphological, genetic, and physiological properties (Christoffels et al., 2000). Finally, septations form to partition the single atrium and ventricle into two atria and two ventricles, and valves arise from the cardiac cushion in the atrioventricular canal that separate the atrium from the ventricles (Mohun et al., 2000; Kolker et al., 2000). The morphological account of heart development and the tissue interactions that initiate cardiogenesis has been well documented from many years of observations using classical embryology. Recently, the molecular events that underlie the morphological processes of cardiogenesis have been extensively studied. For the purposes of this overview, I will focus on transcription factors and their target genes (Bruneau, 2002).

1.3. Molecular studies of cardiac development

Of the cardiac expressed transcription factors, the homeodomain-containing Nkx2-5 protein is the earliest known marker of the heart field in a wide range of evolutionarily diverse species (Lints et al., 1993; Komuro and Izumo, 1993; Tonissen et al., 1994; Bodmer, 1993). *Nkx2-5* gene expression is initiated early during gastrulation as the involuting pre cardiac mesoderm migrates to the dorso-lateral heart patches (Harvey, 1996; Komuro and Izumo, 1993). Cardiac expression continues throughout the heart during development and into adulthood. The importance of this transcription factor in the specification of cardiac tissue was first suggested by *Drosophila* that are mutant for the *Nkx2-5* related *tinman* gene. *Tinman* mutant flies completely lack the dorsal vessel, the insect equivalent of the heart (Bodmer, 1993). This would suggest that *tinman* function is essential for heart development, however analogous experiments in which the mouse *Nkx2-5* gene was knocked out result in the formation of a relatively normal heart tube (Lyons et al., 1995; Tanaka et al., 1999). While cardiogenesis appears to be correctly initiated in the *Nkx2-5* null mouse, a number of cardiac differentiation products are greatly reduced or absent, and the heart tube fails to loop, resulting in death at E9.5 due to

hemodynamic insufficiency (Lyons et al., 1995; Tanaka et al., 1999). Furthermore, experiments in which a dominant negative form of the *Xenopus Nkx2-5* gene was overexpressed by microinjection resulted in a block to cardiac differentiation (Grow and Krieg, 1998). The Nkx2-5 protein has been shown to be important for the expression of many cardiac genes including *ANF* (Durocher et al., 1996), *eHand* (Biben and Harvey, 1997), and *GATA6* (Davis et al., 2000; Molkentin et al., 2000). Thus, while not absolutely necessary for vertebrate heart development, Nkx2-5 appears to play a central role in the differentiation and maintenance of a functional heart

Members of the zinc finger family of GATA transcription factors have also been implicated as early regulators of the cardiogenic pathway. *GATA-4* is expressed in the developing and adult heart and visceral endoderm (Arceci et al., 1993; Kelley et al., 1993), and recent experiments have shown that GATA-4 is sufficient to induce cardiomyocytes in *Xenopus* ectodermal explants (Latinkic et al., 2003). Mouse knockout experiments have also shown that *GATA-4* is required for heart tube formation. Mice lacking a functional *GATA-4* gene do not form a heart tube and die between E8.5 and E10.5 (Kuo et al., 1997; Molkentin et al., 1997). Cardia bifida is observed in *GATA-4*

mutant mice and is likely due to abnormal formation of the underlying endoderm and the subsequent failure of the ventral migration of the bilateral heart patches (Kuo et al., 1997; Molkentin et al., 1997). Somewhat surprisingly, initiation of cardiac gene expression in the early heart patches seems to occur normally in *GATA-4* null mice. These observations suggest that *GATA-4* is not required for specification of cardiomyocytes, but plays a role in later cardiac morphogenesis.

While Nkx2-5 and GATA-4 undoubtedly play important roles in cardiogenesis, these factors do not appear to be absolutely required for initiation of all cardiac gene expression. Many studies have recently emphasized that combinatorial interactions between multiple cardiac transcription factors are crucial for optimal activation of target gene expression. Studies using luciferase assays in cultured cells have demonstrated cooperative interactions between Nkx2-5, the zinc finger protein GATA-4, the T box containing Tbx5, and the ubiquitous serum response factor (SRF) and its cardiac specific cofactor myocardin. These studies demonstrate a synergistic activation of the *atrial natriuretic factor* (*ANF*) and *cardiac* α *actin* promoters by combinations of transcription factors, reaching 1000 fold above background levels for the case of Nkx2-5, GATA-4

and SRF together (Durocher et al., 1997; Durocher and Nemer, 1998; Lee et al. 1998; Lee et al., 2002; Wang et al., 2001a; Bruneau et al., 2001). Parallel experiments have demonstrated physical interactions between Nkx2-5, GATA-4, and SRF on specific regulatory elements which increase binding efficiency and transcription levels of target promoters (Chen and Schwartz, 1996; Durocher et al., 1997; Sepulveda et al., 1998; Lee et al., 1998). While caution is advised in the interpretation of these results since all this work has been done in cell culture systems, it is evident that a number of key factors cooperate in the regulation of early cardiac morphogenesis. Results to be presented in this thesis will implicate another cardiac specific transcription factor, myocardin, as an essential component of the regulatory pathway leading to heart development (Chapter 2).

1.4. Molecular Regulation of Cardiac Chamber Differentiation

During embryonic development, the heart is initially formed as a simple linear tube located at the ventral midline of the organism. During subsequent development the tube undergoes a complex series of movements and tissue remodeling events that lead to formation of the mature four-chambered, septated organ. These morphological events are accompanied by an equally complex series of cellular and molecular processes that lead

different regions of the heart to express different genes and to adopt different physiological properties. The myocardium of the atrial and ventricular chambers contain distinct isoforms of the myosin light chain genes, MLC2a and MLC2v respectively, which contribute to distinct contractile properties of the chambers. When MLC2a replaces MLC2v protein in ventricular cardiomyocytes of MLC2v null mice, a lack of sarcomeric structure and lowered cardiac contractility is observed (Chen et al. 1998). Furthermore, chamber-specific expression of regulators of Ca^{2+} cycling is responsible for differences in contractile properties between the atria and ventricles (Koss et al. 1995, Minamisawa et al. 2003). At the molecular level remarkably little is known about the regulatory mechanisms that result in differential gene expression in different chambers of the heart. From expression studies we know that genes become chamber-specific at different times during cardiac development. This fact alone suggests that a single mechanism is unlikely to be responsible for chamber-specific expression.

The isolation of regulatory sequences for a number of chamber specific genes has resulted in identification of a number of cis-elements and trans-acting factors. Analysis of these sequences in transgenic animals has revealed a couple intriguing insights into

chamber specific gene regulation. First, all of the atrial specific genes analyzed to date appear to arise from inhibition in the ventricle, as opposed to activation strictly in the atrium. For example, the atrial regulatory domain (ARD) of the atrial-specific, quail myosin heavy chain 3 gene seems to be inhibited in the ventricle by the ventricle-specific Irx4 protein bound to the promoter via a vitamin D receptor/retinoic X receptor heterodimer (Wang et al., 2001b). This inhibitory mechanism also seems to hold true for the *MLC2a* gene (Ngyuen-Tran et al., 1999), and the *ANF* gene (Small and Krieg, 2003; see Chapter 4 of this dissertation). Second, it seems likely that currently unidentified transcriptoin factors must be involved in regulation of chamber-specific expression. Analysis of the *MLC2v* regulatory sequences in transgenic mice has identified a 250 bp fragment that drives right ventricular transgene expression (Zhu et al., 1991). Further studies have shown that the ventricle-specificity is dependent on a 28 bp segment that contains an HF-1a site and an AT-rich domain (Ross et al., 1996). None of the factors that bind this region are chamber specific however, making it likely that other factors remain to be identified that can form a complex on the *MLC2v* promoter. Furthermore, we provide evidence in Chapter 4 that *ANF* gene expression is likely inhibited in the ventricle by an as yet unidentified repressor.

This dissertation describes findings on two aspects of transcriptional regulation during cardiac differentiation. First, Chapter 2 will focus on one of the earliest timepoints during cardiogenesis, the initial induction of cardiac differentiation products. We will provide evidence using both gain-of-function and loss-of-function experiments that the SRF cofactor, myocardin, is both necessary and sufficient to activate the expression of a number of cardiac differentiation products (Wang et al., 2001a). Second, Chapter 3 of this dissertation will describe the developmental expression profile of the *atrial natriuretic factor* (*ANF*) gene which becomes confined to the atrial chamber late during heart development. Chapter 4 will describe experiments using transgenic *Xenopus laevis* embryos in which the *ANF* promoter, with mutations of various conserved transcription factor binding sites, was used to drive the expression of a GFP reporter gene. We provide evidence that the binding sites for Nkx2-5 (NKE) and GATA-4 (GATA) are required for the restriction of *ANF* expression to the atrium.

Chapter 2: Myocardin induces cardiac differentiation[1]

3.1. INTRODUCTION

Skeletal muscle differentiation appears to be regulated in part by transcription factors capable of controlling expression of a large number of downstream, muscle differentiation genes. These transcription factors comprise the MyoD family and have many of the properties of "master regulatory" factors. So far, however, the search for equivalent factors involved in regulation of the cardiogenic lineage has proved unsuccessful. While a single gene has not been shown to activate the heart program, a number of important transcription factors are expressed in the pre cardiac mesoderm. *Nkx2-5*, the vertebrate homologue of the *Drosophila tinman* gene, is among the earliest known cardiac-expressed transcription factors. Nkx2-5 has been shown to directly activate the cardiac differentiation products *ANF* and *Cardiac α actin* in HELA cells and cardiac myocytes (Chen and Schwartz, 1996, Durocher et al., 1997). Additionally, in

[1] Andrew Warkman assisted in the cloning of *Xenopus myocardin*, morpholino injection experiments, and Figures 1 and 7.

vitro studies have demonstrated synergistic interactions between Nkx2-5, the zinc finger containing GATA-4, the T box containing Tbx5 and the MADS box gene SRF (Hines, 1999; Durocher and Nemer, 1998; Bruneau et al., 2001). Further studies have demonstrated an importance for the MADS box containing Mef2 factors to heart development, and a number of Mef2 responsive genes have been identified (Black and Olson, 1998).

While the importance of these various factors has been shown in vitro, they are not absolutely required for heart induction in vivo. Experiments using knockouts of these factors in mice have demonstrated a lesser role to cardiogenesis. While mice null for either *Nkx2-5* or *GATA-4* die from cardiac abnormalities due to failure of heart looping or heart patch fusion, respectively, expression of most cardiac sarcomeric genes is initiated properly (Lyons et al., 1995; Molkentin et al., 1997; Biben et al., 2000). Thus, although heart differentiation does not proceed normally in these mice, it appears that cardiac tissue is correctly specified. There are several possible explanations for the relatively normal cardiac induction in *Nkx2-5* and *GATA-4* null mice. First, other related molecules with redundant properties may compensate for their absence. This is a plausible

explanation for the ablation of the GATA transcription factors, since GATA-4, 5 and 6 are often expressed in the same tissues, and they are known to have similar transcription regulating activities (Jiang and Evans, 1996). Redundancy, however, does not seem to explain the Nkx2-5 ablation results, since double knockouts of the related homeodomain proteins *Nkx2-5* and *Nkx2-6* still initiate cardiogenesis and express the majority of cardiac markers (Tanaka et al., 2001). Second, combinatorial interactions between multiple factors could act to increase differentiation gene product levels, whereas transcriptional activation is initiated relatively normally in the absence of a single factor. And finally, an undiscovered cardiac "master regulator" might remain to be discovered, analogous to the MyoD family of skeletal muscle regulators.

While the basis of muscle induction seems to differ between skeletal and cardiac muscle, there are certain similarities and SRF is a key factor in the differentiation of both lineages. SRF is a MADS box transcription factor and is expressed in a wide range of tissues. Activation of SRF-inducible genes is mediated by binding to a CarG box, found in many heart and skeletal muscle promoters. It has recently been determined that myocardin is a potent transcriptional activator and cofactor of SRF that is expressed in

the developing heart and in the adult organ as well as smooth muscle cells (Wang et al., 2001a). Although myocardin itself possesses no DNA binding activity, interaction of myocardin with SRF greatly increases the level of expression of SRF target genes. Indeed, interactions between myocardin and SRF may provide the mechanism whereby SRF responsive elements in heart and smooth muscle-specific promoters are distinguished from those in skeletal muscle promoters. Myocardin has recently been suggested to be necessary and sufficient for activation of SRF dependent genes expressed in smooth muscle (Wang et al., 2003) and the mouse knockout of the myocardin gene results in loss of vascular smooth muscle differentiation (Li et al., 2003). An important role for myocardin in the heart development pathway is supported by studies showing that expression of a dominant negative form of the protein in *Xenopus* embryos is able to abolish heart marker expression (Wang et al., 2001a).

In this dissertation, we report that myocardin is able to activate a large number of myocardial and smooth muscle differentiation genes in non-muscle cells. Injection of *myocardin* mRNA into *Xenopus* embryos results in ectopic expression of cardiac markers in a range of non-cardiac tissues. Furthermore, myocardin is able to activate expression

of myocardial and smooth muscle differentiation markers in animal caps (prospective epidermal tissues) independently of mesoderm or skeletal muscle development programs. We also show that myocardin can function cooperatively with additional cardaic-expressed transcription factors to activate the transcription of a wider range of products than possible alone. Conversely, depletion of myocardin in the developing embryo by antisense morpholino injection abolishes expression of cardiac gene expression suggesting myocardin is also required for induction of cardiac differentiation. To our knowledge, this is the first example where a single factor is necessary and sufficient to induce multiple myocardial markers in the absence of mesoderm. Myocardin appears to be the important intermediate between cardiac induction and differentiation and draws parallels between cardiac and skeletal muscle gene regulation.

2.2. RESULTS

2.2.1. Cloning of *Xenopus myocardin.*

The deduced amino acid sequence of the *Xenopus laevis* myocardin protein is presented in Figure 1, aligned with the mouse and human myocardin protein sequences. *Xenopus* myocardin contains 918 amino acids with a predicted molecular weight of 101.3

kDa. The *Xenopus* myocardin protein is 56% and 57% identical to mouse and human proteins, respectively. The basic region, which is involved in interactions interactions with serum response factor (SRF) is particularly highly conserved (89%), as is the SAP domain (about 90%) which functions during chromatin remodeling (Wang et al., 2001a; reviewed in Aravind and Koonin, 2000).

Figure 1. Protein alignment of *Xenopus*, human and mouse myocardin.

Amino acid alignment of the Xenopus, mouse, and human myocardin proteins is represented in Figure 1. The basic region (which is involved in SRF binding), the SAP domain (involved in chromatin remodeling), and the leucine zipper-like domain (which presently has an unknown function), are depicted by boxes. The Xenopus myocadin protein is 56% and 57% identical to the mouse and human proteins, respectively. The basic region displays 89% identity to the mouse and human basic regions. The three proteins display 90% identity across the SAP domain.

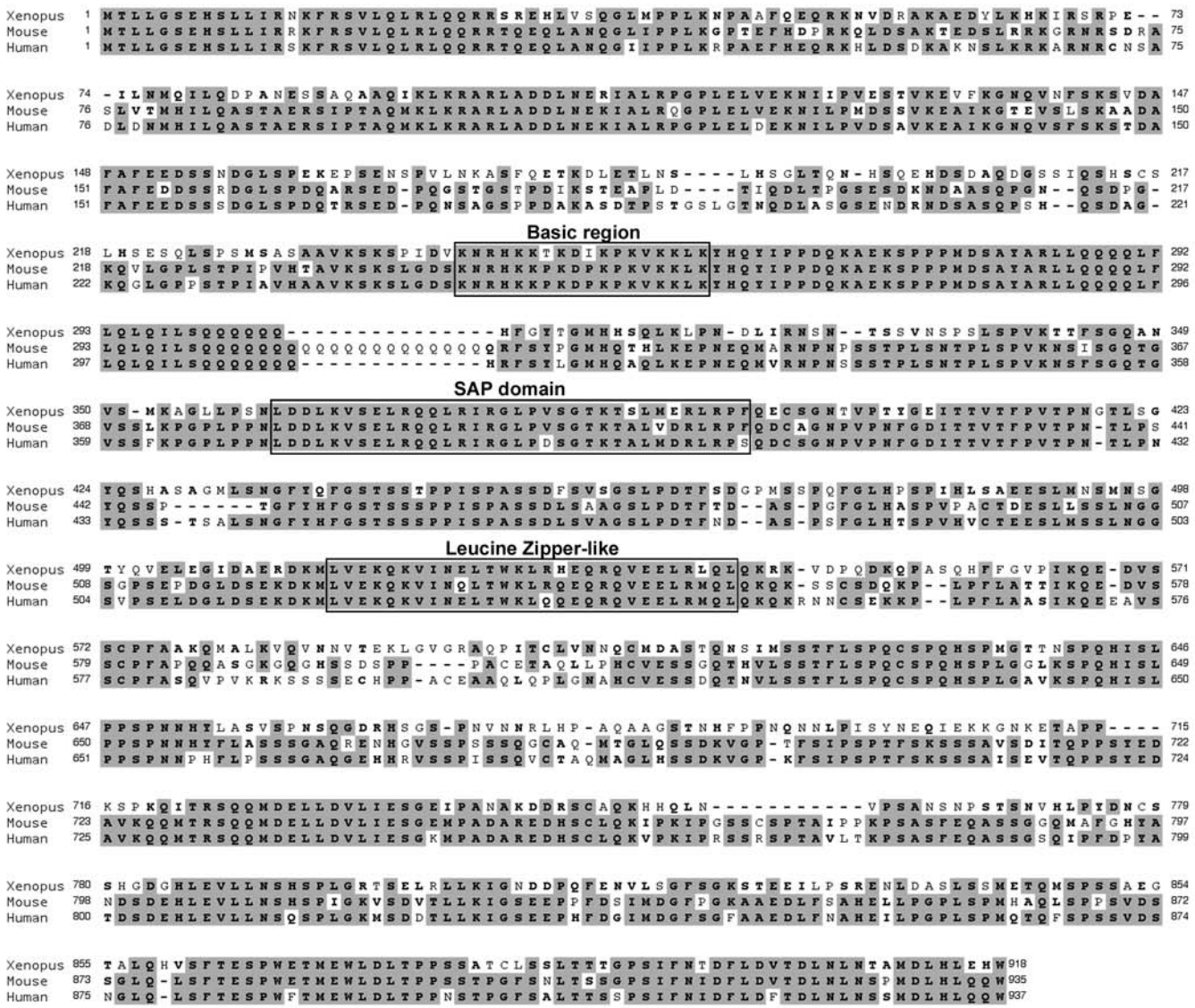


Figure 1

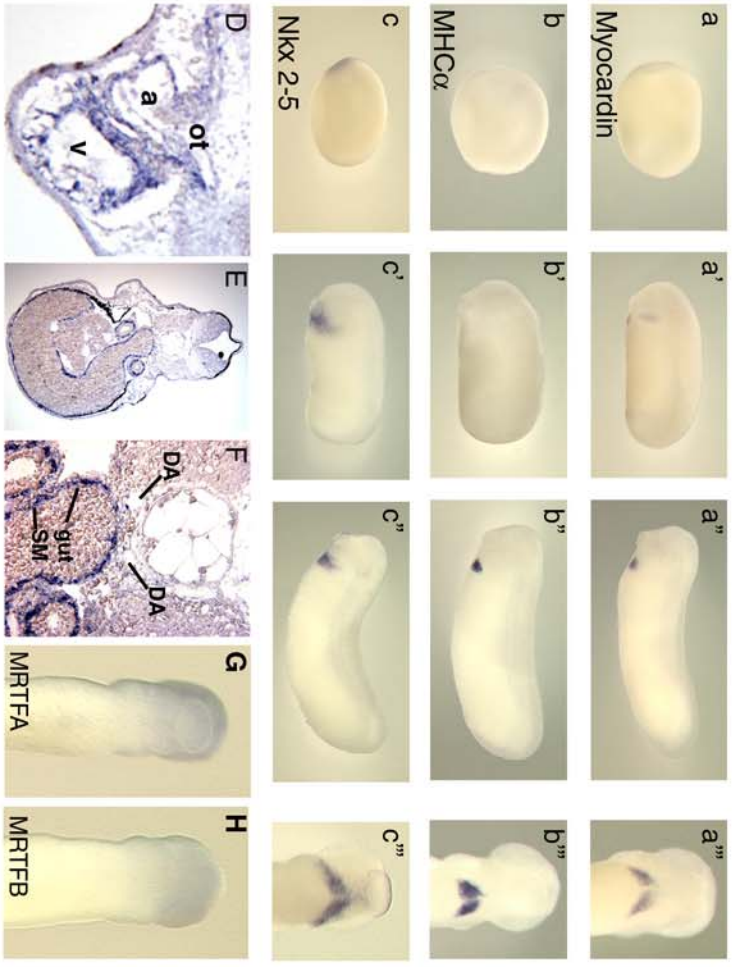
2.2.2. *Myocardin* is expressed in cardiac and smooth muscle cells

The expression pattern of *myocardin* during *Xenopus* development is presented in Figure 2. *Myocardin* transcripts are not detectable in the early neurula embryo (stage 15, Fig. 2a) at a time when transcripts for the precardiac marker, *Nkx2-5*, are abundant (Fig. 2c). *Myocardin* expression is first detected in the late neurula embryo (st. 24) in the pre-cardiac patches of anterior lateral mesoderm (Fig. 2a'). This expression precedes detection of transcripts for *myosin heavy chain-α* (*MHCα*) which is the earliest marker of myocardial differentiation in the *Xenopus* embryo. By stage 27, *myocardin* transcript levels have continued to increase and the domain of expression appears to be identical to that of *MHCα*, but significantly more restricted than the *Nkx2-5* expression domain (Fig. 2a", b", c"). *Myocardin* expression persists during subsequent cardiac development and is visible throughout the atrial and ventricular muscle layers of the tadpole heart (Fig. 2D). By the tadpole stage, *myocardin* transcripts are also visible in visceral smooth muscle cells surrounding the looping gut (Fig. 2E) and in isolated smooth muscle cells adjacent to the forming dorsal aorta (Fig. 2F). Overall, the expression of *Xenopus myocardin* in developing cardiac and smooth muscle tissues closely resembles the expression profile

Figure 2. Expression of myocardin during *Xenopus* development.

The expression of the Xenopus *myocardin* gene (a, a', a", a"') was analyzed by whole mount in situ hybridization and compared to the developmental expression patterns of the cardiac differentiation marker, *MHCα* (b, b', b", b"'), and the early cardiac inducer, *Nkx2-5* (c, c', c", c"'). a.) *Myocardin* is not detected in the early neurula stage embryo (stage 15). a') Myocardin expression is first observed at the early tailbud stage (stage 24) in the mesoderm of the heart patches. This expressin is approximately 24 hrs after the onset of *Nkx2-5* expression in the pre-cardiac mesoderm (stage 15, c). *Myocardin* expression in the tailbud embryo is localized to the pre-differentiated cardiac mesoderm, much more specific than *Nkx2-5*, which is also expressed in the pharyngeal arches (compare a' with c' and a" with c"). *MHCα* expression commences shortly therafter (stage 26) and persists in an identical domain to myocardin at stage 27 (compare a" with b"). a"', b"', c"') Ventral views of stage 27 embryos illustrates the expression of *myocardin* and *MHCα* is localized to very similar domains within the heart patches, while *Nkx2-5* is observed in a more widespread domain. D.) *Myocardin* expression persists into late development as is seen throughout the atrium, ventricle, and outflow tract in a serial section to a stage 45 swimming tadpole. E, F.) *Myocardin* is also expressed in the visceral and vascular smooth muscle as observed by in situ hybridization to sections of a stage 42 embryo. F.) Individual cells surrounding the dorsal aorae are positive for *myocardin*, as are the smooth muscle layer of the looping gut. G, H.) The myocardin related transcription factors, *MRTF-A* and *MRTF-B*, are not expressed in the developing pre-cardiac mesoderm, as assayed by whole mount in situ hybridization.

Figure 2


a
Myocardin
a'
a"
a"'
b
MHCα
b'
b"
b"'
c
Nkx 2-5
c'
c"
c"'
D
ot
a
v
E
F
DA
DA
gut
SM
G
MRTFA
H
MRTFB

reported for the murine *myocardin* gene (Wang et al., 2001a). Two additional *myocardin* related sequences, *Xenopus MRTF-A* and *B* (Wang et al., 2002) are expressed in the *Xenopus* embryo. In situ hybridization analysis of these sequences is shown in Fig. 2G, and H. In each case, the chromogenic detection reaction was continued until non-specific background staining became evident. It is relevant to experiments presented later in this report, that neither *MRTF-A* nor *B*, is expressed at detectable levels in cardiac primordia tissues of the embryo.

2.2.3. Myocardin induces ectopic cardiac muscle gene expression in whole embryos

Previous studies using cells in culture have indicated that myocardin is able to activate markers corresponding to the cardiac and smooth muscle differentiation pathways (Wang Z. et al., 2003; Du et al., 2003; Chen et al., 2002; Wang 2001a, 2002). We have used the *Xenopus* embryo system as an in vivo model to investigate the ability of *myocardin* to regulate expression of heart and smooth muscle specific genes. In these experiments, mRNA encoding myocardin was injected into a single blastomere of an eight-cell embryo. The uninjected side of the embryo serves as a negative control. At various subsequent stages of development, expression of cardiac or smooth muscle

markers was determined using in situ hybridization of the injected embryos. Our results indicate that myocardin is sufficient to induce precocious and ectopic expression of cardiac markers. For example, expression of the cardiac specific differentiation marker, *MHCα*, is normally initiated in the late neurula embryo (st. 25). In myocardin mRNA injected embryos however, high levels of ectopic *MHCα* transcripts are present in stage 14 embryos (Fig. 3B) approximately 24 hours before expression would commence in the heart. Ectopic expression is observed in 65% of myocardin injected embryos (13 / 20). Similarly, precocious and ectopic expression of *cardiac α-actin* is observed in myocardin injected embryos (Fig. 3D) as is expression of *cardiac troponin I*, and the smooth muscle marker, *SM22* (data not shown). Ectopic expression of *MHCα* persists in later stage embryos, and is particularly strong in isolated patches in neural tissues (Fig. 3G) and transverse sections show *MHCα* transcripts apparently within tissues of the eye and neural tube (Fig 3H, I). Neural expression has also been observed for *cardiac α-actin* transcripts (data not shown). Despite extended culturing of myocardin mRNA injected embryos, beating tissue or striated muscle has never been observed at ectopic locations. Sectioning of embryos shows that ectopic expression of cardiac markers appears to be limited to ectodermal and mesodermal tissue layers (Fig. 3E) and we have never observed

cardiac gene transcripts in endodermal tissues, even when myocardin mRNA is specifically targeted to this germ layer.

It is important to note that not all cardiac differentiation markers are induced by injection of myocardin mRNA into the embryo. For example, we have never observed ectopic expression of *myosin light chain-2* (*MLC2*) transcripts in these embryos. This does not seem to be a dose effect, since injection of greater amounts of myocardin mRNA does not result in expression of these markers. The profile of gene expression responsive to myocardin activation will be examined in more detail using animal caps explants (below).

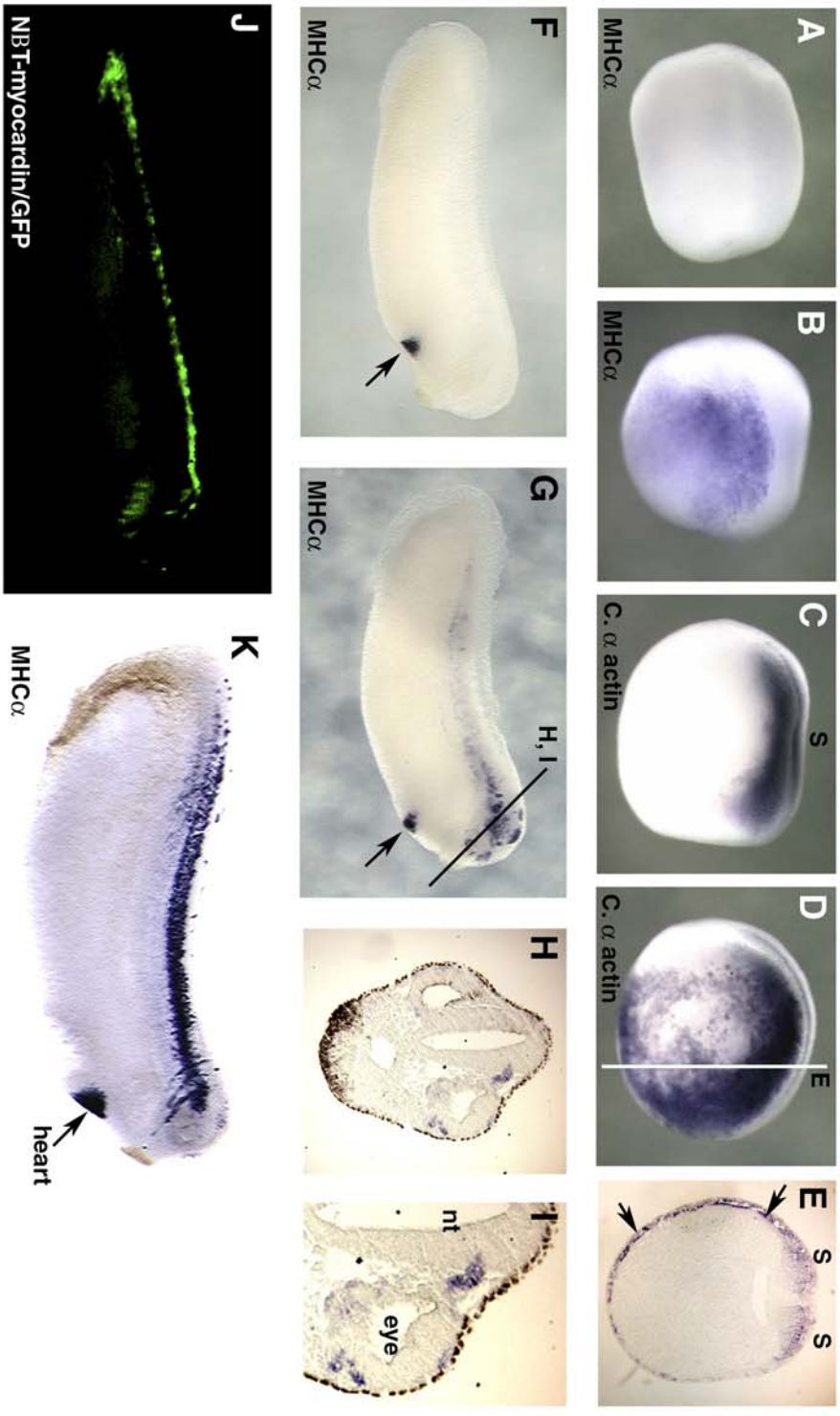
When mRNA is injected into a *Xenopus* embryo, translation of the mRNA commences almost immediately. In the experiments described above, injection occurred at the eight-cell stage, however, activation of tissue specific transcription pathways does not commence until approximately the gastrulation stage of development. It is possible therefore, that myocardin is only capable of initiating cardiac gene expression in ectopic tissues, in the absence of competing developmental programs. To address this question,

we generated transgenic embryos in which transcription of myocardin mRNA was driven by the *neural β tubulin* (*NβT*) promoter. *NβT* is a neural differentiation marker that is specifically expressed in the central and peripheral nervous system (Richter et al., 1988) and a *NβT*-GFP transgene recapitulates the endogenous expression pattern (Amaya and Kroll, 1996 and Fig. 3J). We have assayed embryos expressing myocardin under control of the *NβT* promoter using in situ hybridization for *MHCα* transcripts. As shown in Fig. 3K, *MHCα* expression have been activated throughout the differentiated neural tissues, identical to the pattern of expression indicated by the GFP marker (Fig. 3J). This result indicates that myocardin is able to activate transcription of cardiac specific genes in tissues that are already specified to a neural fate. Transgenic embryos expressing myocardin in neural tissues develop normally and show a full repertoire of reflex responses, indicating that myocardin does not subvert neural regulatory pathways, but apparently acts in parallel to them.

Figure 3. Misexpression of myocardin in the developing embryo.

A-I) 125pg of myocardin mRNA was injected into one cell of an eight-cell embryo and assayed for cardiac markers by whole mount in situ hybridization at various timepoints. The expression of the *MHCα* gene is never observed in stage 15 embryos (A), however injection of myocardin induced widespread expression of *MHCα* at this stage (B). Similarly, *C. α actin* is observed specifically in the pre-somitic mesoderm at stage 15 (C), while myocardin injected embryos display widespread expression of *C. α actin* on the side of injection (D, E). E.) A serial section through the embryo pictured in D shows C. α actin expression in the ectodermal and mesodermal tissue layers. Ectopic cardiac marker expression has never been observed in endodermal derivatives. Myocardin injected embryos also misexpress cardiac markers when cultured to later stages. F.) *MHCα* expression is heart-specific at stage 28 (arrow marks the endogenous heart expression in F and G). G.) When myocardin is injected into dorsal blastomeres at the 8 cell stage, *MHCα* expression is observed in a range of neural tissues at stage 28. H, I.) A serial section through the embryo in G demonstrates *MHCα* expression in tissues of the neural tube (nt) and eye. J, K.) When myocardin expression is driven in the central and peripheral nervous system by the *NβT* promoter in transgenic embryos, ectopic *MHCα* expression is induced in the neural tube and invading optic nerves. A stage 29 *Xenopus* embryo transgenic for *NβT*-GFP and *NβT*-myocardin is observed under fluorescent microscopy for GFP expression (J). *NβT*-GFP / *NβT*-myocardin transgenic embryos display robust ectopic expression of the *MHCα* gene in the neural tube and invading optic nerves as assayed by whole mount in situ hybridation.

Figure 3


A
MHCα
B
MHCα
C
S
C. α actin
D
E
C. α actin
E
S
S
F
MHCα
G
H, I
MHCα
H
I
nt
eye
J
NBT-myocardin/GFP
K
MHCα
heart

2.2.4. Myocardin activates myocardial and smooth muscle differentiation markers in animal cap explants

Since myocardin is able to activate cardiac tissue markers in whole *Xenopus* embryos, we wished to assess its ability to activate myocardial gene transcription in a more defined system. Animal cap explants from the *Xenopus* embryo, consisting entirely of ectodermal tissue, have been widely used to investigate gene expression (Casico and Gurdon, 1987; Grainger and Gurdon, 1989; Howell and Hill, 1997; Tada et al., 1998) and are a convenient alternative to cultured cells. Unmanipulated animal caps differentiate to form epidermal tissue and never normally express cardiac genes (Fig. 4A, lane labeled uninjected). It is important to note that animal cap tissue expresses a significant amount of SRF mRNA (Fig. 4A) and so the essential myocardin cofactor is present in this tissue. The consequence of expressing myocardin in animal cap explants was assayed at stage 12.5, corresponding to the late gastrula stage. As shown in Fig. 4A, myocardin precociously activates a range of myocardial differentiation markers including *MHCα, cTnI* and *cardiac α-actin* (which is also expressed in skeletal muscle). In addition, myocardin activates expression of the smooth muscle differentiation markers *SM actin*, *Calponin H1*, and SM22 (Fig. 4A).

Figure 4A. Myocardin induces cardiac and smooth muscle markers in animal pole explants.

125pg of myocardin mRNA was injected into the animal pole of one-cell *Xenopus* embryos. Animal pole explants were dissected and cultured until stage 12.5 and assayed for cardiac and smooth muslce gene expression by RT-PCR. Uninjected animal caps are ectodermal tissue destined to form epidermis, and never express mesodermal derivatives including cardiac or smooth muscle markers (lane labeled uninjected). Myocardin injected caps however, express a wide range of cardiac and smooth muscle differentiation markers (lane one labeled myocardin), including *MHCα, C. α actin*, *cTnI*, *SM22*, S*M actin*, and *Calp H1*. The myocardin cofactor *SRF* is upregulated in injected caps, as is the MADS box transcription factor *Mef2a*. *Nkx2-5* and *GATA-4* are not observed in myocardin injected animal caps.

Figure 4A

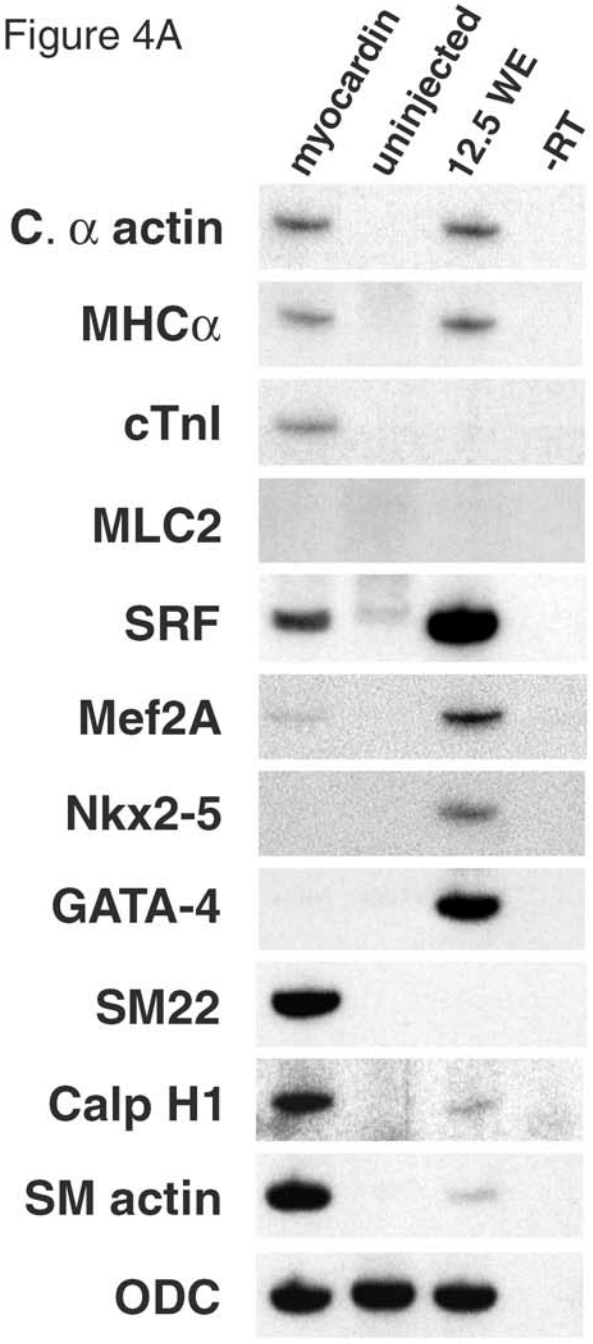
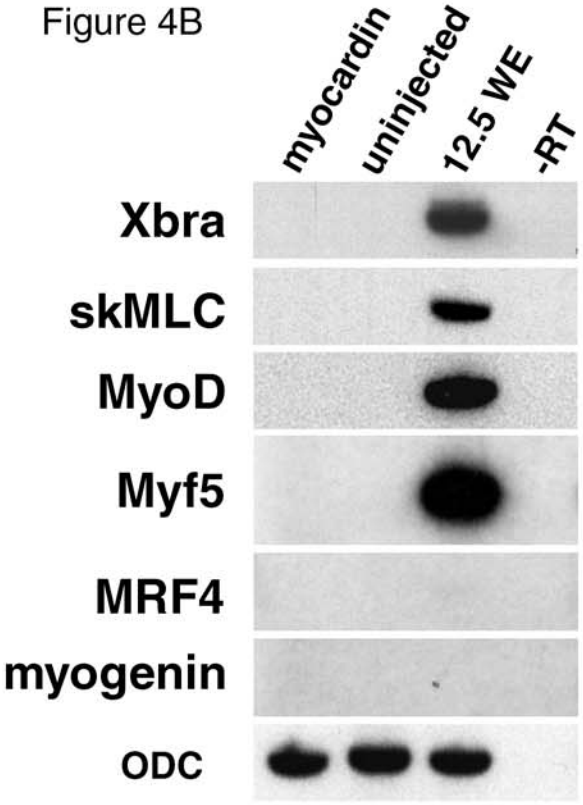

myocardin
uninjected
12.5 WE
-RT
C. α actin
MHCα
cTnI
MLC2
SRF
Mef2A
Nkx2-5
GATA-4
SM22
Calp H1
SM actin
ODC

Figure 4B. Myocardin does not induce genes of the skeletal muscle or mesodermal pathways.

Myocardin injected animal caps were assayed by RT-PCR for the induction mesoderm and the expression of skeletal muscle markers. The mesodermal marker *brachyury* (*Xbra*) is not induced in myocardin injected caps. Myocardin is also unable to activate expression of the skeletal muscle inducers of the MyoD family, *MyoD*, *Myf5*, *MRF4*, or *myogenin*, nor was it able to induce the skeletal muscle differentiation product *skMLC*.

Figure 4B


myocardin
uninjected
12.5 WE
-RT
Xbra
skMLC
MyoD
Myf5
MRF4
myogenin
ODC

As in the whole embryo experiments, myocardin did not activate expression of all myocardial genes. *MLC2* was never expressed in animal caps (Fig. 4A), even when the dose of myocardin was increased (data not shown). Similarly, SRF is not limiting in these experiments since co-injection of SRF mRNA together with myocardin mRNA resulted in identical results (data not shown). It has previously been reported that expression of GATA4 in animal cap explants is sufficient to initiate the complete cardiac differentiation pathway, including formation of beating tissue (Lantinik et al., 2003). In this case, cardiac marker expression is first observed at about stage 28, corresponding to the normal time at which cardiac markers are observed in the intact embryo. To test the possibility that more time is required for expression of *MLC2* we have also examined animal cap explants cultured until stage 28 (data not shown). In all cases, the expression of markers at stage 28 is identical to that observed at stage 12.5, indicating that time of culture is not a significant factor in these experiments.

These results suggest that, although myocardin is able to precociously activate transcription of a subset of myocardial markers, it is not sufficient to initiate the complete

cardiac development program. Investigation of the expression of other cardiogenic genes supports this proposal. Transcription of neither *Nkx2-5* nor *GATA-4* transcription factors is activated in response to myocardin (Fig. 4A). Both of these genes are believed to play an essential role during normal cardiogenesis (Lyons et al., 1995; Tanaka et al., 2000; Molkentin et al., 1997). We note however, that expression of the MADS box transcription factor, *MEF2A*, and the myocardin cofactor, *SRF*, are both activated in animal caps, indicating that at least some cardiac regulatory factors lie downstream of myocardin and may play a role in ectopic activation of cardiac markers. Transcription of the myocardin gene itself however, is not activated in animal caps (data not shown), suggesting that myocardin does not regulate its own expression. Second, induction of cardiac gene expression by myocardin occurs as early as stage 11 in animal caps (data not shown), nearly two days earlier than the normal onset of cardiac differentiation marker expression. These results indicate that the normal cardiogenic program is absent in the animal caps.

Finally, we addressed the possibility that the marker gene expression observed in response to myocardin might be secondary to the formation of mesoderm or the

activation of a general muscle pathway. This is particularly relevant for the *cardiac α-actin* marker, which is expressed in both cardiac and skeletal muscle tissues. First, the presence of myocardin does not cause the animal caps to form mesoderm, as indicated by the absence of the general mesodermal marker, *brachyury* (*Xbra*, Fig. 4B). RT-PCR analysis also indicates that no transcripts are present for the myogenic determination genes *MyoD*, *Myf5*, *MRF4*, or *myogenin*, nor for the skeletal muscle differentiation marker *skMLC*, (Fig. 4B), indicating that myocardin is not inducing the skeletal muscle program in animal caps. This result indicates that the observed marker expression is independent of the mesodermal or skeletal muscle differentiation pathways.

2.2.5. Myocardin interacts cooperatively with Nkx2-5, GATA-4 , and Tbx5.

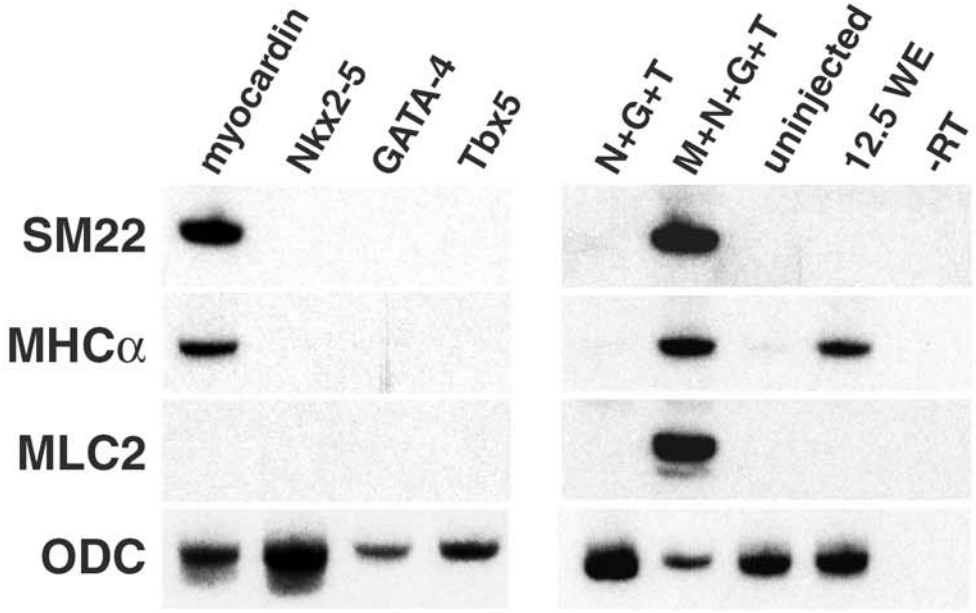
The failure of *MLC2* activation was unexpected since this gene is known to be regulated by SRF, and has been shown to be potently activated by myocardin in transfection assays using COS cells (Wang et al., 2001a). It seems possible that regulation of *MLC2* expression may require additional transcription factors to complement myocardin function. Therefore, we have tested the ability of the transcription factors, GATA-4, Nkx2-5, and Tbx5, all of which are potent regulators of

cardiac gene expression (Durocher et al., 1997; Chen and Schwartz, 1996; Lyons et al., 1995; Tanaka et al., 1999; Bruneau et al., 2001) to cooperate with myocardin in activation of *MLC2* expression. Mixtures of mRNAs encoding all four transcription factors were injected into embryos and animal cap explants were cultured until stage 12.5 when they were assayed for marker gene expression (Fig. 5). Our first observation is that the presence of all four factors failed to activate *MHCα* or *SM22* expression above the levels achieved with myocardin alone. Second, we note that coexpression of all four transcription factors succeeded in activating expression of *MLC2* (Fig. 5), but this activation was not observed in the absence of myocardin. This result suggests that myocardin does indeed cooperate, directly or indirectly, with other transcriptional regulators of cardiac gene expression.

Figure 5. Myocardin acts combinatorially with additional cardiac expressed transcription factors.

While myocardin is sufficient for induction of a number of cardiac and smooth muscle markers in animal caps, myocardin is unable to induce the expression of the *MLC2* gene (lane one labeled myocardin). The injection of 125pg of Nkx2-5, GATA-4, or Tbx5 alone, or the combination of these three factors was also unable to induce *MLC2* or any cardiac gene expression (lanes labeled N, G, T, N+G+T). When 125pg of myocardin was injected along with Nkx2-5, GATA-4 and Tbx5, *MLC2* gene expression was robustly induced (lane labeled M+N+G+T). Myocardin therefore acts cooperatively with other cardiac-expressed transcription factors in the induction of differentiation products.

Figure 5


myocardin
Nkx2-5
GATA-4
Tbx5
N+G+T
M+N+G+T
uninjected
12.5 WE
-RT
SM22
MHCα
MLC2
ODC

2.2.6. Myocardin activity requires both the SAP and basic domains.

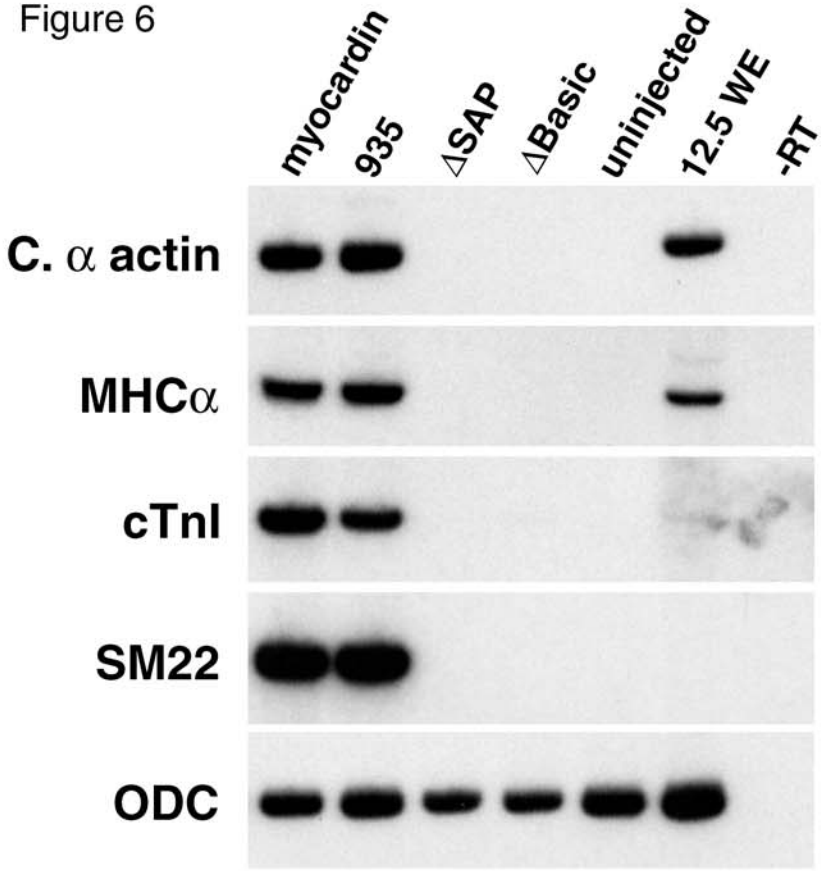
Myocardin is a member of the SAP domain family of transcription factors. The SAP domain is a 35 amino acid motif that is thought to bind DNA and to function in chromatin remodeling, although myocardin itself does not bind DNA directly (Aravind and Koonin, 2000; Wang et al., 2001). In addition, myocardin contains a basic domain close to the N-terminus that is required for interaction with SRF (Wang et al., 2001; Wang et al., 2002). We have tested myocardin constructions containing deletions of the conserved SAP and basic domains for their ability to activate cardiac gene expression in the animal cap assay**.** We have also examined the transcriptional activity of the full length 935 aa myocardin relative to that of a previously published myocardin clone that is missing 128 aa at its N-terminus (Wang et al., 2001; Wang et al., 2002). As shown in Fig. 6B, mutation of either the conserved SAP domain or the basic region abolishes the ability of myocardin to activate transcription of cardiac or smooth muscle genes in *Xenopus* animal cap explants. These in vivo results confirm previous cell culture studies indicating that the SAP and basic domains are essential for myocardin function (Wang et al., 2001). Our results also show that the full length 935 amino acid form of myocardin is

apparently identical to the truncated form in its ability to activate target gene transcription.

Figure 6. The SAP and basic domains are essential for myocardin activity.

125pg of myocardin mRNA with mutations of the SAP domain (involved in chromatin remodeling) or the basic domain (involved in binding to SRF) was injected into the animal pole of one-cell *Xenopus* embryos and caps were cultured until stage 12.5. These mutated constructs have been tested previously in luciferase assays analyzing myocardin function (Wang et al., 2001). These mutated proteins have been shown to be stably expressed. PCR analysis of the injected caps demonstrates that both the SAP and basic domains are required for induction of cardiac markers.

Figure 6


myocardin
935
ΔSAP
ΔBasic
uninjected
12.5 WE
-RT
C. α actin
MHCα
cTnI
SM22
ODC

2.2.7. Myocardin loss of function by morpholino knockdown results in a block to cardiac differentiation

The animal cap experiments described above indicate that myocardin, in the presence of its cofactor, SRF, is capable of activating expression of a wide array of cardiac and smooth muscle differentiation markers. To determine whether myocardin is required for expression of cardiac genes in the developing embryo, we utilized the antisense morpholino method for inhibition of translation of specific mRNAs. Note that only cardiac marker expression was examined in these experiments because smooth muscle differentiation occurs rather late in *Xenopus* development (after about stage 35) and is therefore outside of the window for morpholino interference. Two independent morpholinos were prepared, complementary to non-overlapping sequences within the 5' end of the *Xenopus myocardin* mRNA. A preliminary control experiment was performed to assure knockdown of target gene translation. Constructs containing the 5' end of the myocardin gene linked to the EGFP coding region were injected into one-cell embryos with or without the complimentary morpholino. The morpholino successfully

inhibited translation of the target sequence, as assayed by visible flurorescence, presence of GFP cDNA by RT-PCR, and absence of GFP protein by western blot (Fig. 7A).

Figure 7A. Myocardin morpholino control.

Myocardin morpholino inhibits the translation of a myocardin 5'UTR linked GFP reporter construct. 400pg of 5'UTR-GFP mRNA was injected with or without 10ng of myocardin morpholino and assayed for visible fluorescense (A, B), RT-PCR for GFP transcripts (C), and Western Blot for GFP protein (D). While not affecting GFP mRNA levels (C), myocardin morpholino inhibited visible fluorescence (B), and protein levels (D).

Figure 7A

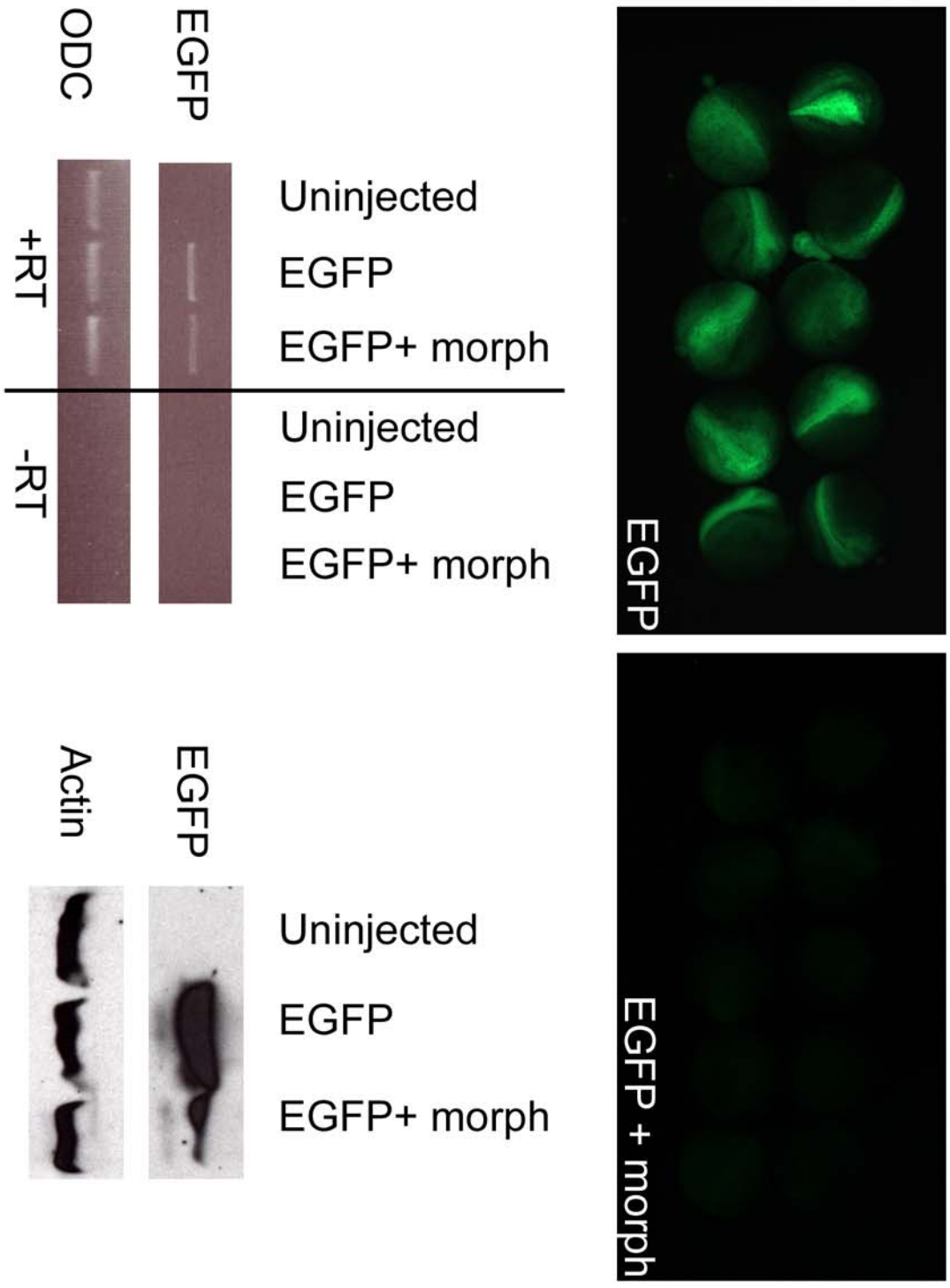

ODC
EGFP
+RT
-RT
Uninjected
EGFP
EGFP+ morph
Uninjected
EGFP
EGFP+ morph
Actin
EGFP
Uninjected
EGFP
EGFP+ morph
EGFP
EGFP + morph

Figure 7B. Myocardin loss-of-function results in inhibition of cardiac differentiation.

Embryos were injected with 10ng of myocardin morpholino into one blastomere at the two-cell stage and cultured until stage 29, when cardiac differentiation markers are normally expressed in the symmetric heart patches. Uninjected control embryos (A, C, E) or myocardin morpholino injected embryos (B, D, F) were assayed by in situ hybridization. Myocardin morpholino inhibited expression of B.) *MHCα,* and D.) *MLC2* expression on the side of injection. F) Myocardin morpholino injection did not affect the expression of *Nkx2-5*. Identical results were obtained from two independent morpholinos (MO1 and MO2).

Figure 7B

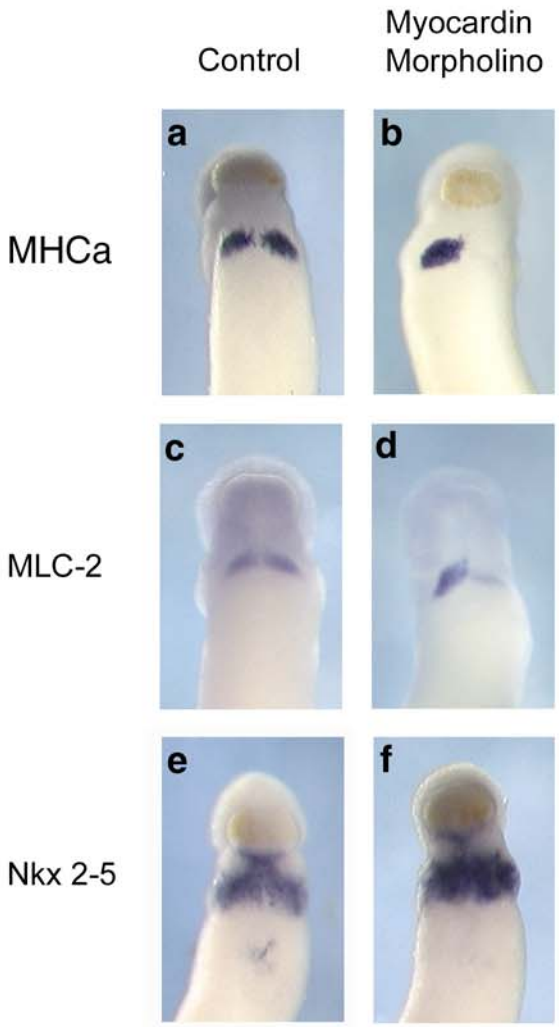


Table 1. Percentage of morpholino injected embryos with inhibited cardiac gene expression.

10ng of myocardin morpholino (MO1 or MO2) was injected into one blastomere at the two-cell stage. Embryos were then cultured to stage 29 and assayed for expression of *MHCα, MLC2*, or *Nkx2-5* by whole mount in situ hybridization. Embryos were scored based on percentage of embryos that exhibited an inhibition of marker expression on the side of morpholino injection. Approximatelyh 5% of unmanipulated embryos exhibit asymmetry of marker expression. Myocardin morpholino significantly inhibited the expression of the differentiation markers *MHCα* and *MLC2*, but did not affect the expression of the cardiac inducer, *Nkx2-5*.

		MHCα	*MLC2*	*Nkx2-5*
	uninj	5% (n=150)	4% (n=50)	1% (n=60)
MO 1	10 ng	57% (n=23)	86% (n=49)	5% (n=55)
MO 2	10 ng	36%(n=135)	49% (n=49)	0% (n=20)

Embryos were injected with myocardin MO1 or MO2 into one cell of a two-cell embryo, so that the uninjected side serves as a negative control. The injected embryos were then raised until stage 28 when they were assayed for cardiac marker gene expression by in situ hybridization. Injection of either MO1 or MO2 results in a significant inhibition of *MHCα* expression on the side of injection (Fig. 7B and Table 1). At a dose of 10 ng for MO1, inhibition of *MHCα* expression was observed in 57% of experimental embryos (13/23). Uninjected control embryos showed asymmetric cardiac marker expression in only 5.4% of embryos (9/150) equivalent to the levels of marker asymmetry observed previously (Grow and Krieg, 1998). Analysis of MO injected embryos with an independent cardiac muscle marker, *MLC2*, also showed inhibition of cardiac differentiation in 86% of embryos (Fig. 7B and Table 1). The independent morpholino sequence, MO2, was also successful in inhibiting expression of *MHCα* and *MLC2*, although the efficiency was less than that observed for MO1 (Table 1). Finally, the expression of the cardiac transcription factor *Nkx2-5* was unaffected by injection of either morpholino (Fig. 7B and Table 1), agreeing with our previous results showing that myocardin is unable to activate these genes. This result has the added value of an

additional negative control, since only myocardin target genes were affected by the antisense morpholino. These results show that myocardin is required for activation of cardiac differentiation in the *Xenopus* embryo.

2.3. Discussion

2.3.1. Tissue specific modulator of SRF activity

Studies of various promoters in transgenic animals or cell culture have made it evident that most cardiac gene regulatory regions contain Nkx2-5 binding sites (NKE), T box binding sites (TBE) and GATA binding sites and are dependent on combinatorial interactions of the associated proteins (Durocher and Nemer, 1998; Lee et al., 2002; Chen and Schwartz, 1996; Bruneau et al., 2001). While these factors play important roles in cardiac development, the MADS box protein SRF seems to play a more general role. In fact, a CarG box (SRF binding site) is required for the expression of most cardiac muscle genes and all known smooth muscle genes (Reecy et al., 1999). An interesting question arises since SRF displays widespread expression throughout the developing embryo yet SRF target genes are only activated in specific subsets of tissue such as the heart and smooth muscle. It has recently been shown that myocardin is a cardiac and smooth

muscle specific SRF co-factor containing a powerful transcription activation domain. Our experiments show that myocardin is necessary and sufficient for cardiac differentiation product expression. Myocardin therefore lends itself to a simple mechanism whereby SRF dependent genes are transcribed specifically in the heart and smooth muscle due to the presence of its crucial cofactor in these tissues.

2.3.2. Myocardin activates ectopic expression of cardiac markers in whole embryos.

These studies show that expression of the myocardin gene is sufficient to activate expression of a range of myocardial marker genes at ectopic locations in the *Xenopus* embryo. Although ectopic expression appeared to be most robust in neural tissues, expression of cardiac markers was observed in a variety of tissues. A single transcription factor has very rarely been observed to activate cardiac differentiation markers ectopically. Overexpression of *Nkx2-5* has been shown to cause an increase in the size of the heart in *Xenopus* or zebrafish (Cleaver et al., 1995; Chen and Fishman, 1996), and high doses of Nkx2-5 in zebrafish activated scattered *MHC* positive cells in ectopic locations (Chen and Fishman, 1996). Similarly, overexpression of GATA-5 in the zebrafish embryo or GATA-4 in Xenopus is sufficient to induce the ectopic expression of

several myocardial markers including Nkx2-5 and in some cases beating tissue is also observed (Reiter et al., 1999; Latinkic et al., 2003). However, myocardin seems to be unique among these cardiac muscle inducers, in that misexpression of low levels of mRNA is sufficient to activate high levels of marker gene expression in a wide range of tissues. Experiments using the *NBT* promoter to drive myocardin expression in fully differentiated neural tissue further illustrates the transcriptional properties of this factor. *MHCα* gene expression is observed at high levels throughout the central and peripheral nervous system as late as the swimming tadpole stage, well after neural differentiation, and the transgenic embryos exhibit a full range of reflex responses and muscular function. This suggests that myocardin is able to induce cardiac markers in the absence of any additional cardiogenic cues without subverting pre-existing regulatory pathways.

2.3.3. Activation of cardiac and smooth muscle genes in animal caps

Within the intact embryo, many tissue-tissue interactions occur that are known to influence the patterns of gene expression that ultimately lead to cardiac development. In order to simplify analysis of the role of myocardin in target gene activation, we have carried out a series of experiments using the *Xenopus* animal cap assay. The animal cap

is naive ectodermal tissue that is fated to form epidermis. Our experiments indicate that myocardin expression in the animal cap is sufficient to activate transcription of a range of myocardial and smooth muscle expressed sequences, including *cardiac α-actin*, *MHCα, cTnI*, *SM22, SM actin* and *CalpH1*. Target gene induction occurs as early as a gastrulation stage embryo (stage 11), much earlier than normal cardiac differentiation, and persists to at least the equivalent of the heart tube stage (stage 30). This powerful inductive property seems to be unique to myocardin, although the induction of cardiac genes in ectodermal explants by GATA4 has recently been observed by Latinkic et al., 2003. In the case of GATA4 however, induction requires nearly 10 fold higher levels of mRNA than is sufficient for induction by myocardin and GATA4 injected caps appear to follow the normal cardiogenic timetable. It seems possible that GATA4 initiates a cascade of events resulting in cardiac differentiation, while myocardin interacts with target gene promoters either directly or indirectly. Indeed, while GATA-4 is expressed in a wide range of non-cardiac and endodermal tissues, much of which never express cardiac markers, the expression of myocardin in the embryo directly precedes and exactly mirrors the expression of the sarcomeric proteins we have shown to be induced in animal caps.

Surprisingly, although *MLC2* is known to be SRF responsive, and is activated by myocardin in COS cells (Wang et al., 2001a; Wang et al., 2002), this gene was not activated by myocardin in animal pole explants**.** Since many cardiac differentiation products are regulated by cooperative interactions between transcription factors, we tested the possibility that combinations of factors are required for *MLC2* induction. Indeed combinatorial interactions between SRF, Nkx2-5, GATA4, and Tbx5 have been shown to be important for *ANF* and *cardiac α-actin* activity (Chen and Schwartz, 1996; Durocher and Nemer, 1998; Lee et al., 2002; Bruneau et al., 2001). Our experiments show that combinatorial interactions alter the inductive properties of myocardin, since co-expression of Nkx2-5, GATA-4, and Tbx5, along with myocardin results in the induction of *MLC2* expression (Fig. 5). No combination of transcription factors is sufficient to induce *MLC2* expression in the absence of myocardin. This result suggests that, while myocardin activity parallels induction of skeletal muscle by MyoD family members in some respects, its modulation of activity by other factors closely resembles cardiac gene regulation. Taken together, myocardin is sufficient for the activation of a wide range of

cardiac and smooth muscle differentiation products, either as "master regulator" or as a component of cooperative activity.

2.3.4. Myocardin loss-of-function and the genetic pathway to heart development.

Since myocardin proved to be sufficient for activation of a wide range of cardiac and smooth muscle markers, we wished to test whether this protein is necessary for cardiac induction in a living embryo. Previous studies using dominant negative versions of myocardin resulted in a loss of heart differentiation in *Xenopus* embryos (Wang et al., 2001a), however mice null for the myocardin gene display a fairly normal heart and die due to loss of vascular smooth muscle differentiation (Ji et al., 2003). The relatively mild cardiac phenotype in the myocardin mouse knockout could be due to redundancy by related factors (MRTF-A and MRTF-B) which are expressed in the developing heart in mice and possess similar transcriptional properties (Wang et al., 2002). Since MRTF-A and MRTF-B are not expressed in the developing *Xenopus* heart (Fig. 2) we were able to test whether myocardin loss-of-function by antisense morpholino injection caused heart defects without the possibility of redundancy. Our results suggest that myocardin is indeed required for the induction of cardiac differentiation in living embryos; expression

of the *MHCα* and *MLC2* genes are dramatically reduced in morphoino injected embryos (Fig. 7B and Table 1). This finding makes it likely that the absence of heart defects in myocardin null mice is in fact due to redundancy by MRTF-A or MRTF-B.

A second observation from our myocardin loss-of-function experiments is that *Nkx2-5* transcription proceeds normally in the absence of myocardin (Fig. 7B and Table 1). Since *myocardin* gene expression commences nearly a full day after that of *Nkx2-5* it is unlikely to be an upstream regulator of *Nkx2-5*. The possibilities remain that *myocardin* is directly or indirectly induced by Nkx2-5 activity, or that myocardin and Nkx2-5 represent two parallel pathways of cardiogenesis. An intriguing question in heart development has been why the expression of Nkx2-5 commences significantly earlier than the genes it has been shown to regulate. Myocardin seems to fit the profile of a crucial intermediate factor that is required for cardiac differentiation. Its expression directly precedes target gene expression by a matter of hours, and it is expressed in a domain that is identical to that of the cardiac differentiation markers. Conversely, Nkx2-5 is expressed in a domain that includes, but is much greater than the differentiated heart patches. It will be of great interest to determine whether *myocardin* is regulated by

Nkx2-5, directly or indirectly, by examining Nkx2-5 null mice and analyzing *myocardin* regulatory sequences. This knowledge will allow for a better understanding of the genetic heirarchy of cardiogenesis.

Chapter 3: Expression of *atrial natriuretic factor* (*ANF*) during *Xenopus* cardiac development[2]

Atrial natriuretic factor is a 28 amino acid circulating peptide with natriuretic/diuretic and vasorelaxant properties (Wilkins *et al*. 1997). Evidence from mouse gene ablation studies (Lyons *et al*., 1995) and from cell culture studies (Durocher and Nemer, 1998) suggest that the Tinman-family homeobox protein, Nkx2-5, directly regulates expression of *ANF*. Indeed, it appears that Nkx2-5 synergizes with the zinc finger transcription factor, GATA-4, in regulation of *ANF* transcription (Durocher *et al*. 1997).

In order to pursue studies of Nkx2-5 / GATA-4 cooperativity and the regulation of chamber specific gene expression, we have isolated the *Xenopus laevis* orthologue of the *ANF* gene. Briefly, a PCR fragment containing the coding sequences of the *Rana catesbiana ANF* gene (Genebank accession # D01043) was used to screen a *Xenopus*

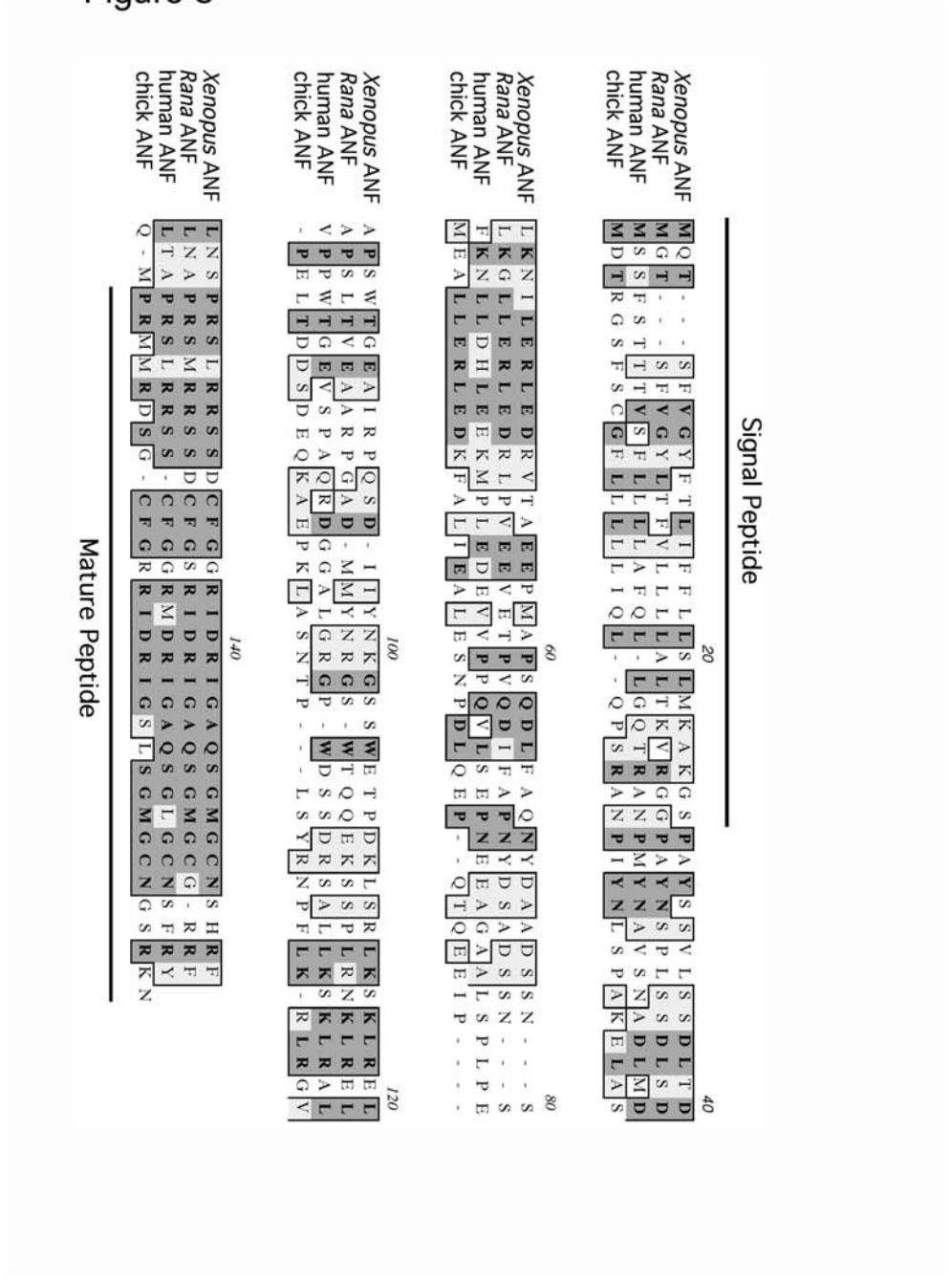
[2] This chapter has been previously published. Small, E.M. and Krieg, P.A. (2000). Expression of atrial natriuretic factor (ANF) during Xenopus cardiac development. *Dev. Gen. Evol*. 210, 638-640.

adult heart cDNA library (Ji *et al*., 1993). Approximately 1/500 of all plaques were positive suggesting that *ANF* transcripts represents about 0.2% of total mRNA in the adult heart. Sequencing of a representative positive clone revealed an open reading frame of 441 bp, which contains the entire coding region of the 147 aa *Xenopus* ANF pre-proprotein. *Xenopus* ANF shares approximately 49% identity to the human and 36% identity to the chicken ANF proteins across the entire proprotein peptide region (aa 24-147, see Fig. 8). The greatest sequence identity, 83% between *Xenopus* and human ANF, is present in the 29 residues at the extreme C-terminal corresponding to the bioactive fragment.

Figure 8.

Alignment of the atrial natriuretic factor predicted protein sequence from *Xenopus*, *Rana* (D01043), human (Nemer *et al*., 1984) and chick (Akizuki *et al*., 1991). The first 23 amino acids of *Xenopus* ANF (overlined) correspond to the signal sequence. The 29 amino acids at the extreme C-terminus (underlined) constitute the mature peptide. The accession number for the *Xenopus laevis* ANF sequence is AF287050.

Figure 8

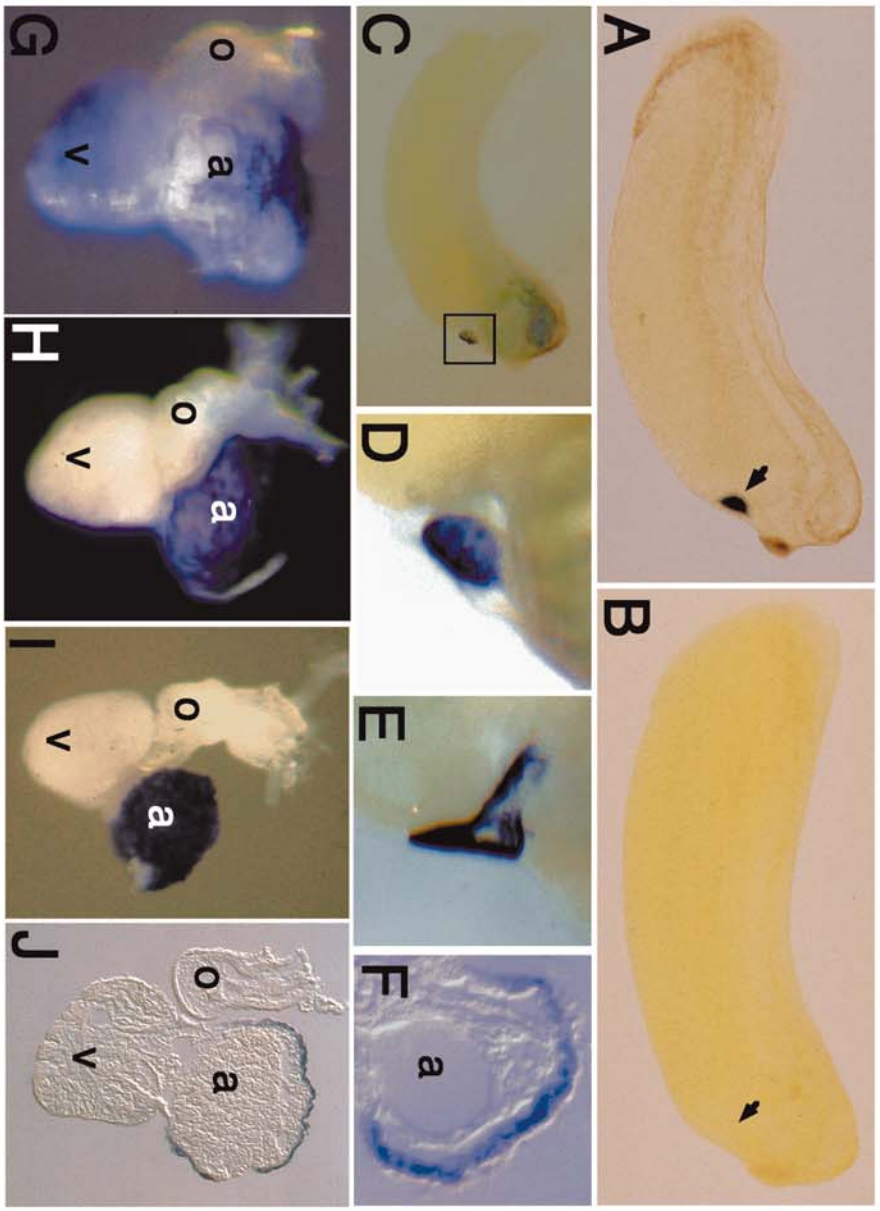

Signal Peptide
Mature Peptide
Xenopus ANF
Rana ANF
human ANF
chick ANF
20
40
60
80
100
120
140

The developmental expression profile of *ANF* was determined by whole mount *in situ* hybridization (Harland, 1991) (Fig. 9B-E). *ANF* is first expressed at the late heart tube stage (st. 32, Nieuwkoop and Faber, 1994), throughout the myocardium. This is several hours later than the expression of all previously described cardiac differentiation markers in *Xenopus*, expression of which is observed simultaneously at the cardiogenic plate stage (about st 27) (Fig 5A and Drysdale *et al*., 1994). *ANF* continues to be expressed in the myocardium of both the atrium and ventricle throughout tailbud and tadpole stages (st. 32-45). At st. 47, a sharp decrease in expression of *ANF* is observed in the ventricle while expression remains intense in the atrium (Fig. 9F-H). This restriction of expression occurs several days after the atrium and ventricle become morphologically distinct (st. 35), yet only shortly after septation of the single atrium into two atria (st. 45) (Kolker *et al*., 2000; Mohun *et al*., 2000). Sectioning through a st. 49 heart (Fig. 9I) confirms that expression of *ANF* is greatest in the myocardial layer of the atrium, although very low levels of *ANF* staining can be detected in the ventricle when viewing the original sections under the microscope. This agrees with the observed low level expression of *ANF* in the ventricle in the rat (Gardner *et al*., 1986). We note that the

Figure 9. Expression of ANF in the developing *Xenopus* embryo.

A) St. 28 embryo showing expression of the cardiac differentiation marker, *Cardiac Troponin I*. B) St. 28 embryo probed with *ANF* showing absence of detectable expression in the heart. (The arrow indicates the heart region in A and B). (C,D) Expression of *ANF* is initiated throughout the entire myocardium at the late heart tube stage (st. 32). The staining visible in the head region in panel C is background. There is no *ANF* expression in the dorsal lymph hearts. E) St. 37 embryo probed with *ANF* showing expression throughout the myocardium into the heart looping stage. (F) Section through atrium of stage 44 heart showing ANF expression in the myocardium but absent from the endocardial cell layer. (G-I) *In situ* hybridization detection of *ANF* transcripts in isolated st. 45, 47 and 49 hearts, respectively, showing the restriction of *ANF* expression to the atrium at approximately stage 47. J) Section through a st. 49 heart, showing restriction of *ANF* expression to the atrial myocardium. The unstained cells within the atrium are blood cells and the entire atrial myocardial layer is expressing *ANF*. a, atrium; v, ventricle; o, outflow tract.

Figure 9



timing of restriction of *ANF* to the atrium in *Xenopus* corresponds temporally to the onset of metamorphosis and the upregulation of *Retinoid X Receptor* (*RXR*α) gene expression (Shi *et al*., 1996). In an attempt to better understand the factors that are involved in the regulation of the *ANF* promoter in an in vivo system, we set out to isolate the 5' regulatory sequence of the *Xenopus laevis ANF* gene for use in a systematic promoter analysis in transgenic frogs. The following sections describe the findings of analyzing the *ANF* promoter.

Chapter 4: Transgenic analysis of the *atrial natriuretic factor (ANF)* promoter: Nkx2-5 and GATA-4 binding sites are required for atrial specific expression of *ANF*.[3]

4.1. INTRODUCTION

The morphological events involved in formation of the vertebrate heart have been well described, but much less is understood about the molecular mechanisms regulating gene expression during cardiogenesis. One class of transcription factors that are known to be important for heart development are homeodomain proteins of the tinman family. In *Drosophila*, absence of tinman function completely abolishes formation of the dorsal vessel, the insect equivalent of the vertebrate heart (Bodmer, 1993; Azpiazu and Frasch, 1993). In vertebrates, a small family of tinman related genes are expressed in the developing heart. The best-characterized member of the vertebrate tinman family is Nkx2-5 (also called Csx) which is amongst the very earliest markers of the cardiac lineage (Harvey, 1996; Komuro and Izumo, 1993). Nkx2-5 continues to be expressed

[3] This chapter has been preiously published. Small, E.M. and Krieg, P.A. (2003). Transgenic analysis of the atrial natriuretic factor (ANF) promoter: Nkx2-5 and GATA-4 binding sites are required for atrial specific expression o f ANF. *Dev. Biol*. 261, 116-131

throughout the myocardial layer during subsequent heart development and in the adult organ. Mice in which Nkx2-5 function has been ablated die at approximately E9.5 due to cardiac insufficiency (Lyons et al., 1995; Tanaka et al., 1999). Although a linear heart tube is formed and beating myocardial tissue is present, the heart tube fails to undergo the looping and morphogenetic movements associated with heart maturation. In addition a number of cardiac genes fail to be expressed, or are expressed at much reduced levels, including *MLC-2v*, *CARP*, *eHand* and *atrial natriuretic factor (ANF)* (Lyons et al., 1995; Tanaka et al., 1999). The relevance of Nkx2-5 function for human cardiac development is evidenced by the fact that a number of congenital heart abnormalities, including atrial septal defects, AV conduction defects, and valvular dismorphogenesis appear to be associated with haploinsufficiency of Nkx2-5 expression (Schott et al., 1998; Rosenthal and Harvey, 1999; Biben et al., 2000).

Nkx2-5 consensus binding sites (NKEs) have been identified in the promoter regions of a number of cardiac expressed genes, including *GATA6* (Molkentin et al., 2000), *cardiac α-actin* (Chen and Schwartz, 1996; Sepulveda et al., 1998), *cardiac troponin I* (Bhavsar et al., 2000), and *ANF* (Durocher et al., 1996). These genes may

therefore be direct targets of Nkx2-5 regulation. In support of this hypothesis, in vitro studies using rat cardiomyocytes, CV1 fibroblast cells, and HeLa cells have shown that Nkx2-5 is able to activate transcription from the *ANF* and *cardiac α actin* promoters (Durocher et al., 1997; Sepulveda et al., 1998). Related cell culture studies strongly imply that Nkx2-5 cooperates with other transcription factors to efficiently activate cardiac gene transcription. For example, examination of the *ANF* and *cardiac α actin* promoters show the presence of GATA sites and SRF consensus binding sites (SREs) in close proximity to the Nkx2-5 binding sites. Cotransfection of both GATA4 and Nkx2-5 into cardiomyocytes, HeLa cells, CV1 cells or 293 cells results in a synergistic activation of *ANF* and *cardiac α actin* promoter activity (Durocher and Nemer, 1998; Sepulveda et al., 1998; Lee et al., 1998). Furthermore, immunoprecipitation experiments suggest a direct physical interaction between the GATA4 and Nkx2-5 proteins. In an extension of these cell culture studies, cotransfection of SRF with GATA4 or Nkx2-5 also shows a synergistic upregulation of *cardiac α actin* promoter expression and the highest levels of promoter activity, approximately 200 -fold over background, are observed when all three factors are present (Sepulveda et al., 2002). Overall, these studies strongly suggest that cooperative interactions between the Nkx2-5, GATA4, and SRF proteins are required for

maximal activity of the *ANF* and *cardiac α actin* promoters and probably other cardiac genes.

Transcription factors of the T-box (Tbx) family have also been shown to play an important role in in cardiogenesis (Bruneau, 2002). Tbx5 mutations are associated with Holt-Oram syndrome, a human disease which results in cardiac and limb defects (Basson et al., 1997; Li et al., 1997; Basson et al., 1999) and mice heterozygous for Tbx5 display cardiac defects resembling those of Holt-Oram syndrome (Bruneau et al., 2001). Furthermore, when Tbx5 is ectopically expressed throughout the entire heart in chicken embryos, chamber development proceeds abnormally (Liberatore et al., 2000). Binding sites for Tbx proteins (TBEs) have been identified in a number of cardiac expressed genes including *ANF* and *cx40* (Bruneau et al., 2001; Habets et al., 2002). Combinatorial interactions apparently can occur between Nkx2-5 and Tbx5 proteins and synergistic activation of the *ANF* and *cx40* promoters by these factors has been observed (Bruneau et al., 2001). In addition, Tbx2 has been implicated in repression of *ANF* expression in the AV canal in transgenic mice (Habets et al., 2002).

Despite these extensive in vitro studies, the relative importance of the Nkx2-5, GATA4, Tbx5 and SRF proteins for *ANF* regulation has not been systematically examined in transgenic animals. To address this issue we have isolated the *ANF* gene from the frog, *Xenopus laevis*, and studied its regulation in transgenic frog embryos. Alignment of the frog and mammalian promoter sequences shows a remarkable degree of conservation of transcription factor binding sites including two SREs, two GATA sites, a TBE and an NKE. The presence of these conserved sequence elements suggests that the transcriptional mechanisms regulating *ANF* expression have been highly conserved in evolutionarily diverse organisms. Our transgenic studies show that the proximal GATA site and the NKE are relatively unimportant for the overall efficiency of expression of the *ANF* promoter, but play an essential role in regulating restriction of *ANF* expression to the atrium.

4.2. RESULTS

4.2.1. Conservation of transcription factor binding sites in the mammalian and frog ANF promoters.

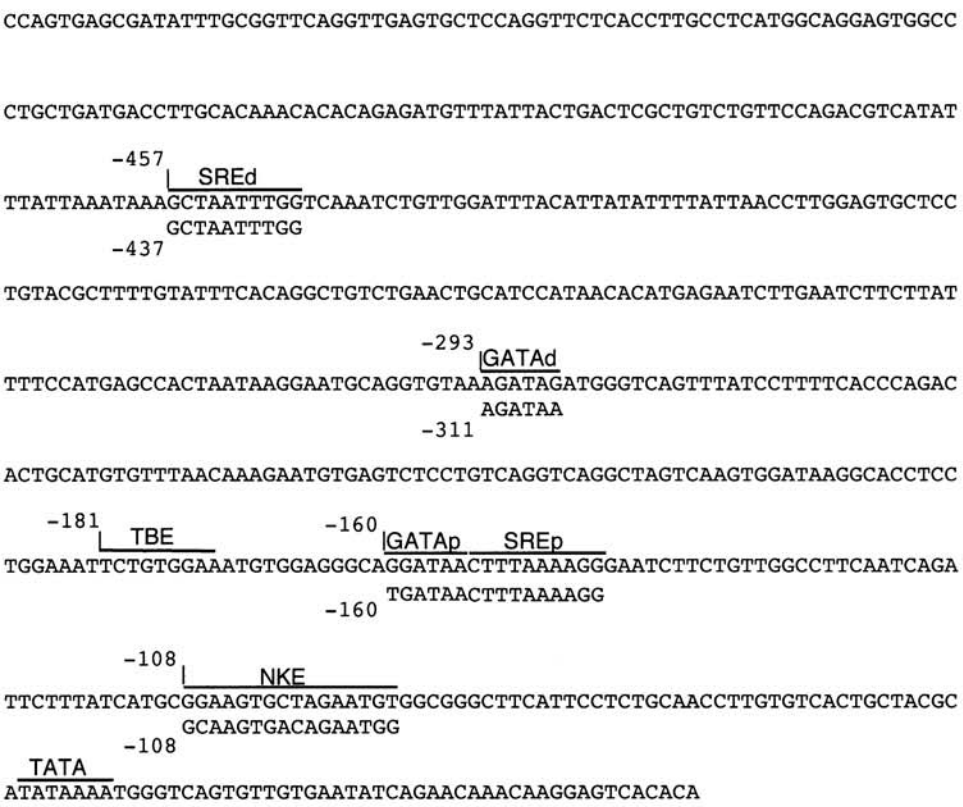
In order to study the spatial and temporal regulation of cardiac gene expression we have isolated a 4.4 kb DNA fragment containing the 5' flanking sequences of the *Xenopus atrial natriuretic factor (ANF)* gene. Figure 10 shows the proximal promoter region of the *Xenopus ANF* gene together with an alignment of the sequence elements conserved in the human *ANF* gene. The first 625 bp of 5' flanking sequences contains a number of transcription factor binding sites and both the sequence and the spacing of these putative regulatory elements is highly conserved between mammals and *Xenopus*. In particular, two SRF binding sites (SRE), two GATA sites, a Tbx factor binding site (TBE) and an NK2 factor binding site (NKE) are located within 350 bases of the start site of transcription. The proximal SRE-like domain, which differs from a consensus SRE by 2 out of 10 bases, and the distal consensus SRE are identical in the human and *Xenopus* genes. The Nkx2-5 binding region (NKE) is 12 of 16 base pairs conserved between human and *Xenopus*. The *Xenopus* 5' flanking region also contains the proximal and distal GATA binding sites that are present in the mammalian *ANF* promoters and a TBE,

although this last element is not precisely conserved in sequence or spacing. Based on the high level of conservation of promoter sequences, it seems likely that very similar regulatory mechanisms will be involved in controlling *ANF* transcription in these evolutionarily divergent organisms.

Figure 10. The 5' regulatory sequence of the *Xenopus ANF* promoter.

The sequence of elements conserved in the human *ANF* promoter are aligned beneath the corresponding frog sequence. The promoter contains an extended Nkx2-5 binding site (NKE) that is identical in 12 of 16 bases. Also conserved are a proximal GATA (GATAp), distal GATA (GATAd) and two SRF binding sites (SREs) termed the proximal SRE (SREp) and distal SRE (SREd). The SREp and SREd are both identical in the human and frog sequences. In addition to the primary sequence, the relative spacing of these binding sites is also highly conserved between the frog and human promoters. Finally, the frog sequence contains a single Tbx binding site (TBE) that is not conserved in location compared with the human promoter.

Figure 10


CCAGTGAGCGATATTTGCGGTTCAGGTTGAGTGCTCCAGGTTCTCACCTTGCCTCATGGCAGGAGTGGCC
CTGCTGATGACCTTGCACAAACACACAGAGATGTTTATTACTGACTCGCTGTCTGTTCCAGACGTCATAT
-457
SREd
TTATTAAATAAAGCTAATTTGGTCAAATCTGTTGGATTTACATTATATTTTATTAACCTTGGAGTGCTCC
GCTAATTTGG
-437
TGTACGCTTTTGTATTTCACAGGCTGTCTGAACTGCATCCATAACACATGAGAATCTTGAATCTTCTTAT
-293
GATAd
TTTCCATGAGCCACTAATAAGGAATGCAGGTGTAAAGATAGATGGGTCAGTTTATCCTTTTCACCCAGAC
AGATAA
-311
ACTGCATGTGTTTAACAAAGAATGTGAGTCTCCTGTCAGGTCAGGCTAGTCAAGTGGATAAGGCACCTCC
-181
TBE
-160
GATAp
SREp
TGGAAATTCTGTGGAAATGTGGAGGGCAGGATAACTTTAAAAGGGAATCTTCTGTTGGCCTTCAATCAGA
TGATAACTTTAAAAGG
-160
-108
NKE
TTCTTTATCATGCGGAAGTGCTAGAATGTGGCGGGCTTCATTCCTCTGCAACCTTGTGTCACTGCTACGC
GCAAGTGACAGAATGG
-108
TATA
ATATAAAATGGGTCAGTGTTGTGAATATCAGAACAAACAAGGAGTCACACA

4.2.2. Spatial and temporal regulation of the *ANF* promoter.

During mammalian development, *ANF* is initially expressed in both the atrial and ventricular regions of the myocardium of the developing heart (Zeller et al., 1987; Habets et al., 2002). Later during development, *ANF* expression becomes limited to the atrial chambers of the heart, with limited expression also observed in trabeculae of the ventricle. In the rat, this restriction occurs at approximately the time of birth (Zeller et al., 1987; Gardner et al., 1986; Argentin et al.,1994). A very similar pattern of *ANF* expression is observed during frog cardiogenesis (Small and Krieg, 2000), with *ANF* initially expressed throughout the myocardium and in the developing outflow tract before becoming restricted to the atrial chambers (Fig. 11A, B). This restriction commences at approximately stage 47 of development, corresponding to about two weeks after fertilization (Fig. 11B). Unlike the mammalian expression pattern, no expression of *ANF* is visible in the trabeculated ventricular myocardium of the frog heart (Small and Krieg, 2000). At no time is expression of *ANF* observed outside of the heart.

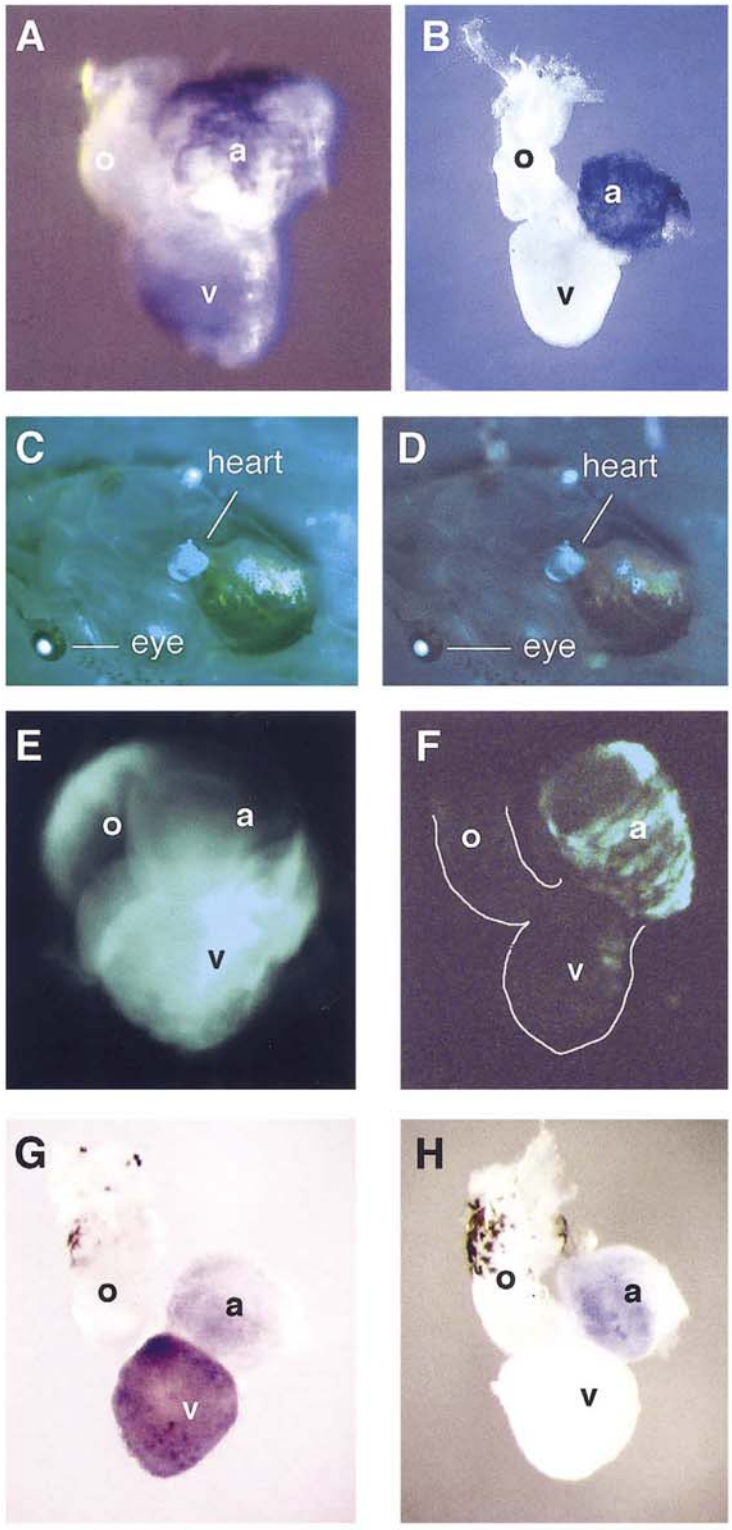
In order to study the regulation of *ANF* transcription in vivo, we have used the *Xenopus* transgenesis system (Kroll and Amaya, 1996; Sparrow et al., 2000a). Preliminary experiments compared the expression of a GFP reporter gene in constructions containing either 3.4 kb or 625 bp of *ANF* 5' flanking sequence. Comparison of more than 20 individual transgenics for each construction showed that the expression levels and profiles were indistinguishable, and both were identical to the expression profile of the endogenous *ANF* gene (data not shown). Based on these observations, the shorter *ANF* promoter construction was used in all subsequent studies. We have compared the pattern of expression of the wild type 625 bp *ANF* promoter-driven GFP transgene to expression of the endogenous *ANF* gene (Fig. 11). Transgene expression is first detected by fluorescence in the heart at approximately stage 34/35 (data not shown). During subsequent development the level of GFP fluorescence increases and is prominent throughout both the atrial and ventricular regions of the myocardium, as shown in Fig. 11C-E. Expression of ANF in the atria always appears much weaker than ventricular expression because the atrial myocardial layer is only one or two cells across, whereas the ventricular myocardium is very thick (Small and Krieg, 2000). Starting at approximately stage 47, GFP fluorescence in the ventricle and the outflow tract begins to

decrease and expression becomes completely restricted to the atria at about stage 49 (Fig. 11F, H). This restriction of *ANF* expression to the atria occurs over a period of approximately five days under standard growth temperature conditions (22° C). Observations using GFP fluorescence as the reporter can be directly confirmed by in situ hybridization against GFP mRNA sequences (Fig. 11G, H) indicating that the transgene expression pattern is not being misrepresented due to GFP protein stability or different transparency of the ventricular or atrial myocardial tissues. Finally, we note that reporter gene expression from the wild type 625 bp *ANF* promoter is restricted to the heart at all stages of development, indicating that the tissue specificity of the promoter has been retained. Overall, the results with the *Xenopus ANF* promoter are consistent with previous transgenic experiments utilizing mammalian promoters (Field, 1988; Knowlton et al., 1995; Habets et al., 2002). These studies showed that all sequences required for cardiac specific expression are located within approximately 600 bp upstream of the transcription start site.

Figure 11. The 625 bp wild-type *Xenopus ANF* promoter fragment recapitulates the endogenous *ANF* expression pattern.

A) In situ hybridization analysis of stage 45 non-transgenic heart showing *ANF* expression throughout the atrium, ventricle, and outflow tract. B) Stage 49 non-transgenic *Xenopus* heart showing restriction of ANF transcripts to the atrial myocardium. C) Stage 44 transgenic embryo showing GFP reporter expression driven by the wild-type 625 bp *ANF* promoter. This is a ventro-lateral view of a living embryo under combined UV and visible light. GFP fluorescence can be observed in the eye, driven from the control *γ-crystallin* promoter and throughout the heart. D) Fluorescent image of embryo in C, highlighting GFP reporter expression. E) Heart of living stage 44 transgenic embryo, with GFP expression driven from the wild-type 625bp *ANF* promoter. Fluorescence is visible throughout the atrium, ventricle, and outflow tract. F) Fluorescent image of dissected stage 48 transgenic heart, showing that GFP reporter expression is now limited to the atria. G) In situ hybridization analysis of dissected stage 45 transgenic heart, showing distribution of GFP transcripts throughout the heart. H) In situ hybridization analysis of GFP transcripts in dissected stage 49 transgenic heart. Note restriction of transgene expression to the atria, confirming the expression pattern observed using fluorescence. Abbreviations: o, outflow tract; a, atria; v, ventricle.

Figure 11



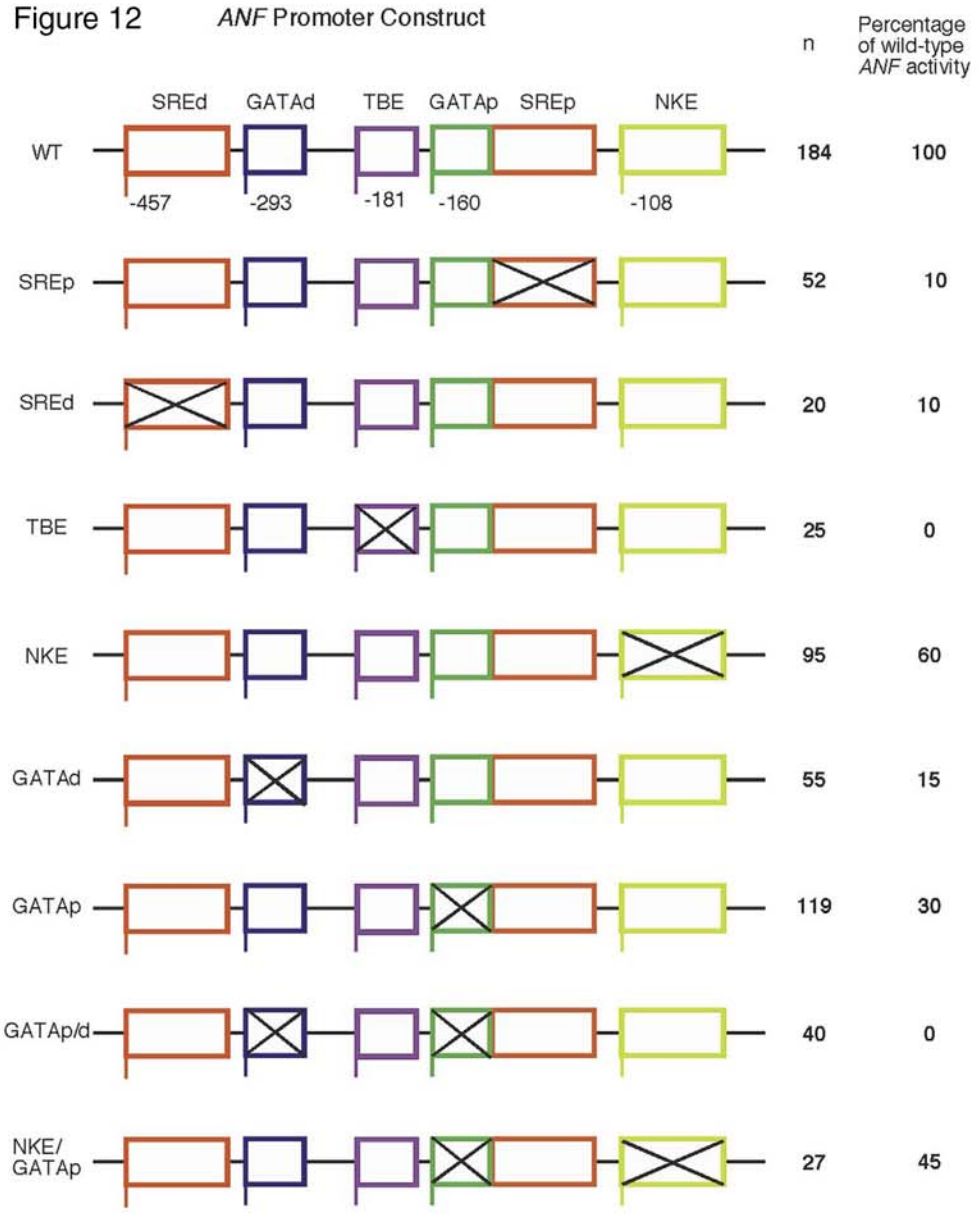
4.2.3. The presence of two SRF binding sites is required for efficient expression of the ANF promoter.

Using expression from the wild type 625 bp *ANF* promoter as a reference, we have undertaken a mutational analysis to determine the relative importance of the different promoter regulatory elements in controlling the efficiency and tissue specificity of *ANF* expression in transgenic embryos. We commenced this analysis with mutations of the SREs, which act as binding sites for SRF. Constructions were prepared in which either the proximal SRE (SREp) or the distal SRE (SREd) were mutated, while taking care to maintain precise spacing between regulatory elements. Using GFP fluorescence as the readout, more than 20 independent F0 transgenic embryos were analyzed for each construction. As shown in Figure 12, mutation of either the proximal or distal SRE results in a dramatic reduction in expression from the *ANF* promoter, to approximately 10% of the level observed from the wild type promoter. Since the SRE mutant promoters resulted in very weak transgene expression levels, no information on the requirements of these sites for temporal or spatial regulation of *ANF* expression was obtained using our assay.

Figure 12. Mutational analysis of the *Xenopus ANF* promoter in transgenic embryos.

The different *ANF* promoter constructions are illustrated at the left of the figure. Numbers on the top line indicate the distance of the regulatory element upstream of the transcription start site. The number of independently generated transgenic embryos is indicated in the first column and the relative activity of the mutant constructions (expressed to the nearest 5 percent) is presented in the second column. This number is calculated as the proportion of mutant transgene embryos showing visible reporter expression relative to the number of wild type transgene embryos showing reporter expression (see Materials and Methods).

Figure 12 *ANF* Promoter Construct



	n	Percentage of wild-type *ANF* activity
WT	184	100
SREp	52	10
SREd	20	10
TBE	25	0
NKE	95	60
GATAd	55	15
GATAp	119	30
GATAp/d	40	0
NKE/ GATAp	27	45

4.2.4. The TBE is essential for efficient expression of the ANF promoter.

Mutation of the single TBE completely eliminates detectable GFP reporter expression during early myocardial development. By stage 45, about 5 days after the normal onset of expression, GFP became visible in the hearts of 30% of transgenic embryos, albeit at much reduced levels relative to wild type (n=18, data not shown). Determination of atrial restriction in these mutants was not possible due to the very low levels of GFP. Based on these results, it appears that a functional TBE is essential for the correct temporal activation of *ANF* expression and also for normal efficiency of the promoter.

4.2.5. A functional Nkx2-5 binding site is required for restriction of *ANF* expression to the atrium.

The *ANF* promoter contains a conserved Nkx2-5 binding site, the NKE, centered at approximately 110 bp upstream of the transcriptional start site. The sequence and spacing of this element, relative to other promoter motifs, is highly conserved between

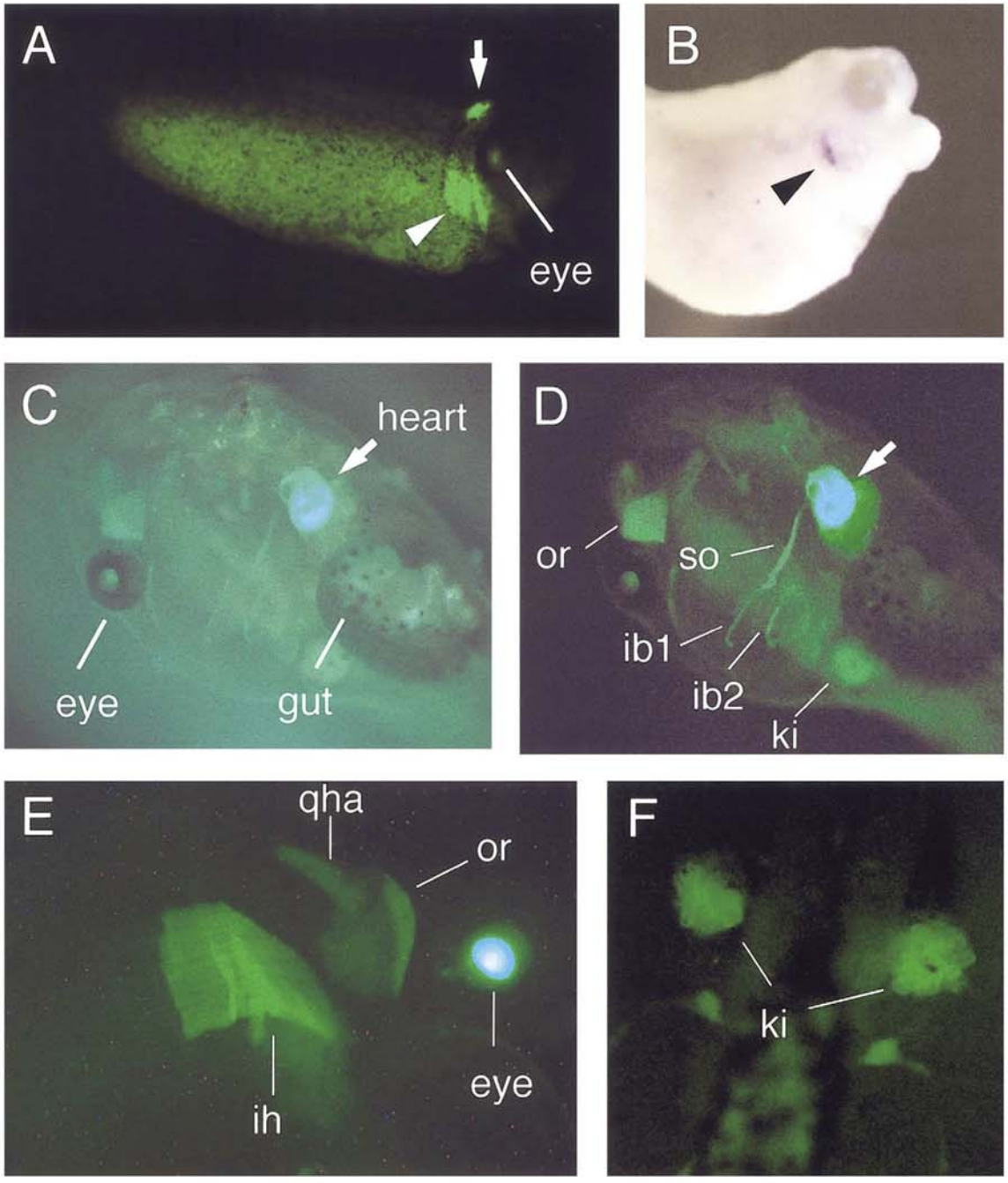
the *Xenopus* and the human *ANF* promoters. As shown in Fig. 12, mutation of the NKE results in a surprisingly minor reduction of reporter gene activity in transgenic frog embryos. Analysis of 95 independent transgenic embryos shows that elimination of the Nkx2-5 binding site reduces detection of GFP expressing embryos to approximately 60% of wild type levels. Apart from this reduction in promoter activity from the NKE mutant promoter, the time of onset of cardiac GFP expression and the initial distribution of GFP fluorescence throughout the atrial and ventricular myocardium appears identical to the wild type sequence (data not shown).

The most striking aspect of the NKE mutant transgenics is the appearance of GFP reporter expression in non-cardiac tissues of the embryo. Ectopic expression is observed in the developing kidneys, facial muscles, and in a group of muscles adjacent to the aortic arch arteries, but never at any other location (Fig. 13). GFP expression outside of the heart was never observed in transgenics carrying the wild type promoter (n = 184) and so it seems extremely unlikely that this expression is due to copy number or position effects at the integration site. In NKE mutant transgenics, expression in developing facial muscles is first detected at approximately stage 32 by visible fluorescence (Fig. 13A) and

by in situ hybridization (Fig. 13B). This is earlier than expression in the heart, which is first detected at about stage 34. Later in development, when muscle morphology is more pronounced, it is possible to identify the facial muscles as the musculus interhyoideus, musculus quadratohyangularis and orbitohyoideus (Sokol, 1977) (Fig. 13C, D, E). Ectopic transgene expression is also observed in the interbranchialis I and II, adjacent to the third and fourth aortic arch arteries, and the subarcualis obliquus (Fig 13C, D). Ectopic expression from the NKE mutant promoter also occurs in the kidneys, as shown by fluorescence in a stage 44 embryo (Fig. 13F).

Figure 13. Mutation of the NKE within the *ANF* promoter results in ectopic transgene expression outside of the heart. All transgenic embryos shown are mutant for the NKE, but identical results are observed for the GATAp mutant promoter. A) Fluorescent image of a living stage 32 transgenic embryo. Ectopic expression of reporter GFP is visible in the developing facial muscles (indicated by an arrow head). Expression in the brain, from the control *γ crystallin* promoter, as indicated by an arrow. Apparent fluorescence in the flank of the embryo is background due to autofluorescence of yolk granules. Cardiac expression of the transgene has not commenced at this stage. B) In situ hybridization detection of GFP reporter gene expression in stage 32 embryo also shows transcripts in developing facial muscles (arrowhead). C) Combined brightfield and UV image of stage 45 transgenic embryo. Ventro-lateral view shows ectopic reporter expression in facial muscles and muscles associated with the third and fourth aortic arch artery. D) Fluorescent image of the tadpole in (C) showing ectopic GFP expression in the interbranchialis I and II (ib1 and ib2) adjacent to the third and forth aortic arch arteries, the subarcualis obliquus (so) muscle and the orbitohyoideus (or) muscle. Expression is also observed in the kidneys (ki). Transgene expression is strong in the heart (arrow). E) Ventral view of the head of a different transgenic embryo shows additional facial muscles expressing GFP. The orbitohyoideus (or) associated with the musculus quadratohyangularis (qha) and the musculus interhyoideus (ih) are indicated. Control *γ-crystallin* promoter driven GFP is observed in the eye. F) Dorsal view of stage 45 transgenic embryo showing ectopic GFP expression in the kidneys (ki).

Figure 13



Significantly, the NKE mutant also shows aberrant expression of the GFP reporter during later stages of heart development. As described above, expression from the wild type transgene becomes completely restricted to the atrial myocardium by stage 49 of development, identical to expression of the endogenous *ANF* gene (Fig 14A, B). In the case of the NKE mutant, GFP fluorescence persists in the outflow tract and is maintained at high levels in the ventricular myocardium (Fig. 14C). The ventricular expression has never been observed to decrease in the NKE mutants and persists for at least three weeks after expression is restricted to the atria in wild type controls (Fig. 14B). Failure of restriction was confirmed by in situ hybridization against GFP mRNA (data not shown).

4.2.6. The distal GATA site is required for basal promoter activity while the proximal GATA site is required for restriction of *ANF* expression to the atrium.

The *ANF* promoter contains two conserved GATA sites at –160 and –293 designated proximal GATA (GATAp) and distal GATA (GATAd) respectively in Fig. 10. To examine the requirement of these elements for *ANF* promoter function in vivo, we have generated promoter constructions containing mutations of either the GATAp, or

GATAd alone, or mutations in both elements. Once again the mutations have preserved the correct spacing of these elements relative to other regulatory sequences. The results of our transgene experiments indicate that the GATAp and GATAd sites have distinct functions in the regulation of *ANF* promoter activity in the frog embryo (Fig. 12). First, the distal GATA site appears to be required for efficient expression from the *ANF* promoter. Mutations in the GATAd element strongly reduce expression of the GFP transgene, to about 10% of wild type levels. This is approximately equivalent to the degree of reduction observed when either SRE was mutated. In all cases where GFP fluorescence was visible, expression from the GATAd mutant promoter was limited to the developing heart. On the other hand, the proximal GATA site appears to be involved in regulation of tissue specific expression of *ANF*. Mutation of GATAp reduced detectable GFP expression to approximately 30% of wild type numbers. Unlike GATAd mutant expression, mutations in the GATAp element resulted in ectopic expression of the GFP reporter in a subset of tissues apparently identical to those observed for the NKE mutant. Furthermore, initial expression of the GATAp mutant in the heart appeared normal, but GFP fluorescence failed to restrict to the atria after stage 47 (Fig. 14D). Again this is indistinguishable from the expression observed with the NKE mutant

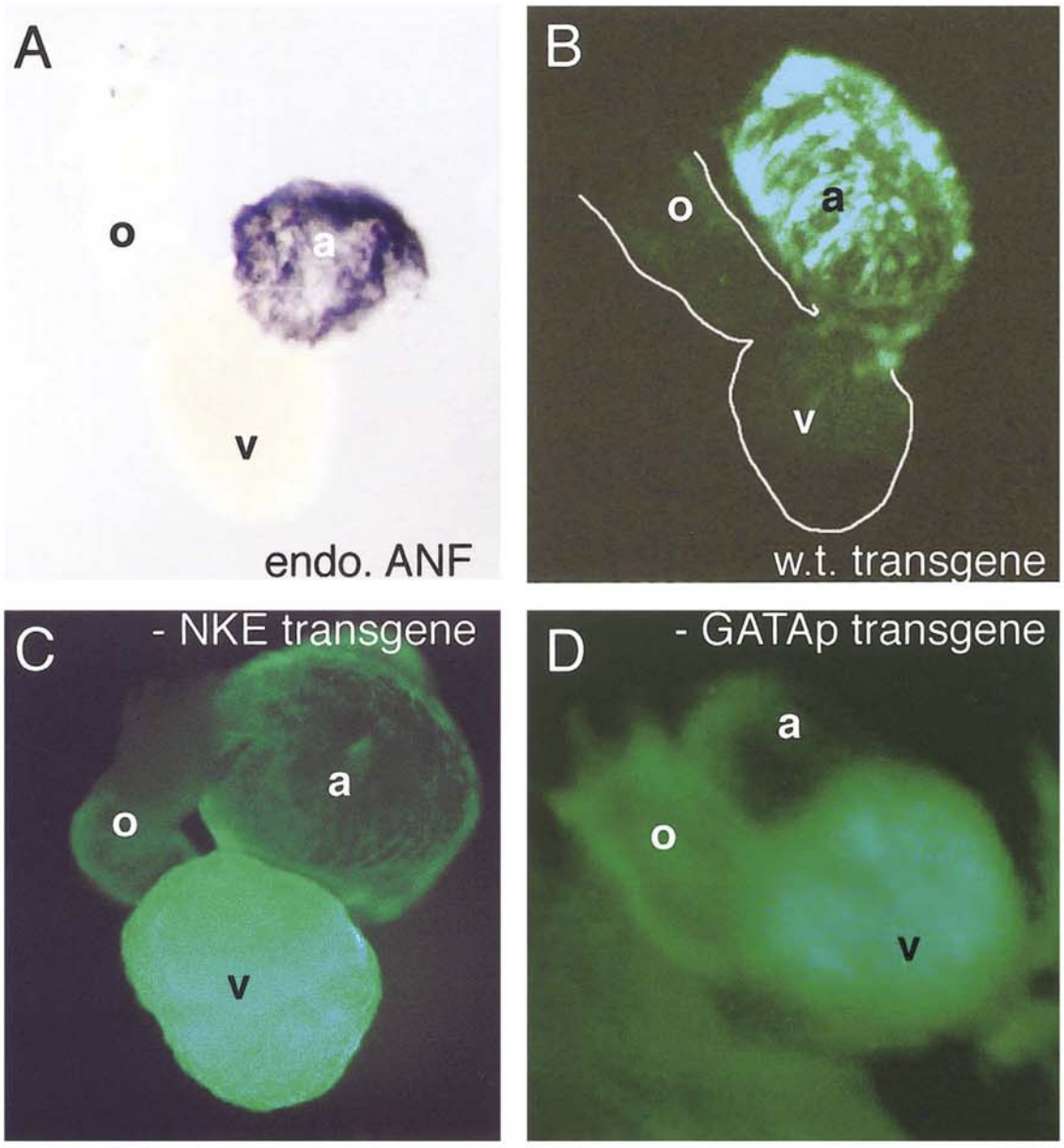
promoter. Finally, as might be expected, mutation of both the proximal and distal GATA sites resulted in a complete absence of detectable GFP transgene expression. Overall, these results indicate that the proximal and distal GATA sites are both required for efficient *ANF* expression, but that the proximal GATA element is also required for regulation of correct spatial expression.

Since mutation of either the proximal GATA site or the NKE, individually, does not abolish the GFP transgene expression, we have tested the activity of a construction containing a double mutation of the NKE and GATAp sites. Surprisingly, embryos transgenic for the NKE and pGATA double mutant promoter still exhibit a relatively high level of GFP transgene expression. As shown in Figure 12, expression from the NKE/GATAp double mutant promoter is observed at approximately 45% the frequency of the wild type control. Note that this is similar to the expression levels observed for either the NKE or the GATAp single mutations. Similarly, the cardiac and ectopic expression pattern of GFP fluorescence driven by the NKE/GATAp double mutant is indistinguishable from that previously observed for the NKE or GATAp single mutants (data not shown).

Figure 14. Mutations of the NKE or proximal GATA (GATAp) element within the *ANF* promoter result in persistent transgene expression throughout the ventricle and outflow tract.

A) In situ hybridization analysis of dissected non-transgenic stage 49 heart, showing restriction of endogenous *ANF* transcripts to the atrium. Note patchy expression of endogenous gene. B) Fluorescent image of GFP expression in dissected stage 49 heart transgenic for the wild-type *ANF* promoter. Reporter expression is restricted to atria and also shows patchy expression. C) Fluorescent image of dissected stage 52 transgenic heart. Mutation of the NKE within the *ANF* promoter results in persistent expression of the GFP reporter throughout the ventricle, and outflow tract. This ectopic expression persists for at least 3 weeks after expression of endogenous *ANF*, or the wild-type *ANF* transgene, has restricted to the atria. D) Fluorescent image of GATAp mutant transgene expression in the heart of a living stage 50 embryo. Expression is visible throughout the ventricle and outflow tract, exactly as observed with the NKE mutant transgene.

Figure 14


A
o
a
v
endo. ANF
B
o
a
v
w.t. transgene
C
- NKE transgene
a
o
v
D
- GATAp transgene
a
o
v

4.3. DISCUSSION

4.3.1. Mutation of SRF binding sites in the *ANF* promoter.

Our first transgenesis experiments focused on the conserved SRF binding sites (SREs) in the ANF promoter, since previous studies have suggested that these elements play a central role in muscle gene regulation (Hines et al.,1999; Wang et al., 2001a). Although the proximal SRE is not a perfect consensus site, previous studies have shown that both the proximal and distal SREs are capable of binding SRF protein. Furthermore, although SRF binds the non-consensus SREp with slightly lower affinity, functional analyses have demonstrated that this site is indeed SRF inducible in cardiac myocytes (Hines et al., 1999). Our transgenic studies showed that mutation of either the proximal or the distal SRE resulted in a severe reduction in expression from the *ANF* promoter, to about 10% of normal levels. These observations are in complete agreement with a model in which SRF proteins form the foundation of a transcription complex containing myocardin, a powerful transcriptional activator that does not itself possess DNA binding activity (Wang et al., 2001a). Cell culture experiments using several different cardiac expressed promoters demonstrate that two SRF binding sites are required for optimal

activation by myocardin (Wang et al., 2001a). With specific reference to activity of the *ANF* promoter however, our results disagree with a previous study using *ANF* promoter constructions in transfected primary cardiac muscle cells, which suggested that the proximal SRE element played only a minor role in basal promoter efficiency (Hines et al., 1999). These differences may reflect species specific variation in the importance of the proximal SRE between mammals and frogs, or may be due to differences between the in vitro and transgenic assays.

4.3.2. A functional Tbx binding site is essential for correct ANF expression.

The mammalian *ANF* promoters contain three potential TBEs. Although the sequence and location of the single TBE in the *Xenopus ANF* promoter is not conserved with any of those in the mammalian promoters, our results demonstrate an important role for both the timing and efficiency of *ANF* promoter activity. Mutation of the TBE results in total absence of detectable GFP in the hearts of transgenic embryos at the normal time of activation. Approximately five days later, GFP reporter expression becomes detectable in about one quarter of the transgenics, but at very low levels. These results suggest that a functional TBE is essential for regulation of both the time of activation and

the efficiency of expression of the *ANF* promoter. This finding is consistent with previous studies showing that Tbx5 potently activates the *ANF* promoter in CV1 cells (Bruneau et al., 2001). Furthermore, levels of Tbx5 seem to be crucial for *ANF* expression. Mice heterozygous for Tbx5 show reduced levels of *ANF* transcripts, while Tbx5 null mice completely lack *ANF* expression (Bruneau et al., 2001). Other studies in mice have reported that Tbx2 is able to cooperate with Nkx2-5 to inhibit AV canal expression of *ANF*. This is believed to be mediated via the TBE at –259, which is adjacent to an NKE (Habets et al., 2002). This pairing of a TBE and NKE is not present in the *Xenopus ANF* promoter.

4.3.3. Mutation of the NKE causes only a slight reduction in *ANF* promoter activity.

The sequence of the single NKE element is almost identical in the *Xenopus* and mammalian *ANF* promoters, suggesting that the regulatory function of this element has been conserved in evolutionarily distant organisms. In transgenic *Xenopus* embryos, mutation of the NKE resulted in only a relatively minor reduction in the number of embryos expressing GFP in the heart, to approximately 60% of wild type levels. This result was initially very surprising, because it appears to contradict the observation that

ANF expression is greatly reduced or completely absent in the Nkx2-5 knockout mouse (Harvey et al., 1999; Tanaka et al., 1999). One explanation might be that, whereas Nkx2-5 protein is essential for *ANF* promoter activity, direct binding of the protein to its target site is not. For example it is possible that protein-protein interactions with either GATA-4 or SRF are sufficient to tether Nkx2-5 to the transcription complex, independent of direct binding to the NKE. This interpretation is in general agreement with several in vitro studies of the *cardiac α actin* and *ANF* promoters, which have suggested that the absence of the NKE does not result in a great reduction in promoter activity (Chen and Schwartz, 1996; Durocher and Nemer, 1998). Indeed, a nearly identical 50% reduction in promoter activity is observed when an ANF promoter with a mutated NKE is transiently transfected into primary cardiac myocytes (Durocher and Nemer, 1998). Other experiments have shown that the DNA binding domain of Nkx2-5 is not required for activity of the *cardiac α actin* promoter, provided that SRF is present (Chen and Schwartz, 1996). Together these studies suggest that Nkx2-5 is indeed an important transcriptional activator for *ANF* expression, but that direct interaction of Nkx2-5 protein with DNA is largely dispensable. It may not be appropriate to generalize this result too far however, since transgenic studies have shown that an Nkx2-5 binding sites is essential

for GATA-6 promoter activity (Molkentin et al., 2000). Overall, these transgenic and cell culture studies suggest that the effect of NKE mutation may be promoter specific. In particular, the consequences for transcriptional activity may be dependent on the presence of binding sites for specific cofactors that could form a transcription complex with Nkx2-5 and help to modulate its activity. Finally, the most striking effect of NKE mutation in transgenic embryos is activation of *ANF* promoter activity in several different tissues outside of the developing heart and the persistence of *ANF* expression in the ventricle and outflow tract. These observations will be discussed below.

4.3.4. Mutation of the GATA binding sites in the *ANF* promoter.

Our transgenic studies indicate that the two GATA sites in the *ANF* promoter exhibit distinct regulatory activities. First, the distal GATA element is essential for efficient *ANF* promoter activity, since mutants lacking this site showed greatly reduced reporter gene expression (approximately 15% of wild type activity). This result is not consistent with a previous cell culture study which suggested that the distal GATA exerts only a modest effect on *ANF* promoter activity (Morin et al., 2001). On the other hand, the proximal GATA element plays a more subtle role in modulating *ANF* activity.

Elimination of this site results in a moderate reduction in cardiac promoter activity, but also allows ectopic expression of the GFP reporter in the facial muscles and in the developing kidneys, apparently identical to the aberrant pattern of expression observed for the NKE mutant. During subsequent development of the heart, expression of the proximal GATA mutant never restricted to the atria, again corresponding to the pattern of the NKE mutant.

4.3.5. Mutation of both the proximal GATA and the NKE sites.

Dual mutation of the proximal GATA site and the NKE within the *ANF* promoter reduces reporter expression to approximately half of wild type activity (Fig. 12). The expression of the double mutant is indistinguishable from both the single NKE mutant, or the proximal GATA site mutant. This result indicates that mutation of both binding sites causes no further reduction in promoter activity or tissue specificity than mutation of either site independently. These results are in apparent disagreement with *ANF* promoter studies in cultured cells, which suggest binding of both Nkx2-5 and GATA-4 are required for efficient transcription (Lee et al., 1998). In fact, mutation of both the GATA and NKE sites nearly eliminated promoter activity in primary cardiac myocytes (Durocher

and Nemer, 1998). Additional cell culture studies indicated that GATA-4 and Nkx2-5 synergize strongly to activate *ANF* expression, resulting in a transcriptional activation of between 25 fold and 200 fold over expression levels observed with one of the factors alone (Durocher et al., 1997; Lee et al., 1998). Our studies in the transgenic embryo do not contradict a possible synergy between the Nkx2-5 and GATA proteins, but they strongly suggest that the corresponding binding sites are not required for this activation. Based on previous studies (Grueneberg et al., 1992; Treisman, 1994; Chen and Schwartz, 1996; Belaguli et al., 2000), the most likely explanation is that SRF protein, binding to the essential proximal and distal SRE sites, facilitates the assembly of a transcription complex that includes both the GATA and Nkx2-5 proteins. This would be similar to the proposed role for SRF in providing a platform for recruitment of the non-DNA binding transcriptional activator, myocardin, to the promoter of cardiac specific genes (Wang et al., 2001a).

4.3.6. Tissue specific regulation of *ANF* expression.

An unexpected result of our transgene experiments was the observation that mutation of either the NKE or the proximal GATA element results in ectopic *ANF*

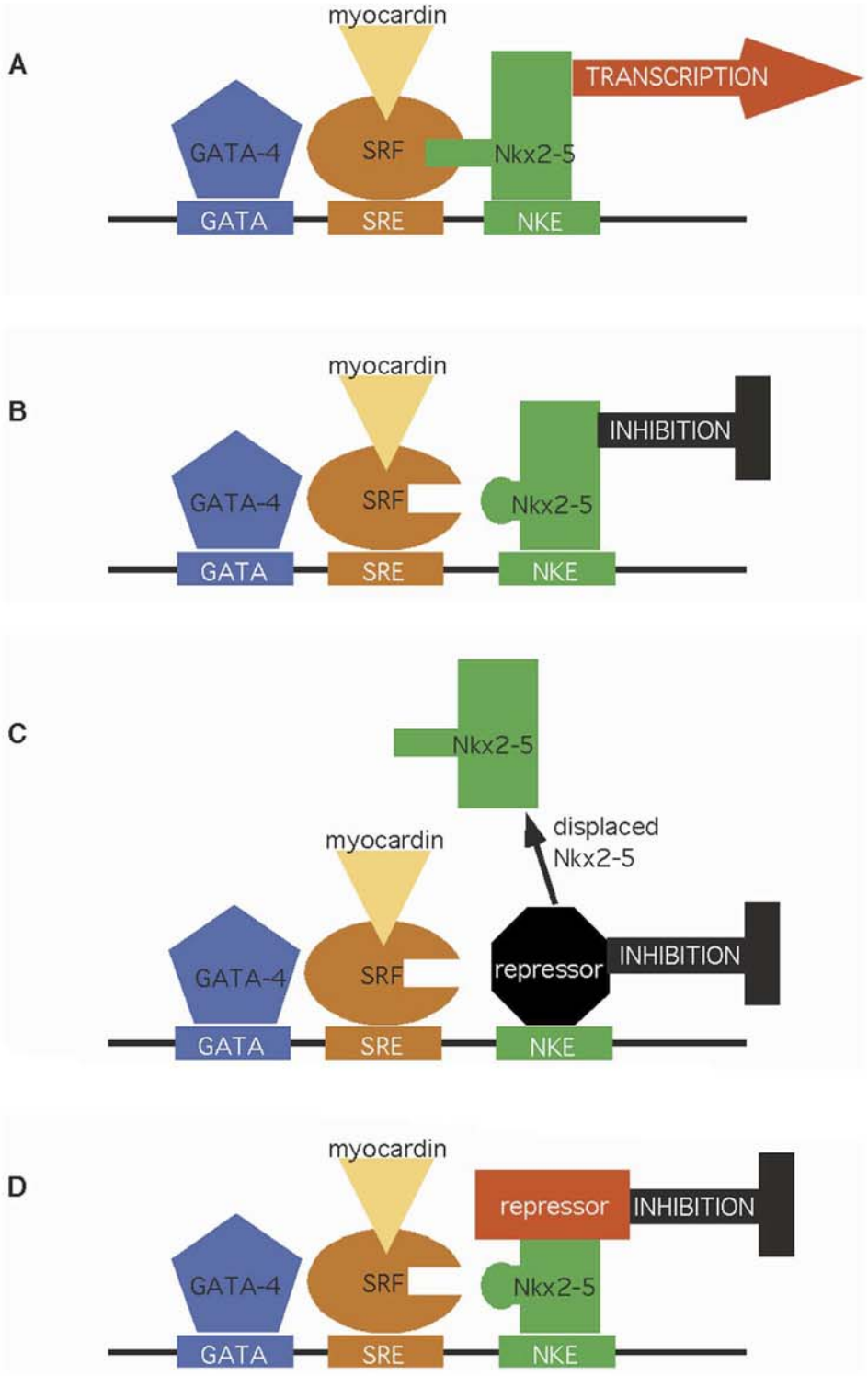
promoter activity. In the wild type promoter therefore, it appears that the NKE and the proximal GATA can play an inhibitory role, preventing *ANF* expression in ectopic tissues. Such an inhibitory role for the NKE is consistent with previous studies suggesting that Nkx2-5, acting together with Tbx2, inhibits *ANF* expression in the atrioventricular canal (AVC) region of the mouse heart (Habets et al., 2002). We note however, that the NKE implicated in the AVC inhibition is located at about -350 in the mouse *ANF* promoter and this element is not conserved in the frog sequence. How might the inhibitory function of the NKE be explained? Within cardiac tissues, it is possible that Nkx2-5 undergoes a post-translational modification that converts it from an activator to a repressor of transcription (Fig. 15B). To explain the observed pattern of *ANF* expression, the modification would be limited to the ventricle and outflow tract tissues. A necessary corollary of this model is that the post-translational modification would also prevent assembly of Nkx2-5 into the SRF transcription complex. If this were not the case, then mutation of the NKE sequences would not have any effect on *ANF* regulation in the transgenics. This model has the advantage that the known interactions between GATA and Nkx2-5 proteins (Durocher et al., 1997; Sepulveda et al., 1998) may continue to be relevant for the inhibitory mechanism.

An alternative explanation for these observations is that, in kidney, facial muscles and the ventricle, the NKE and/or the GATA sites serve as the recognition sequence for a distinct repressor protein (Fig. 15C). Activity of this putative inhibitor in the ventricle and the outflow tract would result in restriction of *ANF* expression to the atria. Mutation of the inhibitor recognition sequence would be expected to prevent inhibitor binding and thereby permit continued *ANF* transcription. Since Nkx2-5 and GATA proteins continue to be expressed throughout all myocardial tissues during development and in the adult heart (Lints et al., 1993; Kasahara et al., 1998; our unpublished observations), this model assumes that the inhibitor would either displace Nkx2-5 from the SRF-based transcription complex (Fig. 15C), or bind directly to Nkx2-5 and completely inhibit its activation properties (Fig. 15D). In general support of this model, Takimoto et al (2000) have shown that persistent over-expression of Nkx2-5 throughout the myocardium increases expression of *ANF* in the ventricle after restriction, possibly by competing with the inhibitor protein for NKE binding. Furthermore, Durocher et al (1996) showed that mutation of the NKE caused an upregulation of *ANF* promoter activity in ventricular myocytes, consistent with an inhibitory role in this tissue.

Figure 15. A model of *ANF* transcriptional regulation by Nkx2-5, GATA-4, and SRF.

A) In the early myocardium and in the mature atria, activation of *ANF* transcription is proposed to be regulated by a complex of Nkx2-5, SRF and co-factor myocardin, and GATA-4. B) Inhibition of *ANF* transcription in the ventricle may occur due to post translational modification of Nkx2-5, which results in exposure of an inhibitory domain within the protein. This modification must also abolish Nkx2-5 - SRF interactions since mutation of the Nkx2-5 binding site within the *ANF* promoter results in loss of inhibition in the ventricle. C) A second inhibitory model relies on a hypothetical repressor protein that becomes expressed (or activated) in the ventricle after stage 47. This repressor protein would bind to the NKE and displace Nkx2-5 from the *ANF* promoter. D) A third possible inhibitory mechanism results from the binding of a ventricle-specific repressor protein directly to Nkx2-5. This interaction between Nkx2-5 and the candidate repressor must abolish the ability of Nkx2-5 to interact with SRF, and therefore, to be tethered to the promoter in the absence of a functional NKE.

Figure 15


A
myocardin
TRANSCRIPTION
GATA-4
SRF
Nkx2-5
GATA
SRE
NKE
B
myocardin
INHIBITION
GATA-4
SRF
Nkx2-5
GATA
SRE
NKE
C
Nkx2-5
displaced
Nkx2-5
myocardin
GATA-4
SRF
repressor
INHIBITION
GATA
SRE
NKE
D
myocardin
repressor
INHIBITION
GATA-4
SRF
Nkx2-5
GATA
SRE
NKE

Somewhat surprisingly, a relatively large number of proteins capable of binding to the NKE have been reported, and several of these may have the potential to act as inhibitors. First, it appears that all members of the NK2 family of homeodomain proteins, and also the NK3 (bagpipe) family proteins, show affinity for a binding site closely related to the 5' - AAGTG - 3' sequence present in the *ANF* NKE (Guazzi et al., 1990, Okkema and Fire, 1994; Chen and Schwartz, 1995; Carson et al., 2000; Steadman et al., 2000; Sparrow et al., 2000b). All known NK2 proteins contain a sequence N-terminal to the homeodomain that has similarity to the engrailed repressor (Smith and Jaynes, 1996) although there is currently no evidence that this domain serves an inhibitory function in vivo. On the other hand, it is clear that NK3 (bagpipe) family proteins may act as transcriptional inhibitors (Steadman et al., 2000). Similarly, it has been reported that the transcription factor COUP-TF1, and homeodomain proteins of the Hmx family, can recognize the Nkx2-5 binding site and may act as transcriptional repressors by opposing the activity of Nkx2-5 (Amendt et al., 1999; Guo et al., 2001). The expression patterns of most of these potential inhibitors have not been reported during the later stages of heart development, although the hmx1 orthologue in chicken,

GH6 appears to be specifically expressed in the ventricular myocardium (Stadler and Solursh, 1994). Expression of the putative repressor may either be restricted to non-atrial tissues, or alternatively, the factor might undergo post-translational modification to assume inhibitor activity in the non-atrial tissues. At present it is not possible to determine whether the same repressor that is acting in the heart is also responsible for inhibiting ectopic *ANF* expression in the facial muscles and kidney, or whether different repressors are acting in the different tissues.

The observation that mutation of the GATA and NKE elements results in persistent expression of *ANF* in the ventricle and outflow tract may help to more generally illuminate the mechanisms by which atrial specific expression of genes is achieved during normal development. In all species examined, *ANF* is initially expressed throughout developing atrial and ventricular myocardium and later becomes restricted to the atria (Zeller et al. 1987; Akizuki et al., 1991; Seidman et al., 1991; Small and Krieg, 2000; Houweling et al., 2002; Habets et al., 2002). Similarly a number of other atrial specific genes, including the quail *slow MyHC3*, mouse *MLC2a* and *MLC1a* (Wang et al., 1996; Kubalak et al., 1994; Lyons, 1994) are initially expressed throughout the heart but

later become limited to the atrial chambers. In the case of *slow MyHC3*, atrial restriction is proposed to require the inhibitory properties of the homeodomain protein Irx4 (Wang et al., 2001b), which is ventricle-specific in all species examined (Bao et al., 1999; Bruneau et al., 2000; Garriock et al., 2001). The repression of *slow MyHC3* by Irx4 in the ventricle is thought to be mediated by protein-protein interactions with a Vitamin D - Retinoid X receptor complex. This complex tethers Irx4 to the vitamin D receptor binding element (VDRE) (Wang et al., 2001b). Since the time of restriction to the atrium varies widely between different cardiac genes it is unlikely that the same factors are involved in regulation all atrial specific genes. However, we propose that inhibition by chamber-specific factors is a general mechanism of restriction of cardiac gene expression. The same families of inhibitory factors mentioned above might be considered as candidates for the ventricular repressors involved in regulation of other atrial specific genes. In the case of the homeodomain proteins, the inhibitors may interact with GATA proteins to stabilize DNA binding.

Chapter 5: Future Directions

5.1. Skeletal muscle specific myocardin-related SRF cofactor

Myocardin is a cardiac and smooth muscle specific cofactor for the ubiquitous serum response factor (SRF), and a potent transcriptional activator. Our work has shown myocardin to be necessary and sufficient for induction of a wide range of cardiac and smooth muscle differentiation products, and this activity is dependent on interactions with SRF. These findings suggests that myocardin may be the means whereby the globally expressed SRF attains a tissue specific functionality, a dilemma that has hampered models of transcriptional regulation for several years. While myocardin is able to activate cardiac and smooth muscle differentiation products, myocardin does not induce skeletal muscle products or mesodermal tissue in general, although SRF is expressed in these tissues as well. Interestingly, we have identified a closely related myocardin-like transcription factor that we have named skeletor. Skeletor is expressed in the somites and skeletal muscle of the body wall and face early during development. We hypothesize that skeletor plays an important role in the induction of skeletal muscle in an analogous manner to the role of myocardin in heart development. It will be straight

forward to carry out gene induction experiments by injecting skeletor into animal caps and assaying for the induction of skeletal muscle genes by RT-PCR. If skeletor specifically induces skeletal muscle, it will be very interesting to determine the domains within the closely related myocardin and skeletor proteins that result in cardiac or skeletal muscle specific induction, respectively. Myocardin and skeletor may belong to a family of tissue-specific SRF cofactors that modulate the inductive capacity of SRF during development.

5.2. Further analysis of downstream targets of myocardin

The experimental method we have used to date has limited our identification of myocardin targets to obvious candidate genes we choose to assay by RT-PCR. We have also been unable to determine the reason why certain genes, such as *ANF*, are not induced in our assay system. *ANF* in particular would seem to be a prime candidate for activation by myocardin in light of the dependence of the *ANF* promoter on SRF binding sites (Small and Krieg, 2003). Indeed, studies using cultured cells have demonstrated *ANF* to be a myocardin target gene (Wang et al., 2001).

In order to identify additional myocardin target genes (and possibly intermediate factors that can induce genes such as *ANF*), we will carry out a micro array screen. In collaboration with Matthew Grow at the University of Indiana Medical School in Indianapolis, we have already constructed gene chips with 88,000 cDNAs from stage 46 *Xenopus* hearts. Screening of the cardiac gene chips with labeled pools of probes from the myocardin-injected or uninjected animal cap samples should identify a number of myocardin-induced genes. Since uninjected animal caps never express cardiac genes there should be no background signal, eliminating the possibility of false positives due to the inclusion of genes with negligible upregulation. This approach is likely to result in large numbers of previously identified myocardin targets, however may also yield a number of unexpected or novel genes that are regulated by myocardin. Ideally, we may also identify additional transcription factors that are induced by myocardin and have the potential to be downstream activators contributing to the complete cardiogenic pathway. This approach will result in a better understanding of the downstream targets of myocardin.

5.3. Identification of upstream regulatory factors for myocardin

In order to place myocardin in a genetic heirarchy it will also be necessary to identify factors that are upstream regulators of myocardin. An analysis of the 5' regulatory region of the myocardin gene can be undertaken to attain this goal. A combination of sequence comparisons with promoters from divergent species and a systematic mutational analysis of conserved regulatory sequences using *Xenopus* transgenesis should result in the identification of important regulatory domains within the myocardin promoter. Since myocardin is among the earliest known cardiac expressed genes, an understanding of myocardin gene regulation will undoubtedly contribute to our general understanding of the earliest stages of heart specification.

5.4. Repression of ventricular cardiac chamber gene expression.

It is evident from our work and the work of several other labs that the differentiation of the atrial and ventricular cardiac chambers is an extremely complex process. Various gene products are differentially expressed between the atria and ventricles, and this restriction of expression occurs at various timepoints in development. For this reason alone, it is unlikely that a single global mechanism controls the

differentiation of the cardiac chambers. Studies from our lab and others however, have demonstrated that atrial-specific expression appears to primarily result from down-regulation in the ventricles at some point during cardiogenesis.

Our analysis of the *ANF* promoter in transgenic *Xenopus* has demonstrated that the binding sites for Nkx2-5 (NKE) and GATA-4 (GATA) act as potential ventricle repressors (Small and Krieg, 2003). This repression could occur via two primary mechanisms. First, post-translational modifications of Nkx2-5 or GATA-4 may result in their transformation into repressors. If a post-translational modification could be observed at the time of *ANF* restriction to the atrium this mechanism would be likely. Second, a ventricle-specific repressor protein that has a high affinity for the NKE or GATA sites could account for the restriction of *ANF* to the atrium. Interestingly, a number of potential transcriptional inhibitors have been shown to bind a consensus NKE and inhibit gene expression in cell culture. Electromobility shift analysis can be undertaken with nuclear extracts from adult atrial or ventricular cardiomyocytes in order to confirm that a different complex exists on the *ANF* promoter in the ventricle.

5.5. Identification of ventricle repressor.

In an attempt to identify the ventricular repressor of *ANF* expression we have begun to focus on candidate cardiac genes. An analysis of potential transcriptional inhibitors was undertaken to determine whether any are expressed in the correct place and time to inhibit *ANF* transcription. Of particular promise, we have shown the NK3 class homeodomain gene *Koza* to be expressed specifically in the ventricle and the muscles associated with the aortic arteries (data not shown). These are the precise tissues in which *ANF* is down-regulated. Further analysis will be required to determine the exact timing of ventricle specificity, but Koza remains a likely candidate for the *ANF* repressor. In order to functionally assess the candidate repressors, we have made a construct that contains the 625 bp *ANF* minimal promoter linked to the luciferase reporter gene. With this construct, we have begun preliminary experiments to determine whether Koza is able to inhibit *ANF* promoter induction by Nkx2-5 or GATA-4 in HeLa or COS cells. If Koza, or another candidate, represses *ANF* activity in cell culture, further experiments could be undertaken to confirm the function of the candidate repressor in vivo. The candidate could be misexpressed throughout the heart in transgenic *Xenopus*, driven by the *myosin heavy chain alpha* (*MHC*α) promoter. The *MHC*α promoter has been isolated

in the lab, and has been shown to drive high levels of reporter gene expression throughout the heart. Whole mount in situ hybridization can be carried out with an *ANF* probe to determine whether *ANF* is downregulated in transgenic embryos. Other atrial-enriched genes can also be assayed by in situ hybridization to determine if Koza plays a more wide spread inhibitory role.

If the candidate gene approach fails, a couple of other approaches could be undertaken. First, gel shifts of the *ANF* promoter using atrial or ventricular nuclear extracts will determine whether different regulatory complexes are associated with the promoter in the atria and ventricles. If different shifts are observed, the ventricular regulatory proteins can be isolated and sequenced. Additional regulators of *ANF* expression, potentially the ventricular inhibitor, could be identified in this manner. Second, a microarray could be screened with cDNA from the atria versus the ventricles to identify differential gene expression between the chambers. We have, in collaboration with Matthew Grow at the University of Indiana Medical School, constructed a gene chip with 88,000 genes isolated from stage 46 *Xenopus* hearts that could be used for this purpose.

The identification of the factor that represses *ANF* (and potentially other atrial specific genes) would be of widespread interest. First, it would corroborate our hypothesis for the regulation of *ANF* tissue specificity. More importantly, it would greatly increase our general understanding of cardiac chamber differentiation as a whole.

Chapter 6: Materials and Methods

6.1. Embryological manipulations

Xenopus embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). *Xenopus* eggs were in vitro fertilized, dejellied using 2% L-cysteine (pH 8.0), and maintained until an appropriate stage in 0.2xMMR. Targeted mRNA injections were performed in 0.4xMMR/4% Ficoll at the 8 cell stage. 125pg, 250pg, or 400pg of various mRNAs were injected in a volume of 4.6nl. Embryos were cultured in 0.2xMMR until a suitable stage and fixed in MEMPFA for assay by in situ hybridization. Embryo injections for use in RT-PCR assays were performed at the one-cell stage into the animal pole.

For animal cap assays, the animal pole explants were dissected from mRNA-injected or uninjected control embryos at stage 8 in 1x NAM. Caps were cultured in NAM/2 (Normal amphibian medum) containing gentamycin until sibling embryos were stage 12.5 or 28, and harvested for RT-PCR analysis.

6.2. Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out using a modification of the protocol by Harland, (1991), using antisense digoxigenin-labeled probes transcribed using a MEGAscript kit (Ambion). For serial sections, embryos were post-fixed in 4% paraformaldahyde for 6 hrs. at RT, and embedded in paraplast. 10 μm transverse sections were cut on a microtome.

6.3. Cloning of *Xenopus laevis myocardin*

Degenerate PCR using primers designed against the conserved amino acid regions WETMEWL and IFNIDF within the human and mouse myocardin sequences was performed on random-primed *Xenopus* frog heart cDNA. After cloning and sequencing of the resulting 99 bp fragment, the remaining portions of the *Xenopus* myocardin cDNA were amplified by 5' and 3' RACE using *Xenopus* frog heart cDNA that was prepared according to the instructions provided with the FirstChoice RLM-RACE Kit (Ambion). The final *Xenopus* myocardin sequence was determined by PCR amplification, cloning and sequencing of the entire myocardin coding region using the Expand High Fidelity

PCR System (Roche), PCR primers within the 5' and 3' UTRs and random-primed *Xenopus* frog heart cDNA as the template.

6.4. RT-PCR

Ten animal cap explants were harvested for each sample and RNA was isolated using Buffer A/ Proteinase K. cDNA was prepared from one half of each RNA sample, and a minus RT negative control sample was prepared from the remaining RNA. $1/50^{th}$ of the cDNA sample was used as template in radioactive RT-PCRs using 0.3 μCi of [α-^{32}P] in a 20μl reaction. RT-PCR cycle number was determined to assure the reaction was in the linear range of amplification. PCR samples were run on non-denaturing 5% acrylamide gels.

6.5. Primers

***ANF*:**

forward: 5'TCTCTGATGAAGGCCAAGG3',

reverse: 5'CAAAACCTGTGGCTGTTGCA3' (Tm=60°C);

Calponin H1:

forward: 5'GCACTGTACGGAAGATCAACG3'

reverse: 5'CGATATCCACTCTGGCACCTT3' (Tm=60°C);

Cardiac α actin (Niehrs et al., 1994) (Tm=63°C);

cardiac Troponin I (Vokes and Krieg, 2002) (Tm=63°C);

***GATA4*:**

forward: 5'TCTGGCCACAACATGTGG3',

reverse: 5'CAGTTGACACATTCTCGG3' (Tm=56°);

***Mef2A*:**

forward: 5'CAGCTCCAGCAGTTCCTAT3',

reverse: 5'TTACACTGAGGCCTAATGCA3' (Tm=56°C);

MHCα:

forward: 5'ACCAAGTACGAGACTGACGC3',

reverse: 5'CTCTGACTTCAGCTGGTTGA3' (Tm=60°C);

***MLC*:**

forward: 5'GAGGCATTCAGCTGTATCGA3',

reverse: 5'GGACTCCAGAACATGTCATT3' (Tm=60°C);

***MRF4*:**

forward: 5’ATCAGCAGGACAAGCCACAGA3’,

reverse: 5’TGTATAGTGCAAGGGTGCCTG3’ (Tm=58°C);

***Myf5*:**

forward: 5’CATGTCTAGCTGTTCAGATGG3’,

reverse: 5’CAATCATGCGCATCAGGTGAC3’ (Tm=58°C);

***MyoD*:**

forward: 5’AACTGCTCCGATGGCATGATGGATTA3’,

reverse: 5’ATTGCTGGGAGAAGGGATGGTGATTA3’ (Tm=60°C);

***myogenin*:**

forward: 5’CCTGAATGGAATGACTCTGAC3’,

reverse: 5’GGCAGAAGGCATTATATGGAA3’ (Tm=58°C);

***Nkx2-5*:**

forward: 5’TCTGAACTCACTGAGGAA3’,

reverse: 5’AGGACTGGTACAGCTATC3’ (Tm=56°C);

ODC (Vokes and Krieg, 2002) (Tm=60°C);

SkMLC:

forward: 5'TGGTCAAAGAGGCATCTGGA3'

reverse: 5'AGTTGTGTCTCTGATGGGATG3' (Tm=60°C);

***SM22*:**

forward: 5’TCCAGACAGTAGACCTGTATG3’,

reverse: 5’GTCGACCGTATCCTGTCATC3’ (Tm=60°C);

SM actin:

forward: 5'ACCACTTACAACAGCATCATG3',

reverse: 5'ACCAATCCAGACGGAGTACTT3' (Tm=60°C);

***SRF*:**

forward: 5’TGCACTGTGCCTGTGTGATTA3’,

reverse: 5’CAGACTCACACAACTTGCACA3’ (Tm=58°C);

Xbra (Vokes and Krieg, 2002) (Tm=60°C).

6.6. Gene depletion by morpholino oligonucleotide injection

Antisense morpholino oligonucleotides (MO1 and MO2) were designed to two independent stretches of sequence in the 5' end of the *myocardin* gene. MO1 was designed entirely in the 5'UTR up to and not including the start codon: MO2 begins at the start codon and includes 21 bp of coding sequence: . The morpholinos were designed

against sequence that was 100% identical between the A and B copies of the *Xenopus* gene in order to inhibit translation of both mRNAs with one morpholino.

A dose curve was performed with 2.5ng, 5ng, 10ng, and 20ng of morpholino, injected in one cell of a two-cell embryo so the uninjected side served as a negative control. A concentration dependent phenotype was observed with increasing percentage of asymmetric heart patches with increasing dose. Subtle non-specific morphological defects were observed with 20ng of injected morpholino, therefore 10ng of MO1 and MO2 was used for all of the following experiments.

6.7. Isolation of the *Xenopus ANF* gene

A PCR fragment containing the coding sequences of the *Rana catesbiana ANF* gene (Genebank accession # D01043) was used to screen a *Xenopus* adult heart cDNA library (Ji *et al*., 1993). Approximately 1/500 of all plaques were positive suggesting that *ANF* transcripts represents about 0.2% of total mRNA in the adult heart. Sequencing of a representative positive clone revealed an open reading frame of 441 bp, which contains the entire coding region of the 147 aa *Xenopus* ANF pre-proprotein.

6.8. Isolation of the *Xenopus ANF* 5' regulatory region and construction of GFP transgenes

A 400bp *Pst*I fragment from 5' terminus of the *Xenopus laevis ANF* coding region was used to probe a *Xenopus* Lambda genomic library (courtesy of Mike King). Approximately 10^6 phage were screened at high stringency (0.2X SSC/0.1% SDS at 65ºC). Positive phage were purified and phage DNA was isolated from liquid culture by cesium chloride gradient, restriction digested with *Eco*RI and subcloned for analysis. Restriction mapping and analysis by Southern blotting against the 5'region of the *Xenopus ANF* cDNA isolated an *EcoRI* fragment that contains 3.4 kb of 5' flanking sequences.

For *Xenopus* transgenics, 3.4 kb and 625 bp fragments of the *ANF* 5' regulatory sequences were amplified using Pfu polymerase and a 5' primer containing a *Sac*I and *Eco*RI linker, respectively:

5'-TCCGGAGCTCGGCTGTCAGTCTCTGGATAA-3'

5'-GGAATTCCCAGTGAGCGATATTTGC-3'

and a 3’ primer containing a *Bam*HI linker:

5’-GTCTGTAAAGATATCACCGCCCTAGGGC-3'.

Both the 3.4kb and 625 bp regulatory sequences were subcloned into the *Sac*I/*Bam*HI or *Eco*RI/*Bam*HI sites respectively of a modified pEGFP1 vector (Clontech).

The pEGFP-625*ANF* clone was used as a template for site-directed mutagenesis using Pfu polymerase. The pEGFP -NKE, -GATAp, -GATAd, -SREp, and -SREd constructions were created using the following oligonucleotides. Mutated regions are underlined.

NKE-*Sma*I: 5’TCCCCCGGGCTGCTCCGAAGGCGGGCTTCATTCCTCTGC-3’

and 5’-TCCCCCGGGGCATGATAAAGAATCTGATTG-3’;

GATAp-*Apa*I: 5’-GTGGGCCCCTTTAAAAGGGAATCTTCTG-3'

and 5’-GTGGGCCCTGCCCTCCACATTTCCACAG-3’;

GATAd: 5’-TGCTTTTACACCTGCATTCCTTATTAGT-3'

and 5’-GATGGGTCAGTTTATCCTTTTCAC;

SREp: 5’-TTATCCTGCCCTCCACATTTCCAC-3’

and 5’-CAAGTGTATAGAATCTTCTGTTGGCCTTCAATCAGA-3’;

SREd: 5’-CCAAACCCGCTTTATTTAATAAATATGACG

and 5’-TCAAATCTGTTGGATTTACATTA-3’

TBE: 5’-TTCGGATGTAATGTGGAGGGCAGGAT-3’;

and 5’-TTTCCAGGAGGTGCCTTATCC-3’

6.9. *Xenopus laevis* transgenesis

ANF promoter plus GFP reporter sequences were excised from the plasmid vector by digestion with *Eco*RI/*Pme*I and the transgene was purified using QIAEX II gel extraction kit (Qiagen). The transgene was then used to generate transgenic *Xenopus* embryos using previously described protocols (Kroll and Amaya, 1996; Sparrow et al., 2000a). Since greater than 90% of *Xenopus* embryos that are transgenic for one construction also co-express a second construction (Huang et al., 1999; Marsh-Armstrong et al., 1999), *γ-crystallin*-GFP expression in the eye was used as a control transgene to assay for insertion of DNA into the genome (Bronchian et al., 1999). Reporter gene expression was detected by fluorescence in live embryos using a Leica Fl-II microscope and MagnaFire digital camera or, in fixed embryos, by whole mount in situ hybridization for GFP transcripts (Harland, 1991).

In presenting the results of transgenic experiments, we are cautiously proposing a system in which activity of different mutant promoters is expressed as a proportion of wild type (wt) 625bp *ANF* promoter activity. This seems reasonable for the following reasons. First, examination of a large number of transgenic embryos has established that detectable expression of the GFP reporter is only observed in 49% (90 of 184) embryos transgenic for the wt *ANF* promoter. Levels of GFP fluorescence vary widely amongst the 49% but it is likely that any promoter mutation that reduces transgene expression will correspondingly increase the proportion of embryos failing to reach the threshold of detectable GFP fluorescence. Second, transgene expression is likely to be influenced by several factors, including the copy number of transgenes inserted, the relative head/tail orientation of transgene insertions and the effects of chromosomal location. However, these effects will average out for both wt and mutant promoters, providing that a sufficient number of independent transgenic embryos are examined for each construction. In order to discourage too much emphasis on the precision of these estimates, relative promoter activity have been stated to the nearest 5%.

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