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MOLECULAR BASIS FOR THE ROLE OF GATA TRANSCRIPTION FACTORS IN CARDIOMYOCYTES

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ABSTRACT

Members of the GATA family of transcription factors are evolutionary conserved DNA-binding proteins that play critical roles in cell differentiation. They share a highly conserved zinc finger domain responsible for specific binding to a consensus (A/T)GATA(A/G) sequence. Three GATA factors, GATA-4, GATA-5, and GATA-6, are expressed in the heart and two of them, GATA-4 and GATA-6, are coexpressed in cardiomyocytes. Given the early embryonic lethality of GATA-4 and GATA-6 null mice, their exact role in cardiomyocytes was unclear. The objective of my doctoral work was to elucidate the role of GATA-4 and GATA-6 in postnatal cardiomyocytes and the molecular basis for their specificity. Since the identification of GATA-4 and GATA-6 target genes would help to elucidate their functions, we inhibited GATA-4 and GATA-6 protein production in postnatal cardiomyocytes and assessed its effect on cardiac gene expression. The results demonstrated that GATA-4 and GATA-6 are required for the maintenance of cardiac gene expression. Importantly, the work showed that GATA-4 and GATA-6 differentially regulate target genes and that this specificity is due, at least in part, to their differential DNA-binding affinity. In addition to differential DNA-binding affinity, we showed that GATA factors also acquire specificity by differential interaction with cofactors, such as MEF2 and Nkx2-5. Thus, GATA factor specificity is achieved at three levels: differential spatio-temporal expression, differential DNA-binding affinity, and differential interaction with cofactors.

The crucial role of GATA-4 and its cofactors in the regulation of contractile actin and myosin protein gene expression suggested that GATA-4 might regulate sarcomere formation. Indeed, we found that, similarly to RhoA GTPase, GATA-4 is essential for sarcomere formation in cardiomyocytes. Moreover, we showed that RhoA acts upstream of GATA-4 and potentiates GATA-4 transcriptional activity. These results suggest that RhoA regulates the expression of sarcomeric proteins and sarcomere formation by inducing the transcriptional activity of GATA-4. Collectively, the work described in this thesis provides important information on the role, regulation, and mechanisms of specificity of GATA transcription factors in cardiomyocytes and in other cells where they are expressed.

RÉSUMÉ

Les facteurs de transcription de la famille GATA jouent un rôle important dans la différenciation cellulaire. Ils sont caractérisés par un domaine de liaison à l'ADN hautement conservé de type doigt de zinc qui lie spécifiquement une séquence consensus (A/T)GATA(A/G). Trois facteurs GATA sont exprimés dans le cœur : GATA-4, GATA-5 et GATA-6, et deux d'entre eux, GATA-4 et GATA-6, sont co-exprimés dans les cardiomyocytes. Étant donné la létalité embryonnaire précoce des souris déficientes en GATA-4 ou GATA-6, le rôle exact de ces protéines dans les cardiomyocytes demeurait inconnu. L'objectif de mes recherches était donc d'élucider le rôle de GATA-4 et GATA-6 dans les cardiomyocytes postnataux, ainsi que de définir la base moléculaire de leur spécificité d'action. Résonnant que l'identification des gènes-cibles de GATA-4 et GATA-6 nous aiderait à déterminer leur fonction, nous entreprîmes de bloquer l'expression de GATA-4 et GATA-6 dans les cardiomyocytes postnataux et de vérifier l'effet de cette inhibition sur l'expression des gènes cardiaques. Ces résultats ont démontré que GATA-4 et GATA-6 sont requis pour le maintient de l'expression de nombreux gènes cardiaques. Des plus, ces résultats ont montré que GATA-4 et GATA-6 contrôlent de façon différentielle l'expression de certains gènes-cibles. Cette spécificité est due, au moins en partie, à une affinité de liaison à l'ADN différentielle de GATA-4 et GATA-6. De plus, nos résultats démontrent que les facteurs GATA acquièrent également une spécificité d'action par leur capacité d'interagir différentiellement avec des cofacteurs, tels que MEF2 et Nkx2-5. Les facteurs GATA acquièrent donc leur spécificité à trois niveaux : patron d'expression différentiel, affinité de liaison à l'ADN différentielle et interaction différentielle avec des cofacteurs.

Le rôle crucial joué par les facteurs GATA et leurs cofacteurs dans la régulation de l'expression des protéines contractiles d'actines et de myosines suggérait que GATA-4 puisse être un régulateur de la formation des sarcomères. En accord avec cette hypothèse, nous résultats démontrent que GATA-4, tout comme la GTPase RhoA, est essentiel pour la formation de sarcomères dans les cardiomyocytes. De plus, nos résultats montrent que RhoA agit en amont de

GATA-4 et induit son activité transcriptionelle. Ces résultats suggèrent que RhoA contrôle l'expression des protéines sarcomériques et la formation des sarcomères en induisant l'activité transcriptionelle de GATA-4. Collectivement, ces travaux apportent d'importantes informations sur le rôle, la régulation et les mécanismes de spécificité des facteurs de transcription de la famille GATA.

ACKNOWLEDGMENTS

I would like to thank my thesis supervisor, Dr Mona Nemer, for her advice, encouragement, criticism, and support throughout my years in her laboratory. She had a profound influence on the development of my scientific personality. I will carry her positive influence throughout my career.

I wish to thank Dr Jacques Drouin for his numerous contributions throughout the various group meetings and especially for his constructive criticism. I would also like to express my appreciation to all the past and present members of the Nemer and Drouin laboratories for their help and comradeship. In particular, I wish to extend my gratitude to Mathieu Arcand, Sophie Caron, Daniel Durocher, Kevin McBride, Steves Morin, Georges Nemer, Pierre Paradis, and George Tsimiklis for their essential scientific contributions to my work as well as their support and friendship; without these individuals, it would have been impossible to have such a great time during all those years. I would also like to thank Michel Chamberland and Lynda Robitaille for their essential expert technical assistance as well as Lise Laroche for her professionalism in assisting the preparation of manuscripts. Additionally, I am grateful to the Fonds de la Recherche en Santé du Québec (FRSQ), Heart and Stroke Foundation of Canada (HSFC), and the National Cancer Institute of Canada (NCIC) for their financial support throughout the course of my studies.

Finally, I would like to thank my family, Gaétane, Yves, and Jean-Sébastien Charron, as well as Julie Lessard, for their love and continuous support.

PREFACE AND CONTRIBUTIONS OF AUTHORS

The present thesis, consisting of five chapters, describes the roles, regulation, and mechanisms of specificity of GATA transcription factors in cardiomyocytes. Chapter I is a literature review which, first, presents the molecular mechanisms underlying GATA transcription factor activity in many tissues and at various developmental stages and, secondly, presents the embryological and molecular processes leading to the formation of the mature four-chambered heart. A particular emphasis is given to the genetic control and transcriptional regulation mechanisms of cardiac gene expression and development. Where appropriate, to ensure the progression of the logical order developed in this second section, the roles and properties of certain relevant transcription factors discussed in the first section will be briefly re-stated.

Chapters II to IV, inclusively, are comprised of three scientific papers: two of them (Chapters II and III) are published papers, whereas Chapter IV is a manuscript submitted for publication. For convenience, reprint copies of Chapters II and III and other published papers are included in the *Appendices* section.

Chapter II, *Cooperative interaction between GATA-4 and GATA-6 regulates myocardial gene expression*, is a multi-authored work in which Dr Pierre Paradis advised and helped me to generate the recombinant adenoviruses. Odile Bronchain cloned the GATA-6 cDNA. Georges Nemer produced Figure 2.8. I generated all the other results and constructions as well as writing the paper under the supervision of Dr Mona Nemer.

Chapter III, *GATA-dependent recruitment of MEF2 proteins to target promoters*, is a co-authored work in which Steves Morin and myself contributed equally. I started this study and trained Steves Morin, a summer student at that time, to finalize it with me. Lynda Robitaille gave technical help with transfections. I wrote the paper under the supervision of Dr Mona Nemer.

Chapter IV, *The cardiac transcription factor GATA-4 is an effector of the small GTPase RhoA and mediates sarcomere reorganization*, is a multi-authored work. I started this study and trained George Tsimiklis, a summer student at that

time, to finalize it with me. Lynda Robitaille gave technical help with transfections. I wrote the paper under the supervision of Dr Mona Nemer.

Finally, Chapter V includes a detailed discussion of the relevance of the findings presented in this thesis and attempts to describe the future prospects of the studies on gene expression regulation by GATA factors. A general bibliography follows and includes the references for Chapters I and V. The references for Chapters II to IV, inclusively, are found at the end of each chapter.

CLAIM TO ORIGINALITY

This thesis was entirely written by me, Frederic Charron, under the supervision of my thesis director, Dr Mona Nemer. The papers presented in Chapters II and III were published by our laboratory and constituted, at the time of publication, original and previously unpublished results. The manuscript presented in Chapter IV was recently submitted. The results presented in this manuscript are original and unpublished.

More precisely, the determination that (i) GATA-4 and GATA-6 are required for the maintenance of cardiac gene expression; (ii) GATA factors recruit MEF2 proteins to synergistically activate MEF2 target genes; (iii) GATA-4 is an essential mediator of sarcomere reorganization in cardiomyocytes; and (iv) RhoA GTPase potentiates the transcriptional activity of GATA factors all represent novel contributions to the field.

Most of the reagents, including adenoviruses, plasmids, GATA-6 antibody, and recombinant proteins, used in these studies were generated by myself and are original. These include the antisense GATA-4 and antisense GATA-6 adenoviruses, which I generated with the help and advice of Dr Pierre Paradis. I also generated the ANF promoter mutations and deletions of the GATA elements, the GATA-4 mutations and deletions, the GATA-6 antibody (with the help of Dr Tony Antakly), and the GATA-4 recombinant proteins.

The original ANF and BNP promoters and the original GATA-1 through GATA-6, MEF2, RhoA wild-type, RhoA N19, and RhoA V14 cDNAs were kind gifts from colleagues. Their contribution is acknowledged in the relevant manuscripts.

CHAPTER I. INTRODUCTION

1 GENE TRANSCRIPTION BY RNA POLYMERASE II

Promoters are DNA sequences responsible for gene expression (reviewed in (Mitchell and Tjian, 1989; Blackwood and Kadonaga, 1998; Zawel and Reinberg, 1992; Buratowski, 1994; Buratowski, 1995)). Eukaryotic promoters are composed of two critical elements: the enhancer and the TATA box. The latter binds the TATA box binding protein (TBP), a polypeptide composing the general transcription factor (GTF) complex, and serves to initiate transcription by RNA polymerase II (RNAP II). Enhancers are composed of *cis*-regulatory DNA elements recognized by specific transcription factors; their role is to regulate gene expression. Transcription factors are typically composed of two domains: a DNA binding domain that recognizes specific sequences within its target promoters and an activation domain that is required for transcriptional activation.

Transcription by RNAP II begins with the binding of transcription activator proteins to their cognate *cis* element (reviewed in (Lemon and Tjian, 2000; Hirose and Manley, 2000)). The activators then recruit the GTFs, which accurately position the RNAP II over the transcription start site. RNAP II then begins transcription, clears the promoter, and enters the elongation phase. Before initiating transcription, RNAP II is hypophosphorylated on its C-terminal domain (CTD), allowing it to interact with the mediator complex, a multi-protein assembly involved in the recruitment of RNAP II to active promoters. After transcription initiation, the polymerase breaks its interactions with transcription initiation factors and its CTD becomes hyperphosphorylated, allowing RNAP II to interact with proteins involved in RNA polyadenylation, capping, and splicing. Finally, directed by DNA sequences at the end of a gene, RNAP II terminates transcription and releases the newly synthesized RNA.

2 THE GATA MOTIF AS A *CIS*-REGULATORY ELEMENT

The GATA *cis*-regulatory element was first defined in analyses of globin gene regulation by three independent groups who showed that many, if not all, globin gene promoters in chicken and human contained at least one copy of the consensus (A/T)GATA(A/G) sequence which was important for maximal globin

promoter activity (Evans et al., 1988; Wall et al., 1988; Martin et al., 1989). The subsequent finding that GATA elements were required for maximal promoter activity of erythroid genes other than globins (Mignotte et al., 1989a; Mignotte et al., 1989b; Zon et al., 1991a; Heberlein et al., 1992; Chiba et al., 1991; Tsai et al., 1991; Aplan et al., 1990) suggested a broader role for these elements in erythroid cells. As will be discussed below, it is now clear that GATA elements are not only restricted to erythroid cell gene expression but are also involved in the regulation of tissue-specific gene expression in numerous other cell types.

An interesting aspect of the GATA elements is the variety of locations where they may be found within genes. In addition to their presence within promoters (either upstream or near the transcription initiation site) and enhancers (usually located 3' in the globin genes), GATA elements are also found in the active core region of the locus control regions (LCRs) of the human α- and β-globin gene clusters (Orkin, 1990; Talbot and Grosveld, 1991; Talbot et al., 1990; Strauss and Orkin, 1992; Strauss et al., 1992; Jarman et al., 1991; Philipsen et al., 1990). LCRs have the property of insulating genes from chromosomal position effects, independent of integration site (Felsenfeld, 1992) and they play a critical role in globin gene expression by facilitating the opening of the chromatin structure, which is necessary for globin gene expression (Schubeler et al., 2000). The LCRs are usually found far upstream of the transcription initiation site and their activity appear to be dependent on GATA elements (Talbot and Grosveld, 1991; Talbot et al., 1990; Strauss and Orkin, 1992; Strauss et al., 1992; Philipsen et al., 1990).

3 THE GATA ELEMENT BINDING PROTEINS

Two independent groups initially cloned the gene coding for the erythroid protein binding to the globin GATA elements, now known as GATA-1 (Evans and Felsenfeld, 1989; Tsai et al., 1989). Subsequently, using low stringency hybridization screening, related genes were cloned in many vertebrate species and named GATA-1 through GATA-6 (Figure 1.1) (Arceci et al., 1993; Evans and Felsenfeld, 1989; Grépin et al., 1994; Kelley et al., 1993; Laverriere et al., 1994; Morrisey et al., 1996; Morrisey et al., 1997a; Suzuki et al., 1996; Tamura et al., 1993; Tsai et al., 1989; Yamamoto et al., 1990). Each of these GATA factor binds

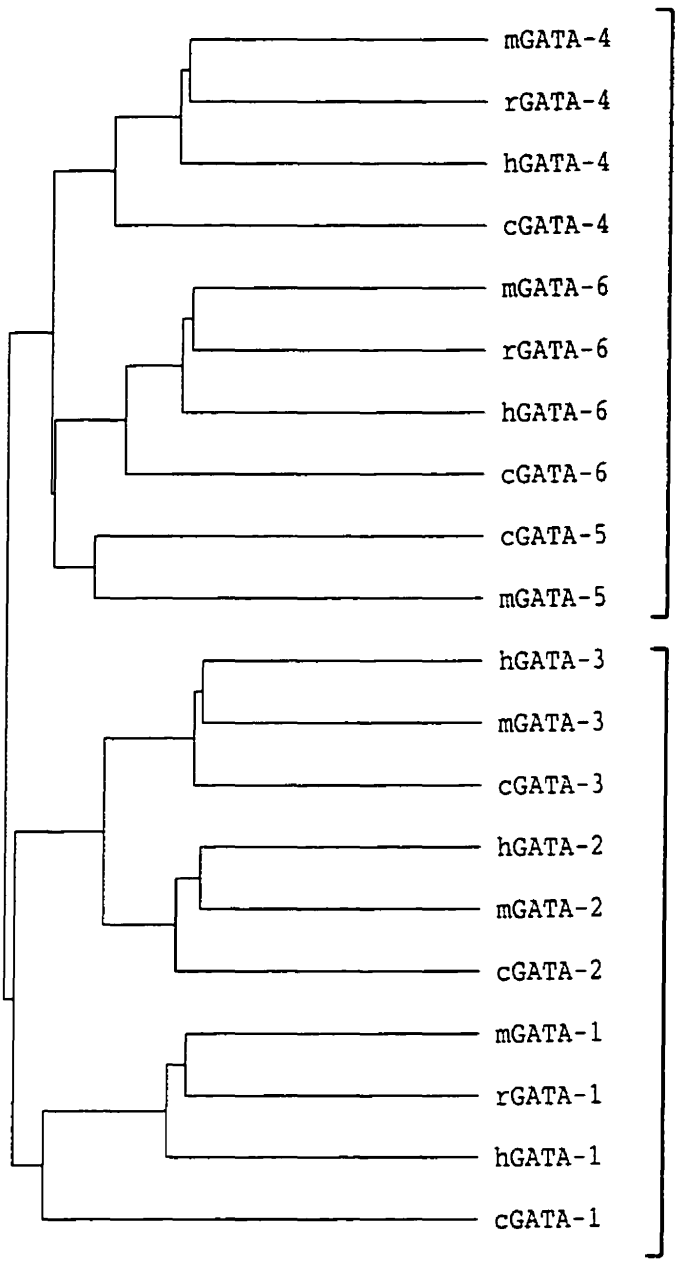


Figure 1.1. Schematic representation of the degree of homology between vertebrate GATA transcription factors. Note that GATA-1, GATA-2, and GATA-3 and GATA-4, GATA-5, and GATA-6 form two high-homology subfamily groups. c chicken; h human; m mouse, r rat.

specifically to GATA elements and exhibits a characteristic pattern of expression during development, as will be discussed below.

3.1 The non-vertebrate GATA proteins

In addition to vertebrates, GATA binding proteins were also identified in lower organisms, where they play diverse roles. However, as this review is mainly intended to discuss the roles of the vertebrate GATA family members, this section will only briefly overview the roles of GATA factors in non-vertebrate species. Nonetheless, throughout this review, relevant studies performed in non-vertebrate species will be presented and put in perspective to work done in vertebrate species.

In unicellular organisms, GATA factors regulate cell fate determination and metabolism. For example, in *S. cerevisiae*, the GATA transcription factor Ash1p is involved in mating-type switching (Bobola et al., 1996; Sil and Herskowitz, 1996) and regulation of pseudohyphal growth (Chandarlapaty and Errede, 1998), while the Gln3p, Gat1p/Nil1p, Dal80p, and Deh1p/Gzf3p GATA factors are involved in nitrogen-catabolic gene expression (reviewed in (Marzluf, 1997)).

In multicellular organisms, GATA factors appear to be especially important for cell type specification. In *C. elegans*, elt-1 is required for the production of epidermal cells (Page et al., 1997), while end-1 specifies endoderm precursors (Zhu et al., 1997; Zhu et al., 1998) and elt-2 is essential for formation of the intestine (Fukushige et al., 1998). In addition, another GATA factor, elt-3, has been identified in *C. elegans* (Gilleard et al., 1999); however, its role remains unknown. Sequence analysis of the *C. elegans* genome revealed that this organism contains 9 potential GATA family members (Clarke and Berg, 1998). Since most of them remain to be functionally characterized, we expect *C. elegans* GATA factors to play even more diverse roles than the ones presented here.

In *Drosophila*, three GATA factors have been described: *pannier* plays a role in embryonic dorsal closure, generation of the medial and lateral dorsal body subdivisions, specification of cardiac cells, bristle determination, and dorsal eye disc development (Gajewski et al., 1999; Heitzler et al., 1996; Ramain et al., 1993; Winick et al., 1993; Maurel-Zaffran and Treisman, 2000; Calleja et al., 2000),

serpent is essential for specification of hematopoietic lineages, development of the fat body (an insect organ analogous to the liver), and differentiation and morphogenesis of the endodermal gut and extraembryonic amnioserosa (Rehorn et al., 1996; Lebestky et al., 2000; Reuter, 1994; Riechmann et al., 1998; Frank and Rushlow, 1996; Sam et al., 1996; Azpiazu et al., 1996; Moore et al., 1998), and *grain/dGATAc* is required for cell rearrangement during organ morphogenesis (Brown and Castelli-Gair, 2000). Together, these studies indicate very diverse roles for GATA transcription factors in non-vertebrates. As will be described below, some of these roles are remarkably conserved in vertebrates.

3.2 The vertebrate GATA proteins

The six vertebrate transcription factors of the GATA family share a highly conserved domain composed of two zinc finger motifs each having the consensus $CX_2CX_{17}CX_2C$ (Figure 1.2). This zinc finger motif represents a relatively ancient protein domain that is conserved in transcription factors from yeast to human and is essential for specific DNA-binding to GATA elements. This domain appears to be restricted to eukaryotic organisms since prokaryotic organisms such as E. coli and M. jannaschii do not contain any sequence homologous to GATA zinc fingers in their genome (Clarke and Berg, 1998). In addition to the two central zinc finger domains, the vertebrate GATA factors are also composed of N- and C-terminal transactivation domains (Figure 1.2). The next section will describe the structure and the role of these domains in more details.

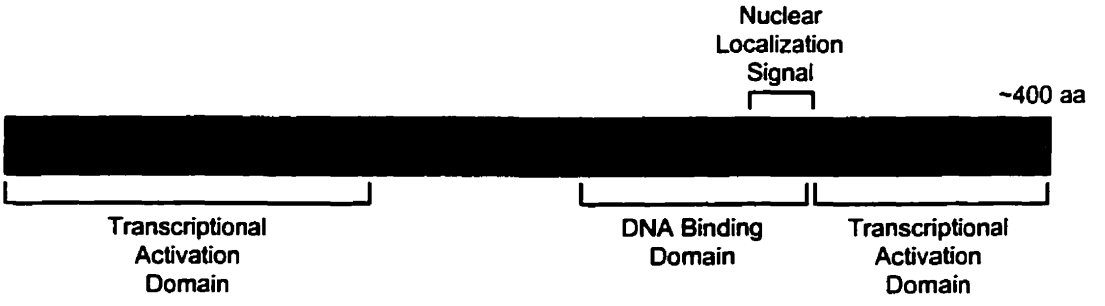
3.2.1 Structure-function analysis of the GATA factors

3.2.1.1 The zinc finger domains

3.2.1.1.1 Roles and structures of zinc fingers

In 1983, the first structural role for zinc in transcription factors was proposed for TFIIIA (Hanas et al., 1983) and further analysis revealed the presence of small zinc-based protein domains termed "zinc fingers" (Miller et al., 1985). These domains all have in common tetrahedral zinc binding sites with four ligands from the side chains of cysteine, histidine, and occasionally aspartate or glutamate (reviewed in (Berg and Shi, 1996)). The role of the zinc atom in a zinc finger is to stabilize the structure of the protein domain.

A



B

```
mGATA-4 RECVNCGAMS TPLWRRDGTG HYLCNACGLY HKMNGINRPL IKPQRRLSAS RRVGLSCANC
rGATA-4 RECVNCGAMS TPLWRRDGTG HYLCNACGLY HKMNGINRPL IKPQRRLSAS RRVGLSCANC
hGATA-4 RECVNCGAMS TPLWRRDGTG HYLCNACGLY HKMNGINRPL IKPQRRLSAS RRVGLSCANC
cGATA-4 RECVNCGAMS TPLWRRDGTG HYLCNACGLY HKMNGINRPL FKPQRRLSAS RRVGLSCANC
mGATA-6 RECVNCGSIQ TPLWRRDGTG HYLCNACGLY SKMNGLSRPL IKPQKRVPSS RRLGLSCANC
rGATA-6 RECVNCGSIQ TPLWRRDGTG HYLCNACGLY SKMNGLSRPL IKPQKRVPSS RRLGLSCANC
hGATA-6 RECVNCGSIQ TPLWRRDGTG HYLCNACGLY SKMNGLSRPL IKPQKRVPSS RRLGLSCANC
cGATA-6 RECVNCGSIQ TPLWRRDGTG NYLCNACGLY TKMNGLSRPL IKPQKRVPSS RRLGLSCANC
cGATA-5 RECVNCGAMS TPLWRKDGTG HYLCNACGLY HKMNGINRPL .KPQKRLSSS RRAGLCCTNC
mGATA-5 RECVNCGALS TPLWRRDGTG HYLCNACGLY HKMNGVNRPL VRPQKRLSSS RRSGLCCSNC
hGATA-3 RECVNCGATS TPLWRRDGTG HYLCNACGLY HKMNGQNRPL IKPKRRLSAA RRAGTSCANC
mGATA-3 RECVNCGATS TPLWRRDGTG HYLCNACGLY HKMNGQNRPL IKPKRRLSAA RRAGTSCANC
cGATA-3 RECVNCGATS TPLWRRDGTG HYLCNACGLY HKMNGQNRPL IKPKRRLSAA RRAGTSCANC
hGATA-2 RECVNCGATA TPLWRRDGTG HYLCNACGLY HKMNGQNRPL IKPKRRLSAA RRAGTCCANC
mGATA-2 RECVNCGATA TPLWRRDGTG HYLCNACGLY HKMNGQNRPL IKPKRRLSAA RRAGTCCANC
cGATA-2 RECVNCGATA TPLWRRDGTG HYLCNACGLY HKMNGQNRPL IKPKRRLSAA RRAGTCCANC
mGATA-1 RECVNCGATA TPLWRRDRTG HYLCNACGLY HKMNGQNRPL IRPKKRMIVS KRAGTQCTNC
rGATA-1 RECVNCGATA TPLWRRDRTG HYLCNACGLY HKMNGQNRPL IRPKKRMIVS KRAGTQCTNC
hGATA-1 RECVNCGATA TPLWRRDRTG HYLCNACGLY HKMNGQNRPL IRPKKRLIVS KRAGTQCTNC
cGATA-1 RECVNCGATA TPLWRRDGTG HYLCNACGLY HRLNGQNRPL IRPKKRLLVS KRAGTVCSNC
Cons.   RECVNCG--- TPLWR-D-TG -YLCNACGLY ---NG--RPL --P--R---- -R-G--C-NC

mGATA-4 QTTTTTLWRR NAEGEPVCNA CGLYMKLHGV PRPLAMRKEG IQTRKRKPKN LNKSKTPAGP
rGATA-4 QTTTTTLWRR NAEGEPVCNA CGLYMKLHGV PRPLAMRKEG IQTRKRKPKN LNKSKTPAGP
hGATA-4 QTTTTTLWRR NAEGEPVCNA CGLYMKLHGV PRPLAMRKEG IQTRKRKPKN LNKSKTPAAP
cGATA-4 HTTTTTLWRR NAEGEPVCNA CGLYMKLHGV PRPLAMRKEG IQTRKRKPKN LNKTKTPAGP
mGATA-6 HTTTTTLWRR NAEGEPVCNA CGLYMKLHGV PRPLAMKKEG IQTRKRKPKN INKSKACSGN
rGATA-6 HTTTTTLWRR NAEGEPVCNA CGLYMKLHGV PRPLAMKKEG IQTRKRKPKN INKSKACSGN
hGATA-6 HTTTTTLWRR NAEGEPVCNA CGLYMKLHGV PRPLAMKKEG IQTRKRKPKN INKSKTCSGN
cGATA-6 HTTTTTLWRR NAEGEPVCNA CGLYMKLHGV PRPLAMKKEG IQTRKRKPKN INKSKACSGN
cGATA-5 HTTNTTLWRR NAEGEPVCNA CGLYMKLHGV PRPLAMKKES IQTRKRKPKN ITKGKTSTGS
mGATA-5 HTATTTLWRR NSEGEPVCNA CGLYMKLHGV PRPLAMKKES IQTRKRKPEN PAKIKGSSGS
hGATA-3 QTTTTTLWRR NANGDPVCNA CGLYYKLHNI NRPLTMKKEG IQTRNRKMSS KSKKCKKVHD
mGATA-3 QTTTTTLWRR NANGDPVCNA CGLYYKLHNI NRPLTMKKEG IQTRNRKMSS KSKKCKKVHD
cGATA-3 QTTTTTLWRR NANGDPVCNA CGLYYKLHNI NRPLTMKKEG IQTRNRKMSS KSKKCKKVHD
hGATA-2 QTTTTTLWRR NANGDPVCNA CGLYYKLHNV NRPLTMKKEG IQTRNRKMSN KSKKSKKGAE
mGATA-2 QTTTTTLWRR NANGDPVCNA CGLYYKLHNV NRPLTMKKEG IQTRNRKMSS KSKKSKKGAE
cGATA-2 QTTTTTLWRR NANGDPVCNA CGLYYKLHNV NRPLTMKKEG IQTRNRKMSN KSKKSKKGSE
mGATA-1 QTTTTTLWRR NASGDPVCNA CGLYFKLHQV NRPLTMRKDG IQTRNRKASG KGKK.KRGSN
rGATA-1 QTTTTTLWRR NASGDPVCNA CGLYYKLHQV NRPLTMRKDG IQTRNRKASG KGKK.KRGSS
hGATA-1 QTTTTTLWRR NASGDPVCNA CGLYYKLHQV NRPLTMRKDG IQTRNRKASG KGKK.KRGSS
cGATA-1 QTSTTTLWRR SPMGDPVCNA CGLYYKLHQV NRPLTMRKDG IQTRNRKVSS KGKK.RRPP.
Cons.   -T--TTLWRR ---G-PVCNA CGLY-KLH-- -RPL-M-K-- IQTR-RK--- --K-------
```

Figure 1.2. A) Schematic representation of a typical vertebrate GATA transcription factor. The CC CC indicates the zinc finger cysteines and the ++ indicates the basic region. B) Alignment of the N- and C-terminal zinc fingers and basic region of the vertebrate GATA factors. The two zinc fingers are underlined and their cysteines are in bold. Cons. consensus; c chicken; h human; m mouse, r rat.

Since their identification in TFIIIA, zinc fingers were found in a wide variety of other proteins (reviewed in (Schwabe and Klug, 1994)). These zinc fingers are mainly thought to be involved in macromolecular interactions. For example, the zinc finger of the steroid and thyroid hormone receptors are involved in DNA-binding (reviewed in (Pabo and Sauer, 1992)), the retroviral nucleocapsid protein zinc fingers are involved in RNA binding (Summers, 1991), and the RING zinc finger domains are involved in protein-protein interactions (reviewed in (Borden, 2000)).

3.2.1.1.2 The GATA DNA-binding C-terminal zinc finger

Numerous studies have shown that the minimal DNA-binding domain of the GATA factors consist of the C-terminal zinc finger core plus the adjacent basic region (Figure 1.2) (Martin and Orkin, 1990; Yang and Evans, 1992; Charron et al., 1999; Morrisey et al., 1997b). In addition to being essential for DNA-binding, this basic region is also thought to direct nuclear localization of GATA factors (Charron et al., 1999; Morrisey et al., 1997b). The solution structure of the minimal DNA-binding domain of chicken GATA-1 complexed to its binding site was determined by nuclear magnetic resonance (NMR) spectroscopy (Omichinski et al., 1993). The overall structure reveals that the C-terminal zinc finger core domain, which is composed of two antiparallel β sheets and an α helix, is located into the major groove. This zinc finger core is followed by a basic region that is composed of a long loop that wraps around the DNA and lies in the minor groove. Most of the specific contacts (7 out of 8) are between the zinc finger core and the major groove and are hydrophobic. The adjacent basic region interacts mainly with the minor groove sugar-phosphate backbone. The overall appearance is analogous to that of a right hand holding a rope, with the rope representing the DNA, the palm and fingers of the hand the zinc finger and the thumb the basic region.

The DNA-binding specificities of the GATA transcription factor family members GATA-1, GATA-2, GATA-3, and GATA-6 have been determined using polymerase chain reaction site selection (Ko and Engel, 1993; Merika and Orkin, 1993; Sakai et al., 1998). GATA-1, GATA-2, and GATA-3 were each shown to bind to the consensus site (A/T)GATA(A/G), with a preference for AGATAA (Ko and Engel, 1993). In addition, GATA-2 and GATA-3, but not GATA-1, were also able to bind

with high affinity to a AGATCT site. A similar study performed with GATA-6 revealed that it binds to a (A/T/C)GAT(A/T/C)A consensus, with a preference order of GATA > GATT > GATC (Sakai et al., 1998). These studies suggest that most GATA factors are capable of mediating transcriptional effects via a common (A/T)GATA(A/G) consensus site, but that some GATA factors may possess additional recognition properties. Indeed, work form our laboratory have shown that differential DNA-binding affinity is one of the mechanisms by which GATA factors regulate distinct downstream target genes: despite that GATA-4 and GATA-6 are co-expressed in cardiomyocytes, the α-MHC gene is preferentially regulated by GATA-4, due to higher affinity of GATA-4 for its promoter GATA element (Charron et al., 1999). These results will be presented and discussed in more details in Chapter II of this thesis.

In addition to serve as a DNA-binding domain, the C-terminal zinc finger is also essential for interactions with many co-factors, as will be discussed in the *Interaction with co-factors* section.

3.2.1.1.3 The GATA N-terminal zinc finger

The mammalian GATA proteins all contain a second N-terminal zinc-binding motif. This motif, which is encoded by a different exon from the C-terminal zinc finger motif, seems to have evolved by duplication of the C-terminal zinc finger motif since most GATA homologues found in non-vertebrate species contain only one zinc finger motif which bears more similarities to the C-terminal than the N-terminal zinc finger motif.

Although early studies focusing on the ability of GATA-1 to transactivate promoters in heterologous cells indicated that the N-terminal zinc finger was not essential (Martin and Orkin, 1990), more physiologically relevant experiments investigating the ability of GATA-1 to induce erythroid differentiation have revealed a critical role for this zinc finger (Weiss et al., 1997).

The solution structure of the N-terminal zinc finger domain of murine GATA-1 was determined by NMR spectroscopy (Kowalski et al., 1999). The overall structure reveals that the backbone fold of the N-terminal zinc finger domain is very similar to that of the C-terminal zinc finger core domain. Although the precise role of this

N-terminal zinc finger motif is controversial, it is clearly different from the C-terminal zinc finger motif. This suggests that the differences in function are due to specific contacts made by functional groups on the side chains of the two fingers. Three functions, which may not necessarily be exclusive of each other, have been attributed to this domain.

3.2.1.1.3.1 Protein-protein interaction

A first function of the N-terminal zinc finger is to mediate protein-protein interaction. Zinc fingers are known for their capacity to interact with other proteins (reviewed in (Mackay and Crossley, 1998a)). For example, the GATA finger-related N-terminal finger of GR mediates homodimerization (Luisi et al., 1991); the transcription factors YY1 and Sp1 interact via their zinc finger domains (Seto et al., 1993); and the zinc finger region of the E1A transactivation domain binds to the TATA box binding protein (TBP) (Geisberg et al., 1994).

For the GATA proteins, it has been shown that the N- and the C-terminal zinc fingers of GATA-1 are able to mediate homodimerization (Crossley et al., 1995). Further studies indicated that the N-terminal zinc finger of GATA-1 does not homodimerize but instead makes intermolecular contacts with the C-terminal zinc finger, suggesting that GATA-1 dimers are maintained by reciprocal N-finger-C-finger contacts (Mackay et al., 1998). Importantly, they show that mutations that impair GATA-1 self-association reduce its ability to activate transcription, especially on promoters containing multiple GATA sites.

Within the last three years a new zinc finger protein family was identified: the Friend of GATA (FOG)/U-shaped (Ush) family (Tsang et al., 1997; Haenlin et al., 1997; Lu et al., 1999; Svensson et al., 1999; Tevosian et al., 1999; Holmes et al., 1999). The founding member of this family, FOG-1 (Friend of GATA-1), was cloned by the yeast two-hybrid system using the N-terminal zinc finger of GATA-1 as a bait (Tsang et al., 1997). As FOG-1 interacts with GATA-1 but not with a mutant lacking the N-terminal zinc finger, it is the first protein found to specifically interact with the N-terminal zinc finger of a GATA factor. Subsequently, the GATA-1 N-terminal zinc finger residues essential for interaction with FOG-1 were identified (Fox et al., 1998). Interestingly, these residues are conserved in the N-terminal

zinc fingers of all GATA proteins known to bind FOG-1, but are not found in the C-terminal zinc fingers, suggesting that these residues account for the particular specificity of FOG-1 for the N-terminal zinc fingers. Moreover, when mapped onto the GATA-1 N-terminal zinc finger three-dimensional structure, these residues form a contiguous surface which is non-overlapping with the putative DNA-binding surface of the N-terminal zinc finger (see below), likely allowing simultaneous DNA-binding and interaction with FOG-1 (Fox et al., 1998; Kowalski et al., 1999). As will be discussed in the *Interaction with co-factors* section, this interaction is essential for synergistic activation of transcription and potentiation of erythroid and megakaryocytic differentiation by GATA-1 and FOG-1.

3.2.1.1.3.2 DNA-binding specificity

A second function of the N-terminal zinc finger is to enhance DNA-binding specificity and stability of the GATA factors (Martin and Orkin, 1990; Whyatt et al., 1993; Yang and Evans, 1992; Trainor et al., 1996). More specifically, Trainor et al. found that the N-terminal zinc finger is required to bind with high affinity a subset of GATA elements harboring the ATC(A/T)GATA(A/G) sequence. These particular GATA elements consist of a consensus GATA element (A/T)GATA(A/G) fused to a partial inverted GATA element GAT (underlined). In these "palindromic" GATA elements, like in every other GATA elements identified, the C-terminal zinc finger binds the (A/T)GATA(A/G) sequence. In addition, in the special case of a palindromic GATA element, the N-terminal zinc finger is involved in binding the partial inverted GAT sequence. These palindromic GATA elements occur very infrequently in vertebrate genes known to be regulated by GATA factors, although such a GATA element is present in the GATA-1 promoter and is conserved amongst species, suggesting functional relevance. Moreover, mutation of the partial GATA sequence significantly decreases GATA-1 promoter activity. These results suggest that the N-terminal zinc finger could be involved in distinguishing between subsets of GATA elements found in various promoters and thereby bringing differential regulation to various GATA-1 targets. For example, the GATA-1 gene is thought to be positively autoregulated once it is activated (Hannon et al., 1991; Tsai et al., 1991). Therefore, the presence of this high affinity GATA element

in the GATA-1 promoter could favor GATA-1 activation and/or autoregulation over the transcription of other targets at a stage when GATA-1 is limiting.

3.2.1.1.3.3 Independent DNA-binding

The studies described above argue that although in some cases the N-terminal zinc finger can contribute to specificity and affinity, it does not bind DNA independently, whereas the C-terminal zinc finger is both necessary and sufficient for binding. However, a study from Pedone et al. showed that the N-terminal zinc finger of GATA-2 and GATA-3 are capable of independent high affinity binding with a preference for the motif GATC; this property is not shared by the N-terminal zinc finger of GATA-1 (Pedone et al., 1997). These results suggest that the DNA-binding properties of the N-terminal zinc finger may help distinguish GATA-2 and GATA-3 from GATA-1 and the other family members and contribute to their functional specificity.

3.2.1.2 The N- and C-terminal transactivation domains

In addition to the two central zinc finger domains, the vertebrate GATA factors are also composed of N- and C-terminal transactivation domains showing low to modest conservation among GATA family members (Figure 1.2). Both the N- and C-terminal domains of GATA-1 are required for maximal transcriptional activity (Martin and Orkin, 1990; Yang and Evans, 1992). The N-terminal GATA-1 activation domain (aa 1-66) is an acidic and serine-rich domain with sequence homology to other acidic activation domains such as that present in the herpes virus transcriptional activating protein VP16 (Mitchell and Tjian, 1989). In contrast, the transcriptional activation domain identified within GATA-3 (aa 30-74) has a neutral charge and shares little sequence homology to previously described transcriptional activation domains, including those within other GATA factors (Yang et al., 1994). Finally, in addition to the C-terminal domain, two transcriptional activation domains were identified in the N-terminal domain of GATA-4 (aa 1-74 and aa 130-177) (Morrisey et al., 1997b; Arceci et al., 1993; Durocher et al., 1997; Charron et al., 1999). These two domains are not present in the GATA-1, GATA-2, and GATA-3 subfamily, but are conserved in the GATA-4, GATA-5, and GATA-6

subfamily, suggesting a conserved mechanism of transcriptional activation within the GATA-4, GATA-5, and GATA-6 subfamily.

3.2.2 Tissue distribution and roles of the GATA transcription factors

The family of GATA proteins can be divided into two subgroups based on tissue distribution and sequence homology (Figure 1.1). The first subgroup comprises GATA-1, GATA-2, and GATA-3, which are prominently expressed in hematopoietic cells. The second subgroup of GATA factors comprises GATA-4, GATA-5, and GATA-6, which are expressed in various mesoderm- and endoderm-derived tissues such as heart, liver, lung, gonad, and gut. The conservation of the expression patterns among orthologs and across species suggests that each GATA factor has conserved properties and roles during development and in adults. The roles of these factors and their expression pattern will be discussed in details in the following sections.

3.2.2.1 GATA-1

In the hematopoietic system, GATA-1 expression is restricted to multipotent hematopoietic progenitor cells, erythrocytes, mast cells, and megakaryocytes (reviewed in (Simon, 1995)). Inactivation of the murine *Gata1* gene results in embryonic lethality between day 10.5 and 11.5 (E10.5-E11.5) of gestation from anemia (Pevny et al., 1991; Fujiwara et al., 1996). Although GATA-1 is expressed in multipotential progenitor cells prior to their commitment, disruption of the *Gata1* gene results in maturation arrest relatively late in both erythroid and megakaryocytic development (Pevny et al., 1991; Fujiwara et al., 1996; Pevny et al., 1995). Indeed, proerythroblasts, but not erythrocytes, are found in these mutant embryos. Moreover, during *in vitro* differentiation of GATA-1 null cells, definitive erythroid precursors are also arrested at the proerythroblast stage and undergo premature cell death by apoptosis (Weiss et al., 1994; Simon et al., 1992; Weiss and Orkin, 1995). These results show that GATA-1 is essential for late erythroid cell differentiation and survival.

In addition to the globin genes, GATA-1 activates the promoter of many other erythroid genes, such as porphobilinogen deaminase (PBG-D), glycophorin B, erythropoietin receptor (Epo-R), SCL/Tal-1, and GATA-1 itself (Mignotte et al.,

1989a; Mignotte et al., 1989b; Rahuel et al., 1992; Zon et al., 1991b; Heberlein et al., 1992; Chiba et al., 1991; Aplan et al., 1990; Tsai et al., 1991). Surprisingly, putative GATA-1 targets such as the globins and other genes whose promoter are activated *in vitro* by GATA-1 are still expressed in GATA-1 null proerythroblasts (Weiss et al., 1994). Since GATA-2 levels in these arrested cells are highly upregulated compared to normal proerythroblasts, it has been proposed that partial compensation by GATA-2 may account for expression of GATA target genes and the initiation of erythroid development to the proerythroblast stage (Weiss et al., 1994). This possibility needs to be tested by examining embryos lacking both GATA-1 and GATA-2.

Outside the hematopoietic system, GATA-1 is also expressed in the Sertoli cells of the testis, starting at the prepubertal stage and lasting during adulthood (Ito et al., 1993; Yomogida et al., 1994; Viger et al., 1998), where it may control target genes like the inhibin α-subunit promoter (Feng et al., 1998). However, the precise role of GATA-1 in postnatal gonads remains to be investigated.

3.2.2.2 GATA-2

In the hematopoietic system, GATA-2 has a distinct but overlapping expression pattern with GATA-1: GATA-2 is expressed in multipotent hematopoietic progenitor cells, immature erythroid cells, mast cells, and megakaryocytes (Simon, 1995). Disruption of the *Gata2* gene results in embryonic lethality at E10-E11, due to anemia (Tsai et al., 1994). Analysis of chimeras generated with GATA-2 null ES cells and *in vitro* differentiation of GATA-2 null ES cells revealed a decrease in multipotent progenitor cells, mainly due to poor proliferative capacity of the GATA-2 mutant cells. This proliferative defect of the progenitors has consequences on all blood cell lineages as erythroid, myeloid, and lymphoid lineages are drastically reduced. Nevertheless, the cells produced appear normally differentiated. Consistent with a potential role in cell proliferation or the choice between self-renewal and differentiation, forced expression of GATA-2 in progenitor cells blocks erythroid maturation (Briegel et al., 1993; Persons et al., 1999). This proliferative capacity of GATA-2 may reflect its ability to regulate the expression of genes that control growth factor responsiveness or its role as a nuclear mediator of growth

factor signal transduction pathways. The role of GATA-2 in the regulation of cell proliferation is evidenced in other cell types such as preadipocytes, where its downregulation correlates with the transition from preadipocytes to adipocytes (Tong et al., 2000). Consistent with a role in promoting proliferation, constitutive expression of GATA-2 (or GATA-3) suppresses adipocyte differentiation, at least in part, through the direct inhibition of the peroxisome proliferator-activated receptor γ (PPARγ) gene expression.

GATA-2 is also expressed in endothelial cells and in the embryonic brain (Lee et al., 1991; Nardelli et al., 1999; Zhou et al., 2000; Bell et al., 1999). However, the role of GATA-2 in these tissues remains elusive.

3.2.2.3 GATA-3

GATA-3 is expressed in a variety of tissues including embryonic brain, embryonic liver, placenta, kidney, adrenal glands, endothelial cells, adipocyte precursors, adult central nervous system (CNS), and adult peripheral nervous system (PNS) (Simon, 1995; George et al., 1994; Tong et al., 2000; Oosterwegel et al., 1992). In the hematopoietic system, GATA-3 is restricted to T lymphocytes and natural killer (NK) cells (Ho et al., 1991)(reviewed in (Kuo and Leiden, 1999)). Disruption of the murine *Gata3* gene is embryonic lethal at E11-E12 and mice display massive internal bleeding, marked growth retardation, severe deformities of the brain and spinal cord, and gross aberrations in fetal liver hematopoiesis (Pandolfi et al., 1995). Further experiments using chimeras between ES cells from RAG-2 null mice (which do not produce mature B and T cells) and GATA-3 null mice clearly demonstrated that GATA-3 is essential for early T lymphocyte development as these mice fail to give rise to thymocytes and mature peripheral T cells (Ting et al., 1996). In addition to serving as a critical regulator of early T cell development, a pivotal role for GATA-3 in $CD4^+$ T helper 2 (Th2) differentiation and Th2-specific cytokine (IL-4, IL-5, IL-6, IL-10, and IL-13) gene expression has been demonstrated *in vitro* and *in vivo* (Zhang et al., 1997; Zheng and Flavell, 1997; Ouyang et al., 1998; Ouyang et al., 2000). Finally, a novel role for GATA-3 in T-cell survival and homing to secondary lymphoid organs was recently proposed

(Yamagata et al., 2000a). Taken together, these results suggest that GATA-3 regulates multiple stages of T cell differentiation and T cell function.

In addition to cytokine genes, GATA-3 is also thought to regulate many other T-cell specific genes, such as the T-cell receptor α, β, and δ (TCR- α, β, and δ) (Leiden, 1993), CD8α (Landry et al., 1993), CD4 (Wurster et al., 1994), and interferon-γ (Penix et al., 1993), consistent with its essential role in T cell function.

The role of GATA-3 in Th2 cytokine gene expression may have pharmacological implications (reviewed in (Ray and Cohn, 1999)). Since Th2 cytokines play a crucial role in the pathogenesis of asthma, it was hypothesized that inhibition of their expression would prevent, or at least partially block, asthma. Indeed, expression of a dominant negative mutant of GATA-3 in T cells led to a reduction in the levels of Th2 cytokines and attenuated key features of asthma, such as airway eosinophilia, mucus production, and IgE synthesis (Zhang et al., 1999).

As described in the *GATA-2* section, GATA-3 is also co-expressed with GATA-2 in adipocyte precursors where it appears to regulate adipocyte differentiation by controlling the preadipocyte to adipocyte transition (Tong et al., 2000).

Recently, a role for GATA-3 in the nervous system has also been shown (Lim et al., 2000). *Gata3* null mice have reduced accumulation of tyrosine hydroxylase (Th) and dopamine β-hydroxylase (Dbh) transcripts, leading to reduced noradrenaline biosynthesis in the sympathetic nervous system. Feeding pregnant mice with catechol intermediates partially rescues the embryonic lethality, showing that noradrenaline deficiency is a cause of death in *Gata3* null embryos.

Finally, GATA-3 haplo-insufficiency was found to cause the hypoparathyroidism, sensorineural deafness, and renal anomaly (HDR) syndrome (Van Esch et al., 2000), implicating GATA-3 in the development of the parathyroids, auditory system, and kidneys.

3.2.2.4 GATA-4

Analysis of cardiac-specific promoters led to the cloning of an additional member of the GATA family, GATA-4, which is expressed in the heart (Grépin et al., 1994)(reviewed in (Charron and Nemer, 1999); see the *Appendices* for a reprint). GATA-4 is expressed in the precardiogenic mesoderm and continues to be

expressed throughout the myocardium and endocardium, where it persists at all stages of heart development (Grépin et al., 1994; Laverriere et al., 1994; Arceci et al., 1993; Jiang and Evans, 1996; Heikinheimo et al., 1994). Transfection studies established that GATA-4 is a potent transactivator of numerous cardiac promoters (Grépin et al., 1994; Durocher et al., 1997; Charron et al., 1999; Ip et al., 1994; Murphy et al., 1997; Di Lisi et al., 1998; Rosoff and Nathanson, 1998; Wang et al., 1998; Thuerauf et al., 1994; Molkentin et al., 1994; Nicholas and Philipson, 1999; Cheng et al., 1999; Rivkees et al., 1999). Moreover, ectopic expression of GATA-4 *in vivo* was shown to activate the transcription of cardiac contractile genes (Jiang and Evans, 1996). These properties are consistent with a key role for GATA-4 in cardiac transcription.

The first evidence for a role of GATA-4 in heart differentiation came from studies in the pluripotent P19 embryonic carcinoma cell line, which provides a model of cardiac differentiation. P19 cells expressing GATA-4 antisense transcripts were unable to achieve terminal cardiac differentiation; conversely, overexpression of GATA-4 in P19 cells markedly potentiated cardiomyocyte differentiation (Grépin et al., 1997; Grépin et al., 1995). Consistent with an important role for GATA-4 in the heart, mice homozygous for a null mutation in the *Gata4* gene are not viable due to the inability of the bilateral cardiac primordia to fuse and form the heart tube (Kuo et al., 1997; Molkentin et al., 1997). Nevertheless, cardiomyocytes expressing differentiation markers are detectable in these mice, raising the possibility that other members of the GATA family may compensate, at least in part, for GATA-4-deficiency during early cardiac development. The roles of GATA-4 in the heart will be further discussed in the *Cardiac development* section.

GATA-4 is also expressed in Sertoli cells of the testis (Arceci et al., 1993; Grépin et al., 1994; Viger et al., 1998; Feng et al., 1998), where its expression is maintained throughout embryonic development and stops at prepubertal stage, coincident with the onset of GATA-1 expression in these cells. Moreover, GATA-4 is expressed in a sexually dimorphic pattern and transactivates, in cooperation with the nuclear receptor SF-1, the Mullerian inhibiting substance promoter, suggesting that it might

be involved in early gonadal development and sex differentiation (Viger et al., 1998; Tremblay and Viger, 1999).

GATA-4 is also expressed, albeit at lower level, in the lung, extraembryonic visceral and parietal endoderm, and embryonic endodermal derivatives, such as the gut and the liver (Arceci et al., 1993; Morrisey et al., 1996; Laverriere et al., 1994). A role for GATA-4 in extraembryonic endoderm formation is supported by the finding that inactivation of *Gata4* in embryonic stem (ES) cells results in a specific block in visceral endoderm differentiation *in vitro* (Soudais et al., 1995). As mentioned, mice homozygous for a null mutation in the *Gata4* gene present an inability of the bilateral cardiac primordia to fuse and form the heart tube (Kuo et al., 1997; Molkentin et al., 1997). The *cardia bifida* phenotype may be due to absence of ventral closure of the embryo and subsequent malformation of the foregut, resulting in the absence of morphogenetic movements required for the fusion of the bilateral primordia. Accordingly, studies with chimeric mice injected with GATA-4 null ES cells further suggest that the cardiac defect is likely due to an endodermal defect (Kuo et al., 1997; Narita et al., 1997). Taken together, these studies suggest a role for GATA-4 in embryonic and extraembryonic endoderm development.

Based on their expression pattern, GATA-4, as well as GATA-5 and GATA-6, have been implicated in the regulation of epithelial cell differentiation in the gut: GATA-6 might function within the proliferating progenitor population, while GATA-4 and GATA-5 might play a role during differentiation to induce terminal-differentiation genes, such as the ones encoding the intestinal fatty acid-binding protein (IFABP), H^+/K^+ ATPase, and the trefoil factor family (Gao et al., 1998; Tamura et al., 1993; Nishi et al., 1997; Al azzeh et al., 2000). Support for a role of GATA factors in the vertebrate gut comes from work in *C. elegans*, where the GATA factor elt-2 is critical for terminal gut differentiation and has been shown to be able to induce ectopic gut gene expression (Fukushige et al., 1998). These results suggest an evolutionary conserved role for GATA factors in gut development between vertebrates and invertebrates (reviewed in (Zaret, 1999)).

Finally, a role for GATA-4 and GATA-6 in the regulation of liver-specific gene expression has been suggested from analysis of the albumin, vitellogenin II, and liver-enriched homeobox (Hex) promoters (Bossard and Zaret, 1998; Davis and Burch, 1996; Denson et al., 2000).

3.2.2.5 GATA-5

The expression of GATA-5 is very dynamic both spatially and temporally: it is expressed in the heart, outflow tract, allantois, lung bud, urogenital ridge, bladder, and gut epithelium during development, and in the small intestine, stomach, bladder, and lungs in the adult (Morrisey et al., 1997a; Laverriere et al., 1994; Nemer et al., 1999). In the heart, GATA-5 is initially expressed in the precardiac mesoderm and developing cardiomyocytes, but then becomes progressively restricted to the endocardium (Laverriere et al., 1994; Morrisey et al., 1997a; Jiang and Evans, 1996).

GATA-5 is a potent transactivator of GATA-dependent cardiomyocyte and endocardial gene promoters (Nemer et al., 1999; Morimoto et al., 1999; Morrisey et al., 1997a) and its ectopic expression in *Xenopus* induces the transcription of cardiac contractile genes (Jiang and Evans, 1996). Loss-of-function studies in an *in vitro* culture model revealed an essential role for GATA-5 in endocardial differentiation that could not be compensated by GATA-4 or GATA-6 (G. Nemer and M. Nemer, unpublished results). These properties are consistent with a role for GATA-5 in cardiomyocyte and endocardial cell differentiation and transcription.

Targeted disruption of the *Gata5* gene in mouse results in females presenting defects in genitourinary tract development (Molkentin et al., 2000a), consistent with its expression in the urogenital ridge (Morrisey et al., 1997a). However, it remains to be defined whether the *Gata5* disruption performed effectively results in *Gata5* null embryos. In sharp contrast to the mild phenotype obtained in *Gata5* null mice, in zebrafish, mutation of the *Gata5* gene causes embryonic lethality and results in *cardia bifida* and gut defects (Reiter et al., 1999), similar to the effect of the inactivation of the *Gata4* gene in mice (Kuo et al., 1997; Molkentin et al., 1997). In addition, these animals display a decrease in the number of myocardial precursors, a reduction of expression of several myocardial genes, including Nkx2-5, GATA-4,

ventricular myosin heavy chain, cardiac troponin T, tropomyosin, cardiac myosin light chain 1, and cardiac myosin light chain 2. Conversely, overexpression of GATA-5 induces the ectopic expression of several myocardial genes and beating cardiomyocytes (Reiter et al., 1999). These results implicate GATA-5 in controlling the growth, morphogenesis, and differentiation of the heart.

Together with the role of GATA-5 in gut gene expression discussed in the previous section, a key role for GATA-5 in endoderm development was recently confirmed in *Xenopus*, where ectopic expression of GATA-5 re-specifies ectodermal and mesodermal cells towards an endodermal fate (Weber et al., 2000). The capacity of GATA-5 to convert all germ layers to endoderm is reminiscent of the role of the GATA factor end-1, which is necessary and sufficient to specify endoderm precursors in *C. elegans* (Zhu et al., 1997; Zhu et al., 1998). These results suggest an evolutionary conserved role for GATA factors in endoderm development in vertebrates and invertebrates.

3.2.2.6 GATA-6

The expression of GATA-6 is also very dynamic: it is expressed in the primitive streak, allantois, visceral endoderm, heart, lung buds, urogenital ridge, vascular smooth muscle, and the epithelial layer of the stomach, small intestine, and large intestine during development, and in the heart, aorta, stomach, small intestine, bladder, liver, and lungs in the adult (Morrisey et al., 1996; Suzuki et al., 1996; Narita et al., 1996; Huggon et al., 1997; Laverriere et al., 1994; Gove et al., 1997). In the heart, GATA-6 is initially expressed in the precardiogenic mesoderm and continues to be expressed throughout the myocardium, where it persists at all stages of heart development (Morrisey et al., 1996; Jiang and Evans, 1996; Gove et al., 1997).

GATA-6 is a potent transactivator of GATA-dependent cardiac promoters (Charron et al., 1999; Nemer et al., 1999; Morrisey et al., 1996) and, like GATA-4 and GATA-5, its ectopic expression in *Xenopus* embryos induces the transcription of cardiac contractile genes (Jiang and Evans, 1996). Other gain-of-function studies in *Xenopus* revealed that GATA-6 could also be a regulator of the cardiogenic field (Gove et al., 1997). Injection of GATA-6, but not GATA-1, mRNA in gastrulating

embryos resulted in a transient block of cardiac differentiation and enhanced proliferation of cardioblasts; after the decay of the injected GATA-6 mRNA, cardiomyocytes resumed differentiation to generate an enlarged heart. This is reminiscent of the proliferative effect of GATA-2 in hematopoietic progenitors (Briegel et al., 1993) and suggests that GATA-6 might regulate proliferation of cardiac progenitor cells. Unfortunately, inactivation of the *Gata6* gene results in early embryonic lethality (E6.5-E7.5), precluding analysis of GATA-6 function in heart development (Morrisey et al., 1998; Koutsourakis et al., 1999).

Nonetheless, analysis of these mutant mice revealed a requirement for GATA-6 in the formation of the extraembryonic visceral endoderm (Morrisey et al., 1998), a result consistent with the expression of GATA-6 in this tissue (Morrisey et al., 1996). In chimeric mice experiments, *Gata6* null ES cells did not contribute to the endodermally derived bronchial epithelium (Morrisey et al., 1998). Moreover, GATA-6 transactivates the thyroid transcription factor-1 (TTF-1) and surfactant protein A (SP-A) promoters, two genes which are expressed in the respiratory epithelium (Bruno et al., 2000; Shaw-White et al., 1999). These results are consistent with a critical role for GATA-6 in endoderm development and respiratory epithelium formation.

In vascular smooth cells, GATA-6 plays an important role in modulating cell proliferation in response to mechanical or mitogenic stimulation (reviewed in (Morrisey, 2000)). The finding that GATA-6 transcript levels are decreased in proliferating vascular smooth muscle cells suggests that GATA-6 expression may inhibit the cell cycle (Suzuki et al., 1996). Accordingly, forced expression of GATA-6 in vascular smooth cells induces the expression of the cyclin-dependent kinase inhibitor p21 and provokes cell cycle arrest (Perlman et al., 1998). *In vivo*, gene transfer of GATA-6 in balloon-injured carotid arteries promoted smooth muscle differentiation and inhibited intimal hyperplasia (Mano et al., 1999). These studies suggest that GATA-6 inhibits vascular smooth muscle cell proliferation and subsequent vascular injury. It will be interesting to determine whether GATA-6 also controls the cell cycle in other tissues.

3.2.3 Regulation of GATA factors

In addition to be expressed in the appropriate spatio-temporal manner, transcription factors are also finely regulated at many other levels. This "fine tuning" of transcription factor activity allows an organism to respond to developmental signals and environmental stimuli. Accordingly, in addition to their tissue- and time-specific expression pattern, GATA factors have also been shown to be regulated at many other levels, allowing them to play specific roles in some cells, but not in others. In this section, the various transcriptional, translational, post-translational, and protein-protein interaction mechanisms used to regulate GATA factor activity will be described in details.

3.2.3.1 Tissue-specific promoters and alternative promoter usage

GATA transcription factors are expressed in a very dynamic pattern, both spatially and temporally. To achieve such a complex expression pattern, many organisms have elaborated highly sophisticated enhancer and promoter regulatory elements for GATA genes. Even though the regulation of GATA gene promoter activity is still poorly understood, a general theme appears to be the utilization of tissue-specific and alternative promoters to control their expression in various tissues.

The best-characterized regulatory regions of a GATA gene are the flanking sequences of GATA-1. Two promoters differentially regulate the GATA-1 gene: the distal promoter specifies the expression of the GATA-1 gene in Sertoli cells, whereas the proximal promoter directs GATA-1 gene expression in the hematopoietic lineages (Ito et al., 1993). The use of more than one promoter to achieve the required tissue-specific expression is not restricted to GATA-1, as GATA-2, GATA-5, and GATA-6 transcription is also controlled by at least two different promoters, which appear to control differential spatial and temporal gene expression (Brewer et al., 1999; Nony et al., 1998; MacNeill et al., 1997). However, the exact promoter fragments required for their tissue-specific expression are not clearly defined and may encompass very large genomic regions, further complicating their analysis. As an example, even a 625 kbp GATA-3 yeast artificial chromosome (YAC) containing 450 kbp and 150 kbp of 5' and 3' flanking sequences, respectively, does not contain the full transcriptional regulatory potential of the endogenous GATA-3 locus (Lakshmanan et al., 1999).

Not only are the GATA genes promoter regions poorly defined, but their *cis*-regulatory elements and their cognate transcription factors are even less known. Again, the regulation of the erythroid GATA-1 promoter is the best understood: the critical regulatory region that recapitulates GATA-1 gene expression in both primitive and definitive erythroid cells consists of 3.9 kbp of the proximal promoter (Onodera et al., 1997). The regulation of this promoter is characterized by a positive feedback loop where GATA-1 regulates its own promoter (Tsai et al., 1991; Nicolis et al., 1991; Schwartzbauer et al., 1992; Meng et al., 1999; Nishimura et al., 2000). In addition, a CACCC element in the proximal promoter region is critical for initiating and maintaining high level of GATA-1 expression, suggesting that a member of the Kruppel-like factor family, such as EKLF, might regulate GATA-1 promoter (Meng et al., 1999; Tsai et al., 1991). However, given that EKLF is itself regulated by GATA-1 (Anderson et al., 2000), these results do not explain the erythroid expression of GATA-1. In the heart, the cardiac promoter of GATA-6 appears to be regulated by the cardiac-specific homeodomain transcription factor Nkx2-5 (Davis et al., 2000; Molkentin et al., 2000b) and the Nkx2-5 promoter was reciprocally found to be regulated by GATA factors (Lien et al., 1999; Reecy et al., 1999; Searcy et al., 1998). Although these results suggest that mutually reinforcing networks regulate GATA transcription factor expression, they do not explain the tissue-specific expression of GATA factors. Thus, much work remains to be done before we can understand the tissue-specific transcriptional regulation of GATA gene expression in sufficient details.

3.2.3.2 Alternative first exon usage

Alternative promoter usage leads to transcripts characterized by alternative first exons. Splicing of these alternative first exons will, in some case, lead to transcripts that will encode functionally distinct isoforms. For example, in the case of GATA-5, the use of an alternative first exon produces a GATA-5 isoform which is composed only of a C-terminal zinc finger and a C-terminal transactivation domain (MacNeill et al., 1997). Although the physiological role of this isoform remains to be determined, *in vitro* assays revealed that it is severely compromised in its transcriptional activation capacity.

3.2.3.3 Alternative translation initiation codon usage

Distinct isoforms of GATA factors are also generated by the use of alternative translation initiation codons. Calligaris et al. reported that at least two isoforms of GATA-1 exist in erythroid cells: in addition to the full length GATA-1, a second GATA-1 isoform results from alternative translation initiation site usage (Calligaris et al., 1995). This new GATA-1 isoform lacks 83 amino acids in the N-terminal transactivation domain and shows a decreased transactivation potential. GATA-6 also possesses two alternative translation initiation codons which result in two GATA-6 isoforms having different transcriptional properties (Brewer et al., 1999). While the shorter isoform corresponds to the GATA-6 protein originally identified, a longer and more active isoform is produced from an upstream AUG initiator codon and leads to a GATA-6 isoform containing a 146 aa-extended N-terminal domain. It is possible that the shorter, less active GATA-1 and GATA-6 isoforms serve as dominant negative proteins which downregulates the longer, more active GATA factors.

3.2.3.4 Phosphorylation

Phosphorylation has been shown to play a crucial role in modulating the activity of transcription factors (reviewed in (Hunter and Karin, 1992)). In murine erythroleukemia (MEL) cells, GATA-1 is phosphorylated on 6 serines within its N-terminal transactivation domain (Crossley and Orkin, 1994). Induction of MEL cell differentiation induces phosphorylation at an additional site, serine 310, which lies in the basic region adjacent to the C-terminal zinc finger. This serine is conserved in mammalian, avian, and amphibian GATA-1 proteins and serves to make direct hydrophobic and hydrophilic contacts with the sugar-phosphate DNA backbone (Omichinski et al., 1993). However, individual or combined mutation of the constitutively or inducibly phosphorylated residues does not affect DNA-binding affinity or specificity, DNA bending, nuclear translocation, or transcriptional activation by GATA-1. In contrast, another group reported that GATA-1 phosphorylation increases during differentiation of the human erythroleukemic cell line K562 and that phosphorylation induces the DNA-binding affinity of GATA-1

(Partington and Patient, 1999). The reasons for these discrepancies are not clear, but may be due to cell type differences.

GATA-2 and GATA-3 are also phosphoproteins (Towatari et al., 1995; Chen et al., 2000a). Stimulation of hematopoietic progenitors with interleukin-3 (IL-3) increases GATA-2 phosphorylation via the ERK MAPK signaling pathway (Towatari et al., 1995). In Th2 cells, cAMP stimulates the phosphorylation of GATA-3, likely by p38 MAPK (Chen et al., 2000a). However, it remains to be determined what is the effect of these induced phosphorylation on GATA-2 and GATA-3 functions. While the role(s) of GATA factor phosphorylation awaits further characterization, it will be interesting to test whether phosphorylation modulates the capacity of GATA factors to associate with other transcription factors.

3.2.3.5 Acetylation

Post-translational modification of histones by acetylation has been intensively studied (reviewed in (Cheung et al., 2000)). However, the discovery of the role of acetylation in the regulation of other types of proteins, such as transcription factors, is relatively recent (reviewed in (Kouzarides, 2000)). Both GATA-1 and GATA-3 have been reported to be acetylated *in vivo* (Hung et al., 1999; Boyes et al., 1998; Yamagata et al., 2000b).

Studies by two independent groups showed that CBP and the CBP-related protein p300, which both possess intrinsic and associated histone acetyltransferase activity (Bannister and Kouzarides, 1996; Ogryzko et al., 1996; Chen et al., 1997; Yang et al., 1996; Spencer et al., 1997), acetylate GATA-1 *in vitro* at the same N- and C-terminal zinc finger lysines which are also acetylated *in vivo* (Hung et al., 1999; Boyes et al., 1998). However, the effect of GATA-1 acetylation on its DNA-binding properties are presently uncertain, with one group reporting that acetylation does not alter DNA-binding and the other reporting that acetylation enhances DNA-binding. Nevertheless, mutation of the acetylation motifs abrogates GATA-1 ability to induce erythroid differentiation, suggesting that acetylation of GATA-1 contributes to regulate its activity.

GATA-3 is acetylated *in vivo* in T cells (Yamagata et al., 2000b). *In vitro*, p300 acetylates GATA-3 at sites mapping to the N- and C-terminal zinc finger domains.

KRR-GATA-3 is a GATA-3 mutant which is hypoacetylated and which functions as a dominant negative construct (Yamagata et al., 2000b; Smith et al., 1995). Expression of KRR-GATA-3 in peripheral T cells results in defective T-cell homing to systemic lymph nodes and prolonged T cell survival after activation, suggesting that acetylation regulates GATA-3 function in the immune system.

Together, these studies indicate that GATA factors are regulated by acetylation. Given that the acetylation sites identified in GATA-1 and GATA-3 are conserved in GATA-2, but not in GATA-4, GATA-5, and GATA-6, these results might suggest a common, specific mode of regulation for the "hematopoietic" GATA factors; conversely, other yet undefined acetylation motifs may exist in GATA-4, GATA-5, and GATA-6 and contribute to regulate their activity.

3.2.3.6 Cleavage by proteases

The importance of proteolysis as a mean of regulating protein activity is now well established (reviewed in (King et al., 1996)). Two GATA factors, GATA-1 and GATA-6, have been reported to be regulated by proteases, albeit by different mechanisms (De Maria et al., 1999; Nakagawa et al., 1997).

The death receptors are transmembrane receptors mediating activation of a specialized group of intracellular proteases named caspases (reviewed in (Green, 1998)). These caspases play a key role in apoptosis by cleaving specific substrates that mediate cell death. The current model accounting for the tight regulation of mature erythrocyte number stipulates that mature erythroblasts participate in a negative-feedback loop to inhibit the formation of mature erythrocytes by activating the Fas death receptor/Fas ligand (Fas/FasL) system in immature erythroblasts (Orkin and Weiss, 1999). De Maria et al. showed that activation of the Fas/FasL death receptor system in erythroid cells leads to selective cleavage and inactivation of GATA-1 and erythrocyte maturation block (De Maria et al., 1999), consistent with the requirement of a functional GATA-1 for erythrocyte maturation. Importantly, expression of a caspase cleavage-resistant form of GATA-1 or treatment with caspase inhibitors restored maturation of Fas/FasL activated cells. These results suggest that caspase-mediated cleavage of GATA-1 is an important control mechanism of erythropoiesis. It will be

interesting to determine whether caspase-mediated cleavage targets other GATA factors in other cellular contexts.

Stimuli which increase the intracellular cAMP concentration trigger the specific degradation of GATA-6 (Nakagawa et al., 1997). In contrast to GATA-1 proteolysis, this effect is mediated by the proteasome and occurs in a protein kinase A (PKA)-dependent manner. Further studies are required to establish the physiological relevance of this observation and whether this is a general aspect of GATA factor regulation.

3.2.3.7 Interaction with co-factors

Gene inactivation experiments of the various GATA factors showed that these proteins each play a unique role and cannot compensate for each other during development, although their expression pattern overlaps in certain tissues. How this uniqueness is acquired at the molecular level remains to be answered. Differences in DNA-binding specificity for GATA elements may play a role, but cannot account for all of the specific functions of GATA factors *in vivo* (Ko and Engel, 1993; Merika and Orkin, 1993; Sakai et al., 1998; Gregory et al., 1996). One hypothesis is that specificity amongst GATA proteins is acquired by differential interactions with co-factors. This section will describe how protein-protein interactions, either with DNA-binding or non-DNA-binding co-factors, may regulate the activity of GATA transcription factors. Some examples where differential interactions provide functional specificity will be presented.

3.2.3.7.1 Interaction with DNA-binding proteins

3.2.3.7.1.1 Homotypic and heterotypic interactions of GATA factors

The first documented protein-protein interaction involving GATA factors was the self-association between GATA factors via their zinc finger region (Yang and Evans, 1995; Crossley et al., 1995). As mentioned in the preceding section, it was shown that GATA-1 participates in homotypic interactions which mediate transcriptional activation of GATA-responsive promoters (Mackay et al., 1998). GATA-1 can also participate in heterotypic interactions with GATA-2 and GATA-3 and the coexpression of GATA-2 with GATA-1 is able to mediate transcriptional activation; however, the coexpression of GATA-5 with GATA-1 cannot (Yang and

Evans, 1995). Whether this is caused by a lack of physical interaction between GATA-1 and GATA-5 or by an unproductive interaction is unknown. Given that mutations that impair GATA-1 self-association reduce its ability to activate transcription - especially on promoters containing multiple GATA sites (Mackay et al., 1998) - a possible role for these interactions could be the formation of higher-order structures among distant regulatory elements that share GATA sites, such as the globin LCRs and downstream globin enhancers and promoters.

In addition, we have shown that GATA-4 and GATA-6 functionally and physically interact in cardiomyocytes (Charron et al., 1999). This interaction, which is essential for the expression of many cardiomyocyte-specific genes, will be presented and discussed in more details in Chapter II of this thesis. Together, these heterotypic interactions might explain, at least partly, why GATA factors cannot compensate for each other.

3.2.3.7.1.2 Interactions with other transcription factor families

3.2.3.7.1.2.1 The Kruppel family: Sp1, EKLF, and YY1

The study of erythroid promoters revealed that GATA elements and CACCC box, which are elements bound by members of the Kruppel family of zinc finger transcription factors, are often situated in close proximity and cooperate with each other to direct tissue-specificity and transcriptional activity (Crossley and Orkin, 1993; Walters and Martin, 1992). These observations suggested a functional interaction between GATA-1 and Kruppel proteins and led to the finding that GATA-1 physically and functionally interacts with two members of the Kruppel family of proteins: Sp1 and Erythroid Kruppel-Like Factor (EKLF) (Merika and Orkin, 1995; Gregory et al., 1996). For this interaction, the C-terminal zinc finger region of GATA-1 and the zinc finger region of Sp1 are required. GATA-2 and GATA-3 are also able to physically interact with Sp1. This interaction may be involved in formation of higher-order structures that bridge the globin LCRs and promoters. These functional interactions occur differentially on target genes; for example, GATA-1 acted in synergy with Sp1, but not EKLF, to activate the pyruvate kinase promoter, while it acted in synergy with EKLF, but not Sp1, to

activate a β-globin-derived promoter. In contrast, both Sp1 and EKLF were able to interact with GATA-1 to activate the glycophorin B promoter (Gregory et al., 1996). More recently, GATA-4 was shown to functionally interact with another Kruppel family member, YY1 (Bhalla and Nemer, 2000). Although GATA-4 and YY1 synergistically activate a cardiac promoter, no direct physical interaction could be detected.

3.2.3.7.1.2.2 The steroid hormone receptor family: GR and ER

The GATA factors also interact with another family of zinc finger proteins, the steroid hormone nuclear receptor family. Transfection of the glucocorticoid receptor (GR) or the estrogen receptor (ER) inhibits GATA-1 activity in a ligand-dependent manner (Chang et al., 1993; Blobel et al., 1995). Although the N-terminal 106 amino acid domain of GR is essential for this effect, it has not been shown whether GATA-1 physically associates with GR. In contrast, ER was shown to physically interact with the zinc finger and the N-terminal activation domain of GATA-1. Knowing the role of GATA-1 in erythrocyte differentiation, the finding that activated GR and ER can interfere with GATA-1 function may provide an explanation for the inhibition of erythroid differentiation by glucocorticoids and estrogens, respectively. However, not all nuclear receptor-GATA factor interactions are inhibitory since GATA-6 and GATA-1 functionally interact with ER to synergistically activate the liver-specific vitellogenin II (VTGII) promoter in an estrogen-dependent manner (Davis and Burch, 1996). Together, these studies suggest that, depending on the promoter and cellular contexts, the steroid receptor-GATA factor interaction may positively or negatively regulate gene expression. Furthermore, given the very diverse effects of steroids on cellular growth and differentiation, the interaction between steroid receptors and GATA factors may be relevant to many tissues.

It is interesting to note that all interactions described so far occur between GATA factors and other proteins harboring zinc fingers. Moreover, when tested, the zinc finger region of the GATA factors always seems to be involved in these interactions, suggesting that the zinc finger region of GATA factors is multifunctional and mediates not only DNA-binding but also protein-protein

interactions (Mackay and Crossley, 1998b). The following sections will describe interactions of GATA factors with other classes of transcription factors.

3.2.3.7.1.2.3 The Ets family: PU.1

GATA-1 interacts with the hematopoietic-specific Ets family member PU.1 (Rekhtman et al., 1999). PU.1 is required for the development of multiple hematopoietic lineages, including B and T lymphocytes, monocytes, and granulocytes (Scott et al., 1994; McKercher et al., 1996). When activated in erythroid precursors (by events such as proviral insertions), PU.1 causes erythroleukemia by blocking erythroid differentiation. It was recently proposed that PU.1 blocks erythroid differentiation by physically interacting with and repressing the transcriptional activity of GATA-1 (Rekhtman et al., 1999). This interaction requires intact DNA-binding domains in both proteins. Furthermore, ectopic expression of PU.1 in *Xenopus* embryos is sufficient to block erythropoiesis, which can be rescued by overexpression of GATA-1. These results suggest that the stoichiometry of GATA-1 and PU.1 may be a crucial determinant governing the process of erythroleukemic transformation. Although this interaction occurs during pathological conditions, it remains to be determined whether the opposing actions of PU.1 and GATA-1 (or other hematopoietic GATA factors) contribute to lineage determination during normal hematopoiesis.

3.2.3.7.1.2.4 The AP-1 family: c-Jun and c-Fos

Two independent studies reported functional interaction between GATA factors and the basic leucine zipper (bZip) AP-1 transcription factors (Kawana et al., 1995; Yamagata et al., 1997). The first study described the cooperative interaction of GATA-2 and AP-1 proteins to synergistically activate the endothelin-1 promoter (Kawana et al., 1995). This promoter contains both GATA and AP-1 sites, although only one of the sites (either GATA or AP-1) is required for synergy. This synergy also works with GATA-1 and GATA-3. Moreover, GATA proteins co-immunoprecipitate with c-Jun and c-Fos *in vivo*. The functional relevance of this interaction remains to be determined.

A subsequent study described that triple synergism between the human T-lymphotropic virus type 1-encoded Tax, GATA-4, and c-Jun is required for

activation of the interleukin-5 (IL-5) promoter (Yamagata et al., 1997). This synergy is phorbol ester (TPA)-dependent, probably due to an increase in c-Jun DNA-binding following protein kinase C (PKC) activation (Boyle et al., 1991). This interaction may play a role during adult T-cell leukemia by leading to constitutive IL-5 gene expression (Yamagata et al., 1997).

3.2.3.7.1.2.5 The NF-AT family: NF-AT3 and NF-ATc1

NF-AT proteins belong to the Rel family of transcription factors, which also includes NF-κB (Rao et al., 1997). These factors are constitutively localized to the cytoplasm, but upon Ca^{2+}-dependent activation of the phosphatase calcineurin, NF-AT proteins are dephosphorylated and shuttled to the nucleus where they bind DNA and modulate gene transcription.

NF-AT3 was shown to interact physically and cooperate with GATA-4 to activate cardiac gene expression in a Ca^{2+}-dependent manner (Molkentin et al., 1998). This interaction requires intact DNA-binding domains in both proteins. Although overexpression of an activated form of calcineurin or its effector NF-AT3 has been shown to induce cardiac hypertrophy in transgenic mice, its remains to be determined whether this effect is dependent on a physical interaction of NF-AT3 with GATA-4.

Another member of the NF-AT family, NF-ATc1, was shown to translocates to the nucleus and interact physically with GATA-2 during calcineurin-induced skeletal myocyte hypertrophy (Musaro et al., 1999). However, it remains to be determined whether the physical interaction of NF-ATc1 with GATA-2 is important for calcineurin-induced skeletal myocyte gene expression and hypertrophy.

3.2.3.7.1.2.6 The homeodomain family: Nkx2-5

The most documented interaction involving a cardiac GATA factor is the finding that GATA-4 and the homeodomain protein Nkx2-5 are mutual cofactors ((Durocher et al., 1997); see the *Appendices* for a reprint). Both genes are early markers of precardiac cells (Heikinheimo et al., 1994; Jiang and Evans, 1996; Kelley et al., 1993; Laverriere et al., 1994; Morrisey et al., 1996; Lints et al., 1993; Schultheiss et al., 1995; Chen and Fishman, 1996) and are essential for heart formation (Kuo et al., 1997; Molkentin et al., 1997; Lyons et al., 1995), but neither

can initiate cardiogenesis. However, overexpression of either Nkx2-5 or GATA-4 in committed precursors enhances cardiogenesis (Jiang and Evans, 1996; Grépin et al., 1997; Chen and Fishman, 1996; Cleaver et al., 1996), suggesting that they require a cardiac-restricted cofactor. Since ANF is a transcriptional target for both GATA-4 and Nkx2-5 (Charron et al., 1999; Durocher et al., 1996), the ANF promoter was used to demonstrate that GATA-4 and Nkx2-5 are mutual cofactors (Durocher et al., 1997; Lee et al., 1998; Shiojima et al., 1999). Subsequently, this cooperativity was also shown to occur on other cardiac promoters, such as α-CA (Sepulveda et al., 1998). Transcriptional synergy involves physical interaction between the homeodomain plus a C-terminal extension of Nkx2-5 and the C-terminal zinc finger plus the adjacent basic region of GATA-4. This interaction seems to be specific for certain GATA proteins since GATA-6 could not substitute for GATA-4 in this interaction (Durocher et al., 1997), suggesting that differential interaction between GATA factors and other proteins may impart functional specificity to GATA factors. More details on the role of Nkx2-5 in the heart will be discussed in the *Cardiac development* section.

3.2.3.7.2 Interaction with non-DNA-binding proteins

3.2.3.7.2.1 The Lim family: RBTN2/LMO2

GATA-1 and GATA-2 interact with RBTN2/LMO2 both *in vitro* and *in vivo* (Osada et al., 1995). RBTN2 is a LIM-domain protein essential for erythropoiesis (Warren et al., 1994). LIM proteins contain zinc-binding domains known to be involved in protein-protein interactions. RBTN2 also interact with the basic helix-loop-helix (bHLH) protein SCL/TAL1, a gene essential for erythropoiesis and hematopoiesis (Shivdasani et al., 1995; Porcher et al., 1996). Knowing the role of GATA-1 and GATA-2 in erythropoiesis and hematopoiesis, respectively, a close functional relationship between RBTN2, SCL and GATA-1 or GATA-2 was suspected. Two-hybrid assays indicated that RBTN2 serves as a bridge between GATA-1 or GATA-2 and SCL/E47 heterodimers, thereby forming a quaternary complex (Osada et al., 1995). Moreover, recent work showed that this complex exists in erythroid cells, binds specifically to bipartite DNA elements composed of a SCL (E-Box) and a GATA sites, and can function in transcriptional activation (Wadman et al., 1997).

These results link RBTN2, SCL, and GATA-1 or GATA-2 together into a transcriptionally active DNA-binding complex and suggest that, during development, variations in the complex equilibrium and in the amount of complex formed may differentially regulate various stages of hematopoietic differentiation.

3.2.3.7.2.2 The FOG/Ush family: FOG-1, FOG-2, and Ush

As mentioned above, FOG-1, the founding member of the FOG/Ush family, was cloned by the yeast two-hybrid system using the N-terminal zinc finger of GATA-1 as a bait (Tsang et al., 1997). FOG-1 also interacts with GATA-2 and GATA-3 via their N-terminal zinc finger domain. During embryonic development, FOG-1 is co-expressed with GATA-1 in the erythrocyte precursors of the yolk sac and fetal liver and later, they are co-expressed in the erythroid and megakaryocytic cells of the adult hematopoietic system. When co-expressed in cell lines, GATA-1 and FOG-1 synergistically activate transcription and cooperate during both erythroid and megakaryocytic cell differentiation (Tsang et al., 1997). Moreover, mice deficient in FOG-1 do not produce megakaryocytes and display arrested erythropoiesis, reminiscent of GATA-1-deficient erythroid precursors (Tsang et al., 1998). Although these findings strongly suggested that FOG-1 acts as a cofactor for GATA-1 in erythrocyte differentiation, the formal proof came from the work of Crispino et al. who generated GATA-1 mutants specifically impaired for interaction with FOG-1 and a compensatory FOG-1 mutant that restores the interaction (Crispino et al., 1999). The GATA-1 mutants failed to rescue erythroid differentiation of GATA-1 null erythroid cells in the absence of the compensatory FOG-1 mutant, indicating that the GATA-1-FOG-1 interaction is essential for GATA-1 function in erythroid differentiation. The importance of the GATA-1-FOG-1 interaction in human megakaryocyte and erythroid development was further supported by the finding that a GATA-1 N-terminal zinc finger mutation which abolishes the ability of GATA-1 to interact with FOG-1 causes familial X-linked dyserythropoietic anemia and thrombocytopenia (Nichols et al., 2000).

Despite the importance of FOG-1 for GATA-1 action, the GATA-FOG interaction cannot explain by itself the specificity among GATA members, as FOG-1 also interacts with GATA-2 and GATA-3. However, given that GATA-2 and GATA-3

appear to function in a FOG-1-independent manner (Tsang et al., 1998), it is possible that other FOG/Ush family members may exist that specifically regulate GATA-2 and/or GATA-3 activity. Moreover, given that the GATA-1 N-terminal zinc finger residues required for interaction between GATA-1 and FOG-1 are highly conserved in GATA-4, GATA-5, and GATA-6 (Fox et al., 1998), it is tempting to speculate that FOG-1 will also be able to interact with these GATA factors. FOG-1 being present in other GATA-expressing organs such as the spleen (GATA-5), the liver (GATA-6) and the testes (GATA-1, GATA-4, and GATA-6), it will be interesting to study its potential role in the development of these tissues in relationship to other GATA factors.

Another member of the FOG/Ush GATA-cofactor family has been recently identified. FOG-2 is expressed predominantly in heart, brain, and testis (Lu et al., 1999; Svensson et al., 1999; Tevosian et al., 1999; Holmes et al., 1999). Its expression in the heart and its physical interaction with GATA-4, GATA-5, and GATA-6 make it a likely cofactor for these GATA proteins in the heart. In support for a role of FOG-2 in the heart, *FOG-2* null mice display cardiac defects characterized by a thin ventricular myocardium, common atrioventricular canal, and the tetralogy of Fallot malformation, in addition to abnormal coronary vasculogenesis (Tevosian et al., 2000). Another group have also reported that *FOG-2* null mice display cardiac defects reminiscent of the tricuspid atresia syndrome, characterized by an absent tricuspid valve and ventricular and atrial septal defects (Svensson et al., 2000); however, this hypothesis still need to be tested directly.

Despite good evidence that FOG proteins are cofactors for GATA proteins, it is not clear whether FOG proteins have a positive or negative transcriptional effect on GATA factors. Together with GATA-1, FOG-1 was initially shown to synergistically activate the 7 kb p45 NF-E2 promoter, but not a minimal GATA-responsive promoter (Tsang et al., 1997). In fact, subsequent studies showed that FOG-1 was even able to repress GATA-1 activation (Fox et al., 1998; Fox et al., 1999; Tsang et al., 1998; Holmes et al., 1999; Turner and Crossley, 1998). Moreover, FOG-2 was shown to either enhance or repress GATA-4 transcriptional activity (Lu et al.,

1999; Svensson et al., 2000). Even genetic evidence support a repressive role of FOG proteins on GATA factors: Ush, a *Drosophila* FOG/Ush family member, antagonizes the activity of the GATA factor Pannier in sensory bristle formation (Haenlin et al., 1997). Thus, the effects of the FOG proteins on GATA-dependent transcription may depend on both the cell types in which they are expressed and on the promoters that they regulate.

3.2.3.7.2.3 The transcriptional integrator/histone acetyltransferase family: CBP and p300

CBP (CREB-binding protein) and p300 are coactivators thought to act as transcriptional integrators, bridging DNA-bound transcriptional activators to the basal transcription machinery (reviewed in (Shikama et al., 1997)). Moreover, CBP and p300 possess intrinsic and associated histone acetyltransferase activity (Bannister and Kouzarides, 1996; Ogryzko et al., 1996; Chen et al., 1997; Yang et al., 1996; Spencer et al., 1997). CBP interacts and cooperates with GATA-1 to activate erythroid promoters (Blobel et al., 1998). The C-terminal zinc finger of GATA-1 and the E1A-binding region of CBP are required for this interaction. A role for this interaction in erythroid differentiation is supported by the observation that expression of E1A in MEL cells blocks differentiation and expression of endogenous GATA-1 target genes, while a mutant form of E1A impaired in CBP binding has no effect.

As described in the *Regulation of GATA factors: Acetylation* section, CBP and p300 acetylate GATA-1 *in vitro* and mutation of the acetylation motifs significantly decreases GATA-1 ability to cooperate with CBP and abrogates its ability to induce erythroid differentiation (Hung et al., 1999; Boyes et al., 1998). These results suggest that acetylation of GATA-1 contributes to regulate its activity. p300 was also shown to interact and cooperate with GATA-5 to activate the ANF promoter (Kakita et al., 1999). However, it remains to be determined whether GATA-5 is acetylated by p300. Moreover, whether the recruitment of CBP/p300 by GATA factors also serves to couple GATA transcription factors to the basal transcription machinery or whether it serves to induce changes in chromatin structure by mean of their histone acetyltransferase activity, or both, remain to be established.

3.2.4 Summary of GATA factor regulation

In summary, the GATA transcription factors are regulated at many levels: (i) tissue-specific promoters and alternative promoter usage ensure appropriate spatio-temporal expression in various tissues; (ii) alternative exon and translation initiation codon usage allow different cell types to generate GATA isoforms having differential properties, such as transcriptional activity or interaction with cofactors; (iii) similarly, specific cleavage by proteases, such as caspases, may alter GATA factor activity by removing a functional domain, while proteolytic degradation may serve to rapidly downregulate the activity of a specific GATA transcription factor; (iv) post-translational modifications, such as phosphorylation and acetylation, may modulate the DNA-binding properties of GATA factors or their capacity to interact with cofactors; (v) many DNA-binding and non-DNA-binding cofactors interact physically and functionally with GATA proteins. Interaction of GATA factors with tissue-restricted cofactors, such as members of the Kruppel (EKLF), Ets (PU.1), homeodomain (Nkx2-5), FOG/Ush (FOG-1 and FOG-2), and GATA (homotypic and heterotypic GATA interactions) families, allows for the generation of a combinatorial code leading to tissue-restricted gene expression and imparts functional specificity to GATA factors. Interaction of GATA factors with ubiquitously or nearly-ubiquitously expressed cofactors, such as members of the AP-1 (c-Jun and c-Fos), steroid hormone receptors (GR and ER), and NF-AT (NF-AT3 and NF-ATc1) families, allows for modulation of GATA factor activity by extracellular stimuli, allowing cells to respond to their environment. Other GATA cofactors, such as LIM proteins (RBTN2), are involved in transcription factor complex assembly and may serve to coordinate the formation of these complexes. Finally, GATA cofactors such as CBP and p300, which possess acetyltransferase activity, may acetylate GATA proteins themselves or other transcription factors at the same promoter. Such interactions could also lead to histone acetylation, resulting in chromatin-remodeling activity by GATA factors. Thus, CBP and p300 may directly modulate GATA factor activity and/or serve as transcriptional integrators, linking the combined effects of many transcription factors to chromatin and/or to the basal transcription machinery.

4 TRANSCRIPTIONAL REGULATION OF CARDIAC GROWTH AND DIFFERENTIATION

This section will review the various stages of vertebrate cardiac development along with genes known to be involved in these events. A particular emphasis will be given to genes coding for transcription factors, given the crucial role that these proteins play in the regulation of tissue-specific gene expression and in the control of cell fate determination. We will focus primarily on the development of the three layers of the heart, namely the myocardium, endocardium, and the pericardium, together with the generation of the cardiac septa and valves. In addition, the combinatorial mechanism underlying cardiac gene regulation, which is emerging as a paradigm in tissue-specific and pathological control of gene expression, will be presented. Finally, we will discuss the emerging role of cardiac transcription factors in two different types of cardiac pathologies, namely hypertrophic cardiomyopathies and congenital heart diseases.

4.1 Cardiac development

Cardiovascular malformations are the largest cause of human birth defects (Hoffman, 1995). The susceptibility of the heart to congenital malformations reflects the complexity of the morphogenetic events involved in its development. Despite the fact that cardiac malformations have been characterized extensively at the anatomical level, the genetic bases for these abnormalities remain largely unknown. However, remarkable progress has been achieved in the identification of genes involved in heart development. It is very likely that the elucidation of the role of these genes could provide a better understanding of the causes of cardiac abnormalities and help prevent or correct congenital heart malformations.

4.1.1 Overview of cardiac development

Cardiomyocyte precursors originate from mesodermal tissue and initially migrate to generate a straight heart tube composed of an outer myocardial layer and an inner endocardial layer, which are separated by an extracellular matrix-rich layer, the cardiac jelly. This heart tube, already patterned into ventricular and atrial domains, will then loop and undergo complex morphogenetic changes to give rise to the

mature multi-chambered heart. The following sections discuss in detail the morphological and molecular events underlying these processes.

4.1.2 Formation of the heart field

During gastrulation, in vertebrates, cardiac precursor cells from the epiblast invaginate through the primitive streak and migrate anterolaterally to form the anterior lateral plate mesoderm (Figure 1.3). This pair of bilaterally symmetrical regions of the embryonic mesoderm, which contains the cardiac precursor cells, is termed the heart field, whereas the two independent bilateral regions are referred to as the cardiac primordia. Subsequently to its formation, the lateral plate mesoderm splits into two layers: the somatic mesoderm, which is composed of skeletal muscle progenitors, and the splanchnic mesoderm, which includes the cardiac precursors for the three layers of the heart tube, namely the myocardium, endocardium, and pericardium (Figure 1.4).

The commitment of these precursor cells to a cardiac fate results from inductive interactions during gastrulation. A major source of inductive signals is the anterior lateral endoderm, which is in contact with, and appears to migrate along, cardiac precursors. Indeed, endoderm ablation experiments have shown that this embryonic layer is required for heart formation. Moreover, the anterior lateral endoderm is able to induce cardiac differentiation of non-cardiac mesoderm explants, suggesting that the anterior endoderm secretes factors that initiate the cardiogenic program in the adjacent mesoderm (Schultheiss et al., 1995; Nascone and Mercola, 1995; Sugi and Lough, 1994). Candidates for endodermal-derived, cardiac-inducing signals are the fibroblast growth factor 4 (FGF4) and the transforming growth factor-β (TGF-β)-family member bone morphogenic protein 2 (BMP2), which are both expressed in the anterior endoderm. In the current model, both BMP2 and FGF4 would be required; BMP2 would specify mesodermal cells to the cardiac lineage whereas FGF4 would promote the proliferation and survival of these specified cells (Lough et al., 1996; Schultheiss et al., 1997).

4.1.3 Transcriptional regulation in the heart field

4.1.3.1 The NK2 family

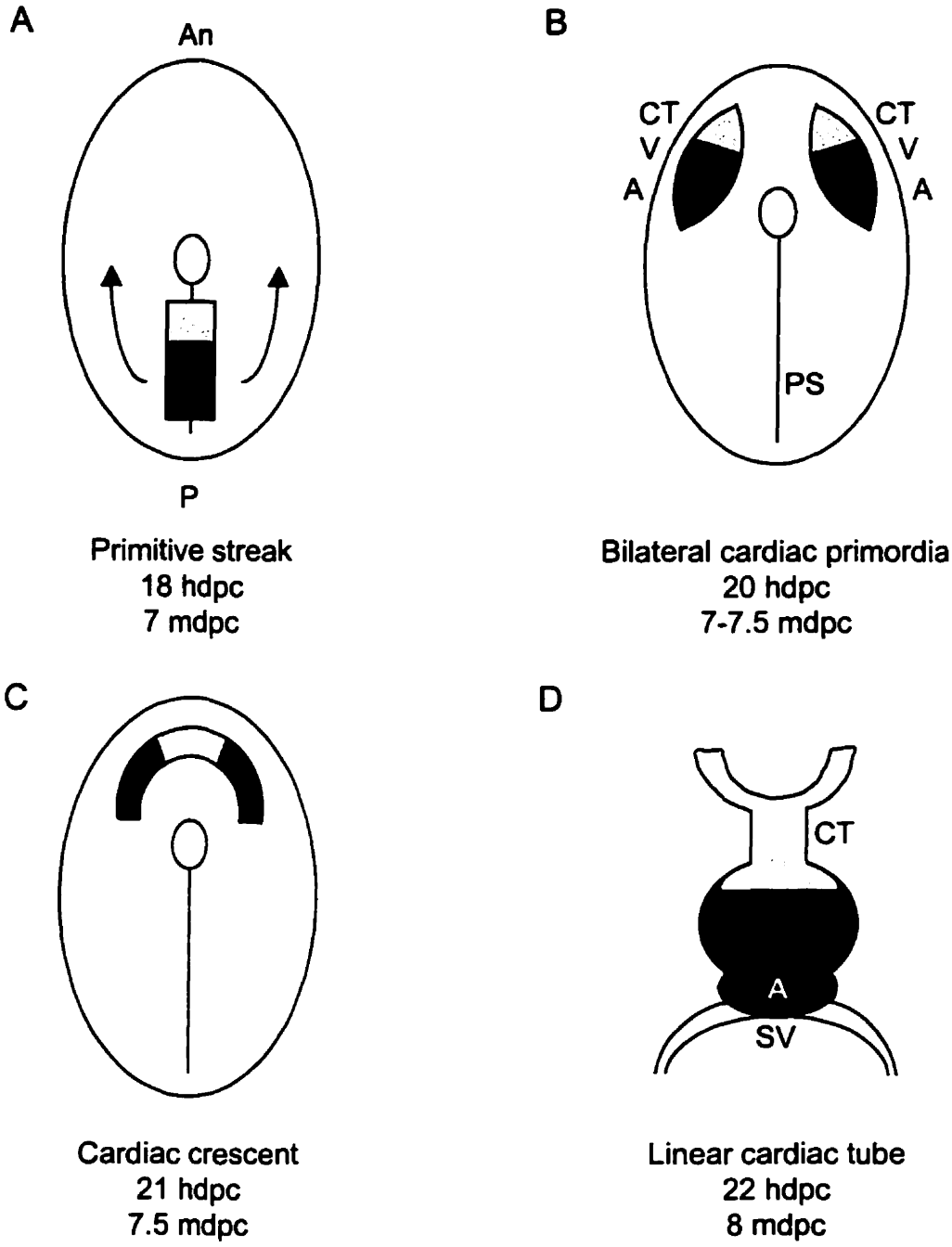


Figure 1.3. Migration of cardiac precursors and generation of the straight heart tube. During gastrulation, cells invaginate through the primitive streak (A) and migrate anterolaterally to form the anterior lateral plate mesoderm (B). The cardiac primordia then fuse at the midline (C) to form the linear heart tube (D). Note that the relative anteroposterior positions of the cardiac precursors in the primitive streak (A) are retained in the heart field (B and C) and in the heart tube (D). hdpc, human days post-coitum; mdpc, mouse days post-coitum; An, anterior; P, posterior; PS, primitive streak; CT, conotruncus; V, ventricle; A, atrium; SV, sinus venosus.

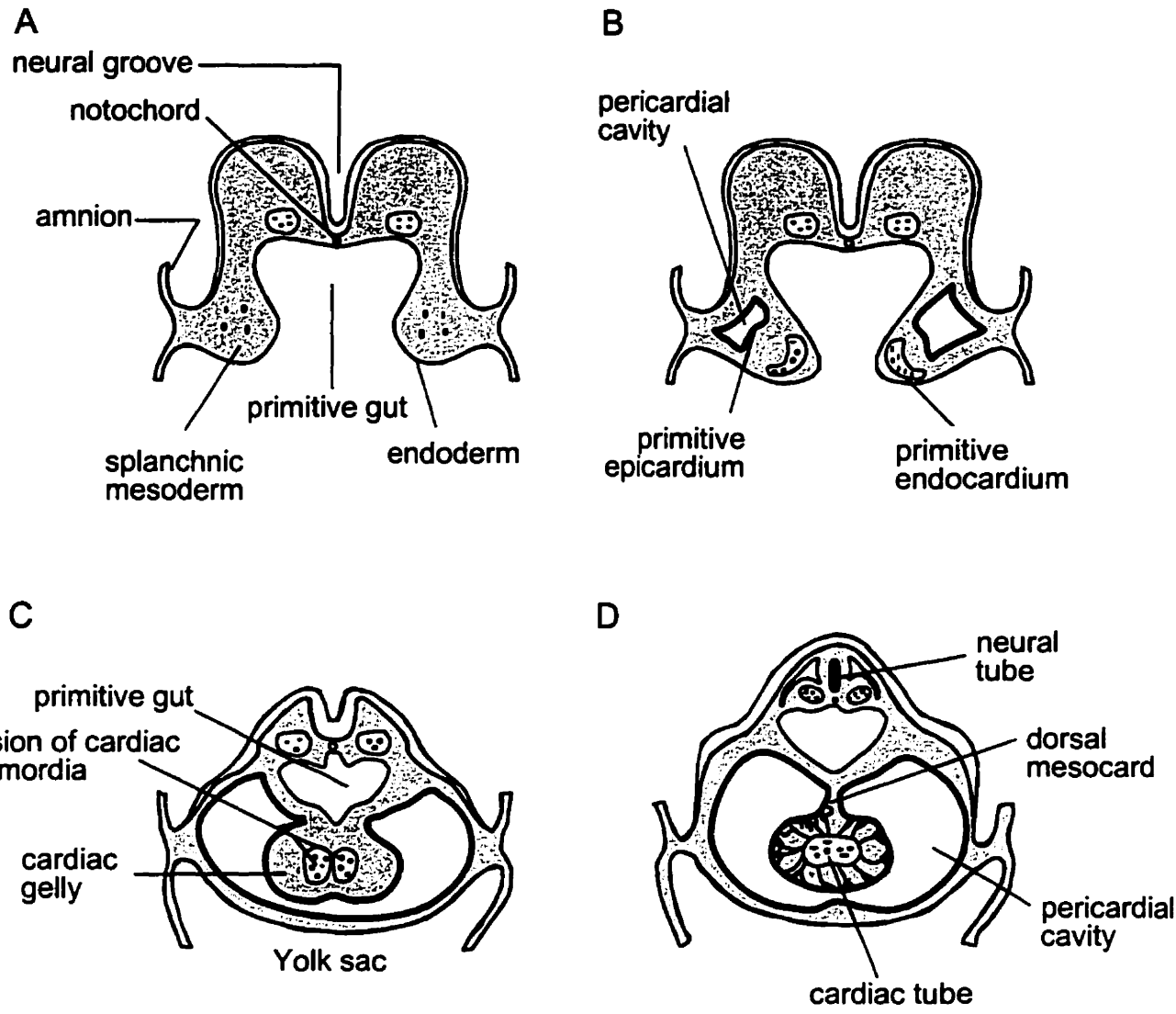


Figure 1.4. Heart tube formation as seen in transverse sections. As the embryo is folding ventrally, the splanchnic mesoderm (A) differentiates into the primitive epicardium, endocardium, and myocardium (B). Ventral folding leads to the fusion of the cardiac tubes (C) into a single tube (D) and also generates the primitive gut.

The mechanisms by which FGF4 and BMP2 induce cardiogenesis remain unknown. However, studies using the fruit fly *Drosophila* have begun to reveal genetic pathways controlling cardiogenesis and suggest that these molecular events are highly conserved across species. In *Drosophila*, the dorsal vessel, a primitive heart-like structure contracting rhythmically and pumping hemolymph through an open circulatory system, is analogous to the straight heart tube of the vertebrate embryos. Formation of the dorsal vessel requires the homeodomain transcription factor *tinman*, which is expressed in the early mesoderm and later in the dorsal vessel (Bodmer, 1993; Azpiazu and Frasch, 1993). *tinman* is thought to specify the formation of the dorsal mesoderm, the tissue from which cardiomyocyte precursors originate. Screens for vertebrate *tinman* homologues led to the identification of the homeodomain transcription factor Nkx2-5 (Table 1.1) (Komuro and Izumo, 1993; Lints et al., 1993).

As mentioned previously, Nkx2-5 activates the transcription of cardiac genes, such as atrial natriuretic factor (ANF) and cardiac α-actin (Durocher et al., 1996; Chen and Schwartz, 1996). Nkx2-5 is expressed in the lateral plate mesoderm and is one of the earliest markers of heart field induction (Harvey, 1996). In mice homozygous for a null mutation in *Nkx2-5*, the heart tube forms but cardiomyocytes do not fully differentiate, as indicated by the downregulation of many cardiac genes, such as genes coding for cardiac contractile protein [ventricular myosin light-chain 2 (MLC2V)], cardiac hormones [ANF, B-type natriuretic peptide (BNP)], and cardiac transcription factors [MEF2C, eHAND, and N-myc)] (Table 1.2) (Tanaka et al., 1999; Lyons et al., 1995). However, in contrast to the *tinman* mutant in *Drosophila*, cardiac mesoderm and cardiomyocytes are still specified. The less drastic effect of the *Nkx2-5* mutation in mice compared to the *tinman* mutation in flies could reflect partial compensation by other *tinman* vertebrate homologues, namely Nkx2-3, Nkx2-7, and Nkx2-8, which are expressed in the cardiogenic mesoderm (Brand et al., 1997; Lee et al., 1996; Buchberger et al., 1996; Evans et al., 1995). Indeed, it was shown that the expression of dominant repressor mutants, which can interfere with all NK2 proteins, completely blocks myocardial gene expression and heart formation (Grow and Krieg, 1998; Fu et al., 1998).

Table 1.1. Transcription Factor Families Involved in Cardiovascular Development

Family	DNA Binding Domain	Binding Site	Family Members	Cardiovascular Expression
NK2	Homeodomain	T(C/T)AAGTG	Nkx2-5 Nkx2-3 Nkx2-7 Nkx2-8	Cardiac progenitors Cardiomyocytes
GATA	C4 zinc finger	(A/T)GATA(A/G)	GATA-4 GATA-5 GATA-6	Cardiac progenitors Cardiomyocytes Cardiac progenitors Endocardial cells Cardiac progenitors Cardiomyocytes Vascular smooth muscle cells
HAND	bHLH	CANNTG	dHAND eHAND	Cardiac progenitors Cardiomyocytes (right ventricle) Neural crest cells Cardiac progenitors Cardiomyocytes (left ventricle) Neural crest cells
MEF2	MADS	(T/C)TA(A/T)$_4$TA(A/G)	MEF2A MEF2B MEF2C MEF2D	Cardiac progenitors Cardiomyocytes Vascular smooth muscle cells Endothelial cells
NF-AT	Rel-homology domain (RHD)	GGAAAAT	NF-AT3 NF-ATc	Cardiomyocytes Endocardial cells

Table 1.2. Loss of Function Phenotypes and Putative Roles of Cardiac Transcription Factors

Gene	Loss of Function Phenotype	Target Genes[a]	Putative Roles
Nkx2-5	•Arrest in cardiac development after looping	ANF, BNP, MLC2V, MEF2C, eHAND, N-myc, Msx2, CARP	•Cardiomyocyte differentiation •Heart tube regionalization
GATA-4	•Cardia bifida (no fusion of the cardiac primordia)	ANF, BNP, α-MHC, β-MHC, cTnI, PDGFRβ	•Cardiomyocyte differentiation •Cardiac primordia fusion •Maintenance of the cardiac phenotype •Hypertrophic response
GATA-6	•Extra-embryonic endoderm defects	GATA-4, ANF, BNP, α-MHC, β-MHC, cTnI, PDGFRβ	•Extra-embryonic endoderm differentiation •Cardiomyocyte progenitor proliferation
MEF2C	•Arrest in cardiac development at the looping stage (absence of the right ventricle) •Vascular and endocardial defects	ANF, Cardiac α-actin, α-MHC, MLC1A, dHAND, angiopoietin-1, VEGF	•Cardiomyocyte differentiation (late stage) •Endocard development
dHAND	•Arrest in cardiac development at the looping stage (absence of the right ventricle) •Neural crest defects	Ufd1	•Heart tube regionalization •Regulation of gene(s) involved in neural crest development
eHAND	•Placentation defects •Arrest in cardiac development at the looping stage	?	?
NF-ATc	•Valve formation and heart septation defects	?	?

[a] Target genes listed are those identified by loss of function studies.

The exact role of Nkx2-5 or other NK2 proteins in the heart is still unclear. Detailed analysis of a null mutation of *Nkx2-5* in mice, as well as analysis of chimeric mice generated from *Nkx2-5* null embryonic stem (ES) cells, suggests that Nkx2-5 is likely required for later stages of myocyte differentiation, such as spatial or asymmetric regionalization (Tanaka et al., 1999). Whether other NK2 proteins are able to substitute or compensate for earlier functions of Nkx2-5 in cardiomyocyte recruitment and/or commitment is presently unknown. Gain-of-function studies in *Xenopus* and manipulations of cardiac induction in chick embryos have shown that Nkx2-5 is able to recruit additional "permissive" cells to the cardiac lineage, thus enlarging the heart field (Cleaver et al., 1996; Chen and Fishman, 1996). These data suggests that Nkx2-5 acts in concert with other factors present in precardiomyocytes to alter the expression of a subset of precardiac cells and affect later stages of differentiation. One such Nkx2-5 collaborator is GATA-4, which interacts physically with Nkx2-5 and cooperatively enhances transcription of Nkx2-5 target genes.

4.1.3.2 The GATA family

GATA-4 expression can be detected in the bilateral cardiac primordia and, together with Nkx2-5, constitutes the earliest markers of heart field induction. Later, GATA-4 transcripts and proteins are detected throughout the myocardium and endocardium and persist at all stages of heart development (Heikinheimo et al., 1994; Grépin et al., 1994). As discussed in the previous section, many studies suggest a role for GATA-4 in cardiomyocyte differentiation and gene expression (Jiang and Evans, 1996; Grépin et al., 1997; Grépin et al., 1995). Consistent with an important role for GATA-4 in the heart, mice homozygous for a null mutation in the *Gata4* gene are not viable due to the inability of the bilateral cardiac primordia to fuse and form the heart tube (Kuo et al., 1997; Molkentin et al., 1997). Nevertheless, cardiomyocytes expressing differentiation markers are detectable in these mice, raising the possibility that other factors, including other members of the GATA family, may compensate, at least in part, for GATA-4-deficiency during early cardiac development.

Within the heart, GATA-4, GATA-5, and GATA-6 are regulated differentially throughout development, with GATA-4 being the predominant transcript in cardiomyocytes at all stages. GATA-6 is also expressed in the precardiac mesoderm and is later found in myocardial and in vascular smooth muscle cells. GATA-5 transcripts are largely restricted to endocardial cells.

Interestingly, the expression of GATA-6 is highly upregulated in GATA-4-deficient mice. Two lines of evidence indicate that both GATA-5 and GATA-6 can indeed substitute for some of the GATA-4 functions in the heart. First, all three factors are potent activators of GATA-dependent cardiac promoters (Durocher et al., 1997), suggesting that, if required, they could compensate for each other with respect to the transcription of several cardiac genes. Second, ectopic expression of GATA-4, GATA-5, or GATA-6 was equally efficient at activating the transcription of cardiac contractile genes *in vivo* (Jiang and Evans, 1996). However, as will be presented in Chapter II, Chapter III, and *Discussion*, other data suggest that each GATA factor fulfills a unique role in heart development. Nonetheless, these results are consistent with critical roles for GATA factors in the differentiation of cardiogenic cells.

4.1.3.3 Combinatorial interaction between GATA-4 and Nkx2-5

The establishment and maintenance of the cardiac phenotype require the activation of cardiac-specific genes in a tightly regulated temporal and spatial manner. An emerging theme in the tissue-specific transcriptional regulation of gene expression is that this process is governed by the combinatorial action of cell-restricted as well as ubiquitous transcriptional regulators (Charron and Nemer, 1999). As stated previously, studies in P19 cells indicated that overexpression of GATA-4 potentiates cardiogenesis only in "permissive" cellular environments, suggesting that GATA-4 action requires a co-factor. In many species, the expression pattern of GATA-4 overlaps with that of Nkx2-5 in the heart-forming region. Interestingly, ectopic expression of Nkx2-5 results in enhanced myocyte recruitment, but is not sufficient to initiate cardiac gene expression or differentiation (Chen and Fishman, 1996; Cleaver et al., 1996), suggesting that Nkx2-5 acts in concert with other transcription factors to specify the cardiac phenotype. The fact

that GATA-4 and Nkx2-5, two of the earliest markers of precardiac cells, are essential for heart formation and that overexpression of either alone cannot initiate cardiogenesis, yet enhances recruitment and/or differentiation of committed precursors (Grépin et al., 1997; Chen and Fishman, 1996; Cleaver et al., 1996), raised the possibility that these proteins may be mutual co-factors. Because ANF was the only shown transcriptional target for both GATA-4 and Nkx2-5 (Grépin et al., 1994; Durocher et al., 1996), it provided a useful tool to investigate potential functional cooperation between GATA-4 and Nkx2-5. Indeed, it was shown that GATA-4 and Nkx2-5 are mutual co-factors, as co-expression of GATA-4 and Nkx2-5 resulted in synergistic activation of the ANF promoter ((Durocher et al., 1997); see the *Appendices* for a reprint). This molecular interaction may provide cooperative cross talk between two pathways that are critical for the early events of cardiogenesis.

4.1.3.4 The GATA-4/Nkx2-5 interaction: a mediator of BMP signaling

Because GATA-4 and Nkx2-5 are the earliest markers of the myocardial cell fate, the identification of the upstream regulators of GATA-4 and Nkx2-5 in the precardiac mesoderm should give important insights on the nature of the inducers of cardiac fate. Interestingly, BMP2, which is able to specify mesodermal cells to the cardiac lineage, also induces Nkx2-5 and GATA-4 expression in anterior lateral mesoderm (Schultheiss et al., 1997). Thus, it is possible that Nkx2-5 and GATA-4 are downstream effectors of BMP2 and the functional interaction between these two factors would be required for cardiomyocyte differentiation. This hypothesis is consistent with the gain-of-function studies that demonstrated that neither GATA-4 nor Nkx2-5 could alone initiate cardiogenesis, although either protein could potentiate it in committed cells. This functional interaction, which potentiates the transcriptional activities of both proteins, would be especially important at low concentrations of GATA-4 and Nkx2-5, a situation that likely occurs in the early moments of cardiac cell fate induction.

4.1.4 Formation of the heart tube

Commitment of splanchnic mesoderm cells to the cardiogenic lineage is followed by their bilateral migration to form the two cardiac primordia, which in turn will

migrate and fuse at the ventral midline to form the primitive cardiac tube (Figure 1.4). The molecular mechanisms underlying these very early morphogenetic events are not well understood, although they are linked temporally to the ventral closure of the embryo. Two genes are known to be required for fusion of the cardiac primordia: *Gata4* in mice and *Gata5* in zebrafish (Kuo et al., 1997; Molkentin et al., 1997; Reiter et al., 1999). As mentioned previously, experiments using chimeric expression of GATA-4 in the endoderm showed that GATA-4 is necessary and sufficient to rescue the *cardia bifida* phenotype, suggesting that GATA-4 plays an essential role in the early cross talk between endodermal and mesodermal layers, which is critical for proper cardiac development.

4.1.5 Heart tube regionalization and looping

As it is forming, the heart tube begins to contract. The first contractions are peristaltic and subsequently become sequential. Along with the functional maturation of the heart, specialization of the contractile protein machinery occurs and chamber-specific contractile genes are detected well before morphological evidence of chamber demarcation. The beating heart tube is organized with an antero-posterior polarity, where the anterior region will become the outflow region of the mature heart and the posterior region will become the inflow region (Figure 1.5). Thus, specific segments of the heart tube are already fated to become, from anterior to posterior, the aortic sac, the conotruncus, the right ventricle, the left ventricle, the atria, and the sinus venosus of the mature heart. Mechanisms controlling heart tube regionalization are poorly understood, and the timing of atrial and ventricular specification is presently controversial. Although fate-mapping studies suggest that atrial and ventricular lineages are specified and separated during gastrulation, work in chicken and zebrafish suggest that the fate of cardiac progenitors can be altered – by retinoic acid treatment, for example – within a specific window of time, raising the possibility that lineage commitment occurs later on (Stainier et al., 1993; Yutzey and Bader, 1995). A homeobox gene, Irx4, has been identified and shown to be expressed only in ventricular myocytes at all stages, making it the only cardiac gene with chamber-specific expression throughout development. Gain and loss-of-function in chick embryos suggest that

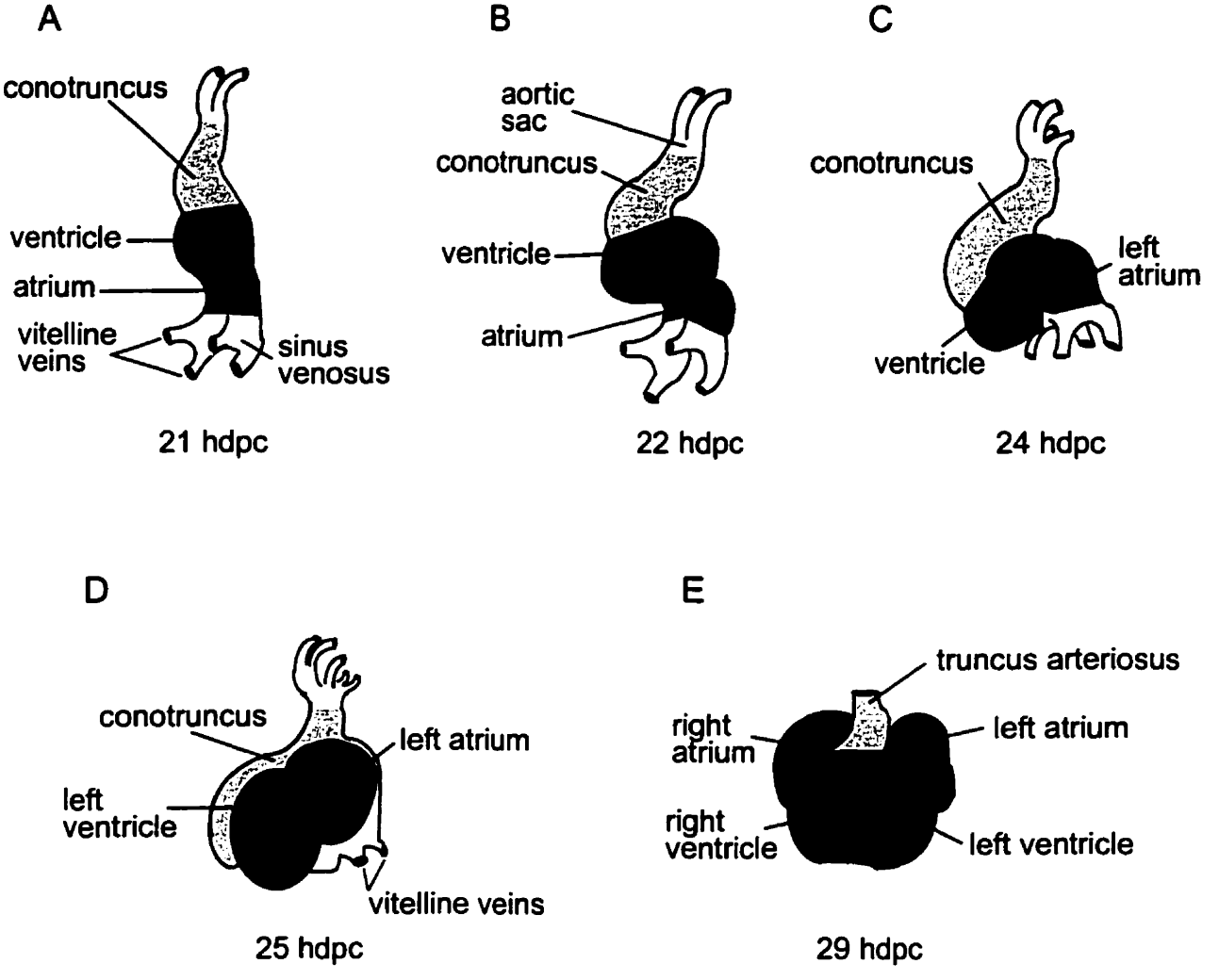


Figure 1.5. Heart tube looping and cardiac morphogenesis. The beating straight heart tube (A) undergoes rightward looping (B and C). By 25 hdpc (D), the atrium and the ventricle are morphologically different and the ventricle is beginning to thicken by growth of the wall and addition of trabeculae. The cardiac jelly separates the myocardium from the endocardium and becomes thicker in the outflow tract and the AV regions, where cushions and valves will form. From the AV cushions and the interventricular and interatrial septa, the atrium and the ventricle divide into right and left chambers (E). (A to D) The developing heart from the left side and (E) a frontal view.

Irx4 serves to impose a ventricular phenotype over a default atrial pathway (Bao et al., 1999).

As it grows, the linear heart tube undergoes rightward looping, which will ultimately bring the already fated regions of the linear heart tube into their mature relative positions. At this stage, the atrial and ventricular chambers start becoming morphologically identifiable. Subsequent growth and maturation of individual chambers result in the mature heart. Which extrinsic or intrinsic factors drive cardiac looping remain to be elucidated. The process clearly depends on a number of concurrent events, which include proper lineage differentiation and differential myocyte proliferation as well as asymmetric signaling.

Laterality signals governing asymmetric heart looping probably originate from the asymmetric process of egg fertilization (reviewed in (Harvey, 1998)). A few cell divisions later, the TGF-β family member Vg1 seems to coordinate the elaboration of the left/right axis signaling pathway. According to the current model, Vg1 would upregulate the expression of a member of the hedgehog family, Sonic hedgehog (Shh), specifically on the left side of the embryo. Via its receptor, Patched (Ptc), Shh would induce the expression of the TGF-β family members nodal and lefty-2, which are expressed in the caudal region of the forming heart tube, where the first cardiac asymmetries are seen. The homeodomain transcription factor Pitx2 lies downstream of nodal and lefty-2 and is expressed along the left side of the forming heart tube, with expression persisting in the left side of the atria and ventricles during looping. Remarkably, expression of Pitx2 in right-sided cardiac primordia induces bilaterally symmetrical hearts. These results suggest that the asymmetric signaling cascade regulating heart morphogenesis converges on Pitx2, the currently most downstream gene involved in the laterality pathway.

The timing of heart looping also coincides with initiation of the embryonic circulation, which can also interfere with rotational movements directly or as a result of alterations in myocyte growth and/or proliferation. Gene inactivation studies have linked two other families of transcription factors to looping phenotypes.

4.1.5.1 The HAND family

eHAND and dHAND are two transcription factors of the basic helix-loop-helix (bHLH) family expressed in the precardiac mesoderm (reviewed in (Srivastava, 1999)). Later, dHAND is expressed throughout the straight heart tube and becomes restricted predominantly to the future right ventricle during looping. In contrast, eHAND expression is restricted to the anterior and posterior regions of the heart tube, which are fated to become the conotruncus and left ventricle, respectively. Finally, in the mature heart, dHAND and eHAND display complementary expression in the right and left ventricles, respectively.

Inactivation of the *dHAND* gene in mice results in embryonic lethality at the looping stage (Srivastava et al., 1997). Interestingly, these mice fail to develop the segment of the heart tube that will form the right ventricle, consistent with the predominant expression of dHAND in this segment.

Inactivation of the *eHAND* gene results in early embryonic lethality due to placentation defects, making analysis of its role in cardiac morphogenesis ambiguous (Firulli et al., 1998; Riley et al., 1998). Nevertheless, chimeric analysis (that rescue the placentation defect) suggests that, like dHAND, eHAND is also required to ensure proper cardiac looping. However, it is not clear whether these looping defects are due to an intrinsic effect of the *HAND* genes on cardiomyocytes or are due to defects of other structures essential for proper cardiac development, such as the neural crest, which is defective in *dHAND* null mice.

4.1.5.2 The MEF2 family

A second family of transcription factors has also been linked to heart looping. Myocyte enhancer binding factor 2 (MEF2) family members, MEF2A, MEF2B, MEF2C, and MEF2D, belong to the MADS [MCM1, agamous, deficiens, and serum response factor (SRF)] box family of transcription factors (reviewed in (Black and Olson, 1998)) and are enriched in striated myocytes. MEF2 factors activate transcription through a conserved AT-rich motif found in the promoter of many skeletal and cardiac muscle genes. MEF2C and MEF2B are the earliest members expressed in the heart field, where their transcripts are detected shortly after GATA-4 and Nkx2-5. Later, MEF2A and MEF2D transcripts are also found in the developing heart. In *Drosophila*, the single *MEF2* gene is not required for the initial

specification of cardiac cells as the dorsal vessel forms normally in *MEF2* mutants (Lilly et al., 1995; Bour et al., 1995; Ranganayakulu et al., 1995). However, terminal muscle differentiation is not achieved, as evidenced by the lack of contractile gene expression in both cardiac and skeletal myocytes. This phenotype, together with the finding that the *MEF2* gene is a downstream transcriptional target of *tinman* (Gajewski et al., 1997), indicates that MEF2 controls late stages of cardiomyocyte differentiation

Consistent with this conclusion, null mutation of the *MEF2C* gene in mice results in developmental arrest at the looping stage (Lin et al., 1997). However, as these mice exhibit complex vascular malformations that impair blood circulation and embryonic growth, it is not clear whether the arrest at the looping stage is due to a direct effect of the *MEF2C* mutation on cardiomyocytes or due to defects in vascular development. Also notable are defects in endocardial development likely due to reduced myocardial expression of angiopoietin-1 and vascular-endothelial growth factor (VEGF) (Bi et al., 1999). Other cardiac differentiation markers, including cardiac α-actin, α-MHC, ANF, and myosin light-chain 1A (MLC1A), were also downregulated, indicating that MEF2C is essential for the transcription of a subset of myocyte genes. Surprisingly, the promoters of most of these MEF2C targets contain no or low affinity MEF2 binding sites and they are not significantly activated by MEF2 proteins in heterologous cells, suggesting a dependence on a cardiac-enriched cofactor for MEF2 action. In Chapter III, we provide evidence that MEF2 proteins are recruited to their target gene promoter by physical interaction with GATA-4 and that this interaction synergistically activates transcription. These results unravel a novel pathway for transcriptional regulation by MEF2 proteins.

4.1.6 Endocardium and endocardium derivatives development

The endocardium, a cellular layer lining the interior of the heart, has a developmental origin distinct from the vascular endothelium. During development, endocardial progenitors are localized at the periphery of the cardiogenic field and they become surrounded by the two myocardial layers of the linear tube as the two cardiac primordia fuse (Figure 1.4) (Lee et al., 1994; Sugi and Markwald, 1996). The cellular and molecular mechanisms of endocardial differentiation are unclear,

and the transcription factors involved in this process are only beginning to be defined. In the heart, two transcription factors, NF-ATc and GATA-5, are restricted to endocardial cells. As described in the next section, gene inactivation studies have implicated NF-ATc in valve formation, a specialized function of the endocard during development. Loss-of-function studies in an *in vitro* culture model revealed an essential role for GATA-5 in endocardial differentiation that could not be compensated by GATA-4 or GATA-6 (G. Nemer and M. Nemer, unpublished data). In addition, GATA-5 preferentially transactivates the promoter of endocard-specific genes, such as endothelin-1 (Nemer et al., 1999). These results support a crucial role for GATA-5 in endocardial differentiation and gene expression.

4.1.6.1 Valve formation and heart septation

The endocardium is essential for the generation of heart valves and membranous septa. Cardiac cushions, which consist of localized swellings in the cardiac jelly, are the primordia for the valves and septa. During development, endocardial cells undergo an epithelial to mesenchymal transformation while migrating to the cushions and proliferating there. Migration of the endocardial cells occurs in response to secreted molecules. Many evidence support a role for the TGF-β family members TGF-β1, TGF-β2, and TGF-β3 in this process. Consistent with a role for the TGF-β signaling pathway in the migration of endocardial cells to cushions, it was shown that the type III TGF-β receptor is essential for endocardial cell transformation and migration (Brown et al., 1999).

Atrioventricular (AV) canal cushions, which appear as a constriction between ventricular and atrial chambers of the looping heart, expand and fuse in the midline to partition the AV canal into right and left sides. The orifices will ultimately become the tricuspid and mitral valves that connect the septated atrial and ventricular chambers. The formation of the outflow tract valves contains many similarities to that of the AV canal formation; however, in this case, they arise from outflow tract cushions and further develop into the aortic and pulmonary valves (Eisenberg and Markwald, 1995).

Interatrial and interventricular septa, which divide the atria and the ventricle into right and left chambers, have multiple origins. A cardiac muscle wall is first

elaborated between the left and right sides of the atria and ventricles. Then, portions of the interatrial and interventricular septa arise from growth of the endocardial cushions toward the anterior and posterior directions of the looped heart, respectively. Many transcription factors have been shown to be involved in septation and myocardial growth. Mice homozygous for a null mutation in genes encoding the retinoic acid X receptor α (RXRα), N-myc, TEF1, Wilms tumor (WT1), and neurofibromatosis (NF1) display ventricular septal defects and ventricular wall hypoplasia (reviewed in (Rossant, 1996)). However, in most cases, the precise role of these transcription factors in cardiac development and their transcriptional targets remain to be determined.

4.1.6.1.1 The NF-AT family

NF-ATc, a member of the NF-AT family, has been shown to play a crucial role in valvular and septal development. During early development, NF-ATc expression is restricted to the endocardium, with higher expression in the endocardium of the AV canal and the outflow tract (de la Pompa et al., 1998; Ranger et al., 1998). Interestingly, inactivation of the NF-ATc gene in mice completely prevents formation of the pulmonary and aortic valves. Moreover, the interventricular septum and the tricuspid and mitral valves are defective in these mice. Remarkably, these defects correlate with regions that express higher levels of NF-ATc. Given that another member of the NF-AT family, NF-AT3, is known to interact with the GATA family members GATA-4, GATA-5, and GATA-6, it is tempting to speculate that the only two known endocardium-specific transcription factors, NF-ATc and GATA-5, may cooperate in endocardial differentiation and/or valve formation.

4.1.7 Pericardium development

The pericardium is the outer layer of the heart and contributes to form the vascular and connective tissues within the heart. The pericardium originates from a population of cells near the sinus venosus that migrates in a posteroanterior fashion to cover the developing heart and penetrates into the ventricular chamber walls to form the vessels of the coronary arteries. The epicardial α4-integrin and the myocardial VCAM-1 cell adhesion molecules are both required for adhesion of the pericardium to the myocardium (Kwee et al., 1995; Yang et al., 1995; Gurtner

et al., 1995). Transcription factors expressed and/or involved in the differentiation of the pericardium remain unknown. Analysis of the promoter regulatory elements specifying the epicardial expression of the α4-integrin gene could give insights into the mechanisms involved in epicardial cell development.

4.2 Transcription factors involved in hypertrophic cardiac growth

4.2.1 The calcineurin/NF-AT pathway

Soon after birth, cardiomyocytes lose their ability to proliferate and respond to growth stimulation by increasing their size, but not their number, a process known as cardiac hypertrophy. This process is characterized by cytoskeletal remodeling and the re-induction of a fetal genetic program, where many genes that were downregulated postnatally are re-induced. *In vitro*, many hypertrophic stimuli, such as endothelin-1 (Et-1), angiotensin II, the α-adrenergic agonist phenylephrine (Phe), stretch, and others, are able to induce cytoskeletal remodeling and the re-induction of the fetal genetic program in isolated cardiomyocytes. However, the nuclear events that they trigger to genetically reprogram the heart and establish cardiomyocyte hypertrophy are still unclear. Pharmacological manipulations have implicated many signal transduction pathways in cardiomyocyte hypertrophy; however, the transcriptional downstream effectors of most of them have not yet been identified clearly. One exception to this is the calcineurin/NF-AT signaling pathway, where it was shown that NF-AT3, a downstream effector of calcineurin (discussed earlier), is able to interact and cooperate with GATA-4 to activate cardiac gene expression in a Ca^{++}-dependent manner (Molkentin et al., 1998). Moreover, overexpression of an activated calcineurin or its effector NF-AT3 has been shown to induce cardiac hypertrophy in transgenic mice. Of note, in this model, cardiac hypertrophy induced by activated calcineurin was inhibited by the calcineurin inhibitor cyclosporin. Cyclosporin was also shown to prevent the development of cardiac hypertrophy in transgenic mice harboring cardiac-specific overexpression of a variety of mutant sarcomeric proteins (Sussman et al., 1998). However, cyclosporin is not able to inhibit the induction of hypertrophy in conventional genetic (spontaneously hypertensive rats; SHR) and hemodynamic overload (aortic banding) rodent models of cardiac hypertrophy (reviewed in

(Walsh, 1999)). These results raise the question of whether the calcineurin/NF-AT signaling pathway is involved in only a subset of cardiac hypertrophy.

4.3 Transcription Factors and Congenital Heart Diseases

The most common types of congenital heart diseases (CHD) are those related to incomplete septation of the atria, ventricles, or AV canal. Mutations in the genes coding for two transcription factors, Tbx5 and Nkx2-5, have been identified in families with a high incidence of atrial or ventricular septal defects. Dominant mutations of the *Tbx5* gene, a T-box transcription factor, cause the Holt-Oram syndrome, which is characterized by a developmental disorder affecting the heart and the upper limbs (Li et al., 1997; Basson et al., 1997). The most frequent cardiac abnormalities found in these patients are atrial and/or ventricular septal defects and conduction defects. These abnormalities correlate with the expression of Tbx5 in the heart and limbs during development. Most of the mutations identified in the *Tbx5* gene result in premature stop codons, preventing synthesis of the full-length protein.

Dominant mutations in the *Nkx2-5* gene were shown to be linked to septal defects and AV conduction abnormalities in four different families (Schott et al., 1998). The three mutations identified are predicted to affect DNA-binding and two of them result in truncated proteins.

AV canal, atrial, and septal defects are also present in 60% of patients with deletions or inverted duplications of chromosome 8p23.1. Haploinsufficiency of the *Gata4* gene has been reported in most patients with CHD associated with 8p23.1 monosomy (Pehlivan et al., 1999).

The dHAND transcription factor may also be involved indirectly in a different type of CHD, the DiGeorge syndrome. The most common cardiac defects presented by DiGeorge syndrome patients are conotruncal defects (persistent truncus arteriosus) and interruption of the aortic arch, which are both due to neural crest defects. More than 80% of affected individuals have microdeletions of chromosome 22q11, suggesting that one or more genes regulating neural crest cells may map to this region. Accordingly, many efforts have been directed at identifying the gene(s) localized in that region that could be responsible for the DiGeorge syndrome,

without success. However, based on the idea that dHAND is required for the survival of cells of neural crest-derived branchial and aortic arch arteries, but does not itself map to 22q11, it was hypothesized that dHAND could regulate the expression of a gene involved in the DiGeorge syndrome and mapping to chromosome 22q11. Indeed, a screen for dHAND targets led to the cloning of the *Ufd1* gene, a mouse homologue of a yeast gene involved in the degradation of ubiquitinated proteins and which maps to 22q11 (Yamagishi et al., 1999). Interestingly, Ufd1 is expressed in virtually all tissues affected in the DiGeorge syndrome, making it a good gene candidate for the DiGeorge syndrome.

4.4 Summary

Remarkable progress has been achieved in the identification of genes involved in heart development. NK2 and GATA transcription factor families, and more specifically Nkx2-5 and GATA-4, appear to play critical roles in heart field generation and cardiomyocyte differentiation. Later, fusion of the two cardiac primordia at the ventral midline to form the heart tube requires GATA-4. Although two transcription factor families, the HAND and the MEF2 families, have been linked to heart tube looping, it is not known whether they exert a direct intrinsic effect on cardiomyocytes or whether the developmental arrest observed at the looping stage in mice deficient for these genes is due to a defect in another tissue essential for proper heart development. However, a member of the homeodomain transcription factor family, Pitx2, has been shown to directly regulate asymmetric heart morphogenesis, although its role in cardiomyocytes remains undefined. Shortly after the initiation of cardiac looping, the cardiac valves and septa, which originate from endocardial cells, begin to form. NF-ATc, a member of the Rel-family of transcription factors that is specifically expressed in endocardial cells, is essential for valve formation and heart septation. However, the genes involved in the development of the two other layers of the heart, the endocard and the pericard, are still unknown.

The combinatorial regulation of gene expression is clearly emerging as a paradigm for cardiac-specific gene expression under normal and pathological cardiac conditions. The best-characterized example of cardiac combinatorial gene

regulation is the functional and physical interaction of Nkx2-5 and GATA-4, which could be important in mediating the cardiac-inducing activity of BMP2. In some pathological cardiac conditions, the calcineurin/NF-AT pathway appears to be involved in the regulation of cardiac gene expression; its effects may be exerted via interaction with GATA-4. As will be presented in Chapter IV and in the *Discussion*, other lines of evidence are also consistent with a role for GATA factors in cardiac hypertrophy.

Finally, the identification of mutations or haploinsufficiency in the transcription factors Nkx2-5, Tbx5, or GATA-4 in patients with congenital heart defects suggests that further investigation of the role of these transcription factors could benefit our understanding of the causes of cardiac abnormalities greatly.

5 HYPOTHESIS AND OBJECTIVES OF THIS WORK

As depicted in this introduction, the study of cardiac transcription is a useful means to understand how cardiogenesis and cardiac homeostasis are controlled. This, in turn, will help us understand the profound genetic and cellular changes that are associated with cardiac diseases.

The study of cardiac-specific gene transcription led our laboratory to the cloning of two cardiac transcription factors: GATA-4 and GATA-6. These two transcription factors are the only GATA factors expressed in postnatal cardiomyocytes. Given the embryonic lethality of GATA-4 and GATA-6 null mice, the postnatal role of these transcription factors in cardiomyocytes remained unknown. Thus, the first objective of my doctoral work was **to elucidate the role of GATA-4 and GATA-6 in postnatal cardiomyocytes.** We reasoned that the identification of GATA-4 and GATA-6 target genes would help to elucidate their functions. Indeed, this work established that GATA-4 and GATA-6 are required for the maintenance of cardiac gene expression and that GATA-4 and GATA-6 differentially regulate target genes (see Chapter II). This led me to the second objective of my doctoral work, which was **to determine the molecular basis for GATA factor specificity,** and allowed us to show that target gene specificity is due, at least in part, to GATA-4 and GATA-6 differential DNA-binding affinity.

In addition to differential DNA-binding affinity, GATA factors acquire specificity by differential interaction with cofactors. The only example reported yet is the cooperative interaction between Nkx2-5 and GATA-4, but not GATA-6 (Durocher et al., 1997). In Chapter III, we show that functional specificity is also imparted to GATA factors by their differential capacity to recruit and synergize with MEF2 cofactors. In addition, the recruitment of MEF2 proteins by GATA factors describes a novel pathway for transcriptional regulation by MEF2 factors and will be relevant to help to understand the mechanisms underlying cardiac gene transcription and development. More globally, this interaction provides a molecular paradigm for elucidating the mechanisms of action of MEF2 proteins in many tissues.

The third objective of my doctoral work was **to characterize the role and regulation of GATA-4 in cardiomyocyte sarcomere formation.** Sarcomeres are contractile units that consist of highly organized actin and myosin filaments. Many studies, including ours, have established a crucial role for GATA-4 in the regulation of the expression of the contractile actin and myosin protein genes in cardiomyocytes, suggesting that GATA-4 might play a role in cardiomyocyte sarcomere formation. Indeed, we found that overexpression of GATA-4 in cardiomyocytes induces sarcomere formation. Conversely, inhibiting GATA-4 expression blocked the sarcomere-inducing activity of hypertrophic stimuli, such as endothelin-1 (Et-1) and phenylephrine (Phe). These results are very similar to the effect of the small GTPase RhoA in cardiomyocytes and suggested that RhoA and GATA-4 may function in the same signaling pathway. Accordingly, we find that RhoA potentiates GATA-4 transcriptional activity. These results identify for the first time a signaling pathway converging on a transcription factor to control sarcomere reorganization and suggest that RhoA, in addition to its direct effect on myofibrillar assembly, also regulates the expression of sarcomeric proteins and sarcomere reorganization by inducing the transcriptional activity of GATA-4, thereby linking the regulation of transcription by RhoA and its effects on the cytoskeleton.

Thus, the main goal of my doctoral work was to characterize the roles, mechanisms of specificity, and regulation of GATA transcription factors in the heart. A better understanding of the molecular basis for the role of the GATA

proteins and their cofactors will help to understand how they regulate various steps of cardiac development and homeostasis. More globally, the information obtained from the study of GATA transcription factors in the heart will likely help to understand the molecular mechanisms underlying the function of GATA factors in other tissues.

CHAPTER II. COOPERATIVE INTERACTION BETWEEN GATA-4 AND GATA-6 REGULATES MYOCARDIAL GENE EXPRESSION

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FOREWORD

Given the embryonic lethality of GATA-4 and GATA-6 null mice, the postnatal role of these transcription factors in cardiomyocytes remained unknown. Thus, one of the objectives of my doctoral work was to elucidate the role of GATA-4 and GATA-6 in postnatal cardiomyocytes. Since we reasoned that the identification of GATA-4 and GATA-6 target genes would help to elucidate their exact functions, we developed an adenovirus-mediated antisense strategy to specifically inhibit GATA-4 and GATA-6 protein production in postnatal cardiomyocytes and assessed their effect on cardiac gene expression. This work established that GATA-4 and GATA-6 are required for the maintenance of cardiac gene expression. Importantly, this work also showed that GATA-4 and GATA-6 differentially regulate target genes and that this specificity is due, at least in part, to their differential DNA-binding affinity.

ABSTRACT

Two members of the GATA family of transcription factors, GATA-4 and GATA-6, are expressed in the developing and postnatal myocardium and are both equally potent transactivators of several cardiac promoters. However, several *in vitro* and *in vivo* lines of evidence suggest distinct roles for the two factors in the heart. Since identification of the endogenous downstream targets of GATA factors would greatly help to elucidate their exact functions, we have developed an adenovirus-mediated antisense strategy to specifically inhibit GATA-4 and GATA-6 protein production in postnatal cardiomyocytes. Expression of several endogenous cardiac genes was significantly downregulated in cells lacking GATA-4 or –6 indicating that these factors are required for the maintenance of the cardiac genetic program. Interestingly, transcription of some genes like α- and β-MHC was preferentially regulated by GATA-4 due, in part, to higher affinity of GATA-4 for their promoter GATA element. However, transcription of several other genes including ANF and BNP was similarly down-regulated in cardiomyocytes lacking one or both GATA factors, suggesting that GATA-4 and –6 could act through the same transcriptional pathway. Consistent with this, GATA-4 and –6 were found to co-localize in postnatal cardiomyocytes and to interact functionally and physically to provide cooperative activation of the ANF and BNP promoters. The results identify for the first time *bona fide in vivo* targets for GATA-4 and GATA-6 in the myocardium. The data also show that GATA factors act in concert to regulate distinct subsets of genes suggesting that combinatorial interactions among GATA factors may differentially control various cellular processes.

INTRODUCTION

The vertebrate GATA transcription factors share a highly conserved domain composed of two zinc fingers (39). This domain is responsible for specific binding to a consensus WGATAR element. Based on sequence homology and tissue distribution, the vertebrate GATA family can be divided in two subgroups. The first subgroup is composed of GATA-1, -2, and -3. These three GATA factors are expressed in the hematopoietic system and are essential for normal hematopoiesis (36;37;39;40;44). The second subgroup is composed of GATA-4, -5, and -6 which are differentially expressed in the heart and gut (26). Within the heart, GATA-5 is restricted to the endocardium (22) whereas GATA-4 and -6 are expressed in the developing and postnatal myocardium (14;18;21;33).

Several lines of evidence have implicated GATA-4 in diverse developmental processes including survival, differentiation, and/or migration of cardiomyocyte precursors. For example, *in vitro* experiments using embryonic stem cells showed that GATA-4 is essential for survival of cardioblasts and terminal cardiomyocyte differentiation (15;16). *In vivo*, inactivation of the GATA-4 gene is embryonic lethal at day 9.5 post-coitum (dpc) due to failure of the GATA-4 null mice to develop a primitive heart tube (25;31). Unfortunately, the requirement for GATA-4 at early stages of heart development has precluded analysis of its role in the postnatal myocardium either *in vitro* or *in vivo*. Nevertheless, two lines of evidence suggest that GATA-4 may play a critical role in postnatal cardiac transcription: GATA elements were found to be essential for activation of some cardiac promoters in adult myocardium (17;19;28;30) and GATA-4 was shown to functionally and physically interact with NFAT-3 which would implicate it as a mediator of the calcineurin-dependent hypertrophic process in the myocardium (32).

Analysis of GATA-6 in the myocardium have so far been more limited but the available data are consistent with a role for GATA-6 in myocardial development and gene expression. Thus, axis disruption experiments in *Xenopus* showed that transcription of GATA-6, much like GATA-4 and -5, correlates with specification of cardiac progenitors and ectopic expression of either of those factors activate the α-myosin heavy chain (α-MHC) and the cardiac α-actin (c. α-actin) genes (21).

Moreover, co-transfection experiments in heterologous cells showed that GATA-6 is as potent as GATA-4 in transactivating cardiac genes harboring GATA elements in their promoter, like cardiac troponin C (cTnC) and atrial natriuretic factor (ANF) (9;33). Interestingly, Gove *et al.* have reported that, in *Xenopus* embryos, GATA-6 over-expression blocks differentiation and stimulate proliferation of heart precursors (12). Recently, the inactivation of the GATA-6 gene in mice was reported to be embryonic lethal at 7.5 dpc, precluding analysis of its role in the heart (34). Finally, the inability of GATA-6 to compensate for GATA-4 in the GATA-4 null mice suggest that GATA-4 and GATA-6 would be playing different roles *in vivo*.

The molecular basis for the differential roles of GATA-4 and -6 in the myocardium remain largely unknown, but could occur via at least three non-exclusive mechanisms: differential expression of GATA-4 and GATA-6 in subsets of cardiomyocytes, differential affinity of GATA factors for GATA elements and therefore different *in vivo* target genes, or differential interaction of GATA-4 and -6 with co-factors. Support for this latter possibility was recently provided by Durocher *et al.*, who showed functional and physical interaction between GATA-4, but not GATA-6, and the cardiac homeodomain protein Nkx2-5 (9;10). However, the relative affinities of GATA-4 and -6 for their DNA binding sites have not been determined and whether GATA-4 and -6 localize differentially in the heart and target distinct genes is presently unknown.

In order to address these issues, we have developed antibodies specific for the different cardiac GATA factors and analyzed at the cellular level the localization of GATA-4 and -6 in the myocardium. We also developed an adenovirus-mediated antisense strategy to specifically inhibit GATA-4 and GATA-6 protein production in neonatal cardiomyocytes and assess its effect on cardiac gene expression. The results indicate that several endogenous cardiac genes, including atrial natriuretic factor (ANF), B-type natriuretic peptide (BNP), α-myosin heavy chain (α-MHC), β-myosin heavy chain (β-MHC), cardiac troponin I (cTnI), and the platelet-derived growth factor receptor β (PDGFRβ), are down-regulated in cardiomyocytes lacking either GATA-4 or GATA-6, suggesting that these genes are *bona fide* targets for

both GATA-4 and GATA-6. Interestingly, the α- and β-MHC genes are preferential targets for GATA-4 likely due to the higher affinity of GATA-4 for their promoter GATA element. Remarkably, GATA-4 and GATA-6 co-localize in postnatal cardiomyocytes and interact functionally and physically to provide cooperative activation of the ANF and BNP promoters. These results suggest that GATA factors are involved in the maintenance of the cardiac phenotype and that expression of cardiac genes is controlled by combinatorial interactions of the different GATA proteins.

MATERIALS AND METHODS

Plasmids and Adenoviral Vectors. The recombinant replication-deficient adenovirus type 5 (Ad5) expressing antisenses directed specifically toward GATA-4 or -6 were generated using the cloning system developed and generously provided by F.L. Graham (29). Briefly, a 358 bp *Eco*RI/*Hind*III fragment encoding the extreme N-terminal portion of rat GATA-4 and a 359 bp *Xba*I/*Stu*I fragment from the 5'-untranslated region (UTR) of rat GATA-6 were subcloned respectively into *Hind*III/*Eco*RI and *Eco*RV/*Xba*I between left-end adenoviral sequences in Ad5 shuttle vector pΔE1sp1B/CMV/BGH, a plasmid generously provided by B.A. French (1). This plasmid was constructed by inserting the 1276 bp *Bgl*II/*Pvu*II fragment (containing the CMV IE promoter, polylinker, and bovine growth hormone polyadenylation signal) from pcDNA3 (Invitrogen Corp., San Diego, CA) between the *Bgl*II/Klenow-blunted *Cla*I sites of the polylinker in Ad5 shuttle vector pΔE1sp1B (6). Each shuttle vector was co-transfected into 293 embryonic kidney cell line with pJM17 that contains a circularized *dl*309 adenoviral genome to generate replication deficient viruses with substitution of the Ad5 E1 genes for the antisense GATA-4 or GATA-6 sequences (AS4 and AS6). The virus Ad5/CMV/nls-LacZ carrying an expression cassette in which the CMV IE promoter transcribes sequences encoding the SV40 large T-antigen nuclear localization signal fused to the *E. coli lacZ* reporter gene was used as control (Ctl, a generous gift from B.A. French) (11). Putative Ad5 clones were plaque-purified, screened for antisense inserts,

propagated, isolated, and tittered according to the protocol of Graham and Prevec (13), to produce viral stocks with titers > 2 x 10^9 PFU/ml.

Wild-type rat ANF and BNP reporter plasmids and wild-type rat GATA-4 expression vector (pCG-GATA-4) were described previously (14). The various deletions or mutations of the ANF promoter and GATA-4 cDNA were performed by PCR or by the Altered Sites *in vitro* mutagenesis system (Promega Corp., Madison, WI) as described by the manufacturer. The polyhistidine-tagged GATA-4 constructs used for *in vitro* transcription and translation were generated by insertion of the *Xba*I-*Bam*HI fragment of the corresponding pCG-GATA-4 construct into the *Nhe*I-*Bam*HI or *Nhe*I-*Bgl*II sites of pRSETA (Invitrogen Corp.). The rat GATA-6 cDNA was cloned by PCR and sub-cloned into the pcDNA3 expression vector. All constructs were confirmed by sequencing.

Neonatal cardiomyocyte preparation, infection, and transfection. Primary cultures of cardiac myocytes were prepared from 4-day-old Sprague-Dawley rats with small modifications to previously described methods (4). Essentially, ventricles and atria of ~60 to 72 hearts were digested four to five times, 15 min each, in Joklik's modified Eagle's medium (Canadian Life Technologies Inc.), containing 18 mM HEPES (pH 7.4), 0.1 % collagenase (~250 units/ml, Worthington Biochemical Corp.), and 5 µg/ml of DNase I (~2000 units/mg, Boehringer Mannheim Canada). The enzymatic digestion was stopped with fetal bovine serum (FBS, Qualified grade, Canadian Life Technologies Inc.), and the undigested tissue removed by filtration through a nylon mesh (pore size 100 µm). Cardiomyocytes were purified by three pre-plating of 20 min each to remove residual non-myocytes by differential adhesiveness, then plated at a density of 0.5 x 10^6 cells/35-mm dish (Primeria, Falcon) and cultured 16 to 24 h in Dulbecco's modified Eagle's medium (DMEM, Canadian Life Technologies Inc.) containing 10 % FBS. The following day, the media was changed for serum-free hormonally defined medium (SFHD) as previously described (4).

Transfections were carried out using calcium phosphate precipitation 24 h after plating. For basal ANF promoter activity, cardiomyocytes were transfected with 6 µg of a wild-type or mutant ANF-luciferase reporter. At 36 h post-

transfection, cells were harvested and luciferase activity was assayed using a Berthold LB 953 luminometer.

Serial dilutions of recombinant Ad5 (Ctl, AS4, or AS6) were prepared in OptiMEMI (Canadian Life Technologies Inc.). Cardiomyocytes were exposed to 200 µL of OptiMEMI containing 0.5 x 10^6, 2 x 10^6, or 8 x 10^6 PFU of recombinant Ad5 for 30 min at 25°C and 2 ml of SFHD was added to each petri. The medium was changed 16 h later with fresh SFHD. The cells were kept for 3 to 5 days with the medium changed every 24 h with fresh SFHD. Just before changing, aliquots of the medium were taken for ANF concentration determination.

β-Galactosidase detection. One day after infection, cardiomyocytes were fixed in 0.5 % glutaraldehyde-phosphate buffered saline (PBS) for 10 min, washed twice 30 min with PBS containing 0.02 % NP-40 and stained with 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 1 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Boehringer Mannheim Corp.), 0.01 % deoxycholic acid and 0.02 % NP-40 in PBS for 1 to 2 h at 25°C in the dark. The stained cardiomyocytes were washed with PBS and 2 ml of 70 % glycerol was added to each petri. The stained cells were kept at 4°C until photographed.

RNA extraction and Northern blots. Total RNA was isolated from cardiomyocytes by the guanidium thiocyanate-phenol-chloroform method as previously described (3). RNA was denatured with formaldehyde and formamide, size-fractionated on a 1.2 % agarose gel as previously described and transferred to a nylon membranes (MSI, Westborough, MA) by capillary blotting with 20 x SSC and cross-linked to the membrane with the UV Stratalinker 2400 (120 mjoules, Stratagene). Blots were hybridized with random prime-labeled rat cDNA probes for GATA-4, GATA-6, ANF, and GAPDH. The GATA-4 and -6 cDNA fragments are the same used to generate the AS4 and AS6 adenoviruses. The ANF cDNA was described (5) and the GAPDH cDNA was generously provided by Dr. P. Jolicoeur. Blots were exposed in PhosphorImager cassette and analyzed with ImageQuant (Molecular Dynamics).

Cell cultures and transfections. HeLa or L cells were grown in DMEM supplemented with 10% FBS. Transfections were carried out using calcium

phosphate precipitation 24 h after plating. For over-expressions, 800 000 L cells were plated per 100 mm petri dish and transfected with 40 μg of pCG, pCG-GATA-4, pCG-GATA-4 mutants, or pCDNA3-GATA-6 expression vector. At 36 h post-transfection, cells were harvested and nuclear extracts were prepared as previously described (14). Nuclear extract protein concentrations were quantitated by the Bradford assay (Bio-Rad Laboratories, Hercules, CA).

For ANF promoter transactivation assays, 100 000 HeLa cells were plated per 35 mm petri dish and transfected with 3 μg of ANF-luciferase reporter plasmid and 1 μg of GATA expression vector. For synergy, 200 ng of GATA-6 and 200 ng of wild-type or mutant GATA-4 expression vectors were used. The total amount of DNA was kept constant at 4 μg. At 36 h post-transfection, cells were harvested and luciferase activity was assayed.

Electrophoretic mobility shift assays (EMSAs). Binding reactions were performed in 20-μl reaction mixtures containing 3 μg of nuclear extracts from L cells over-expressing GATA-4 or GATA-6 in a buffer containing 12 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 60 mM KCl, 4 mM Tris-HCl (pH 7.9), 0.6 mM EDTA, 0.6 mM DTT, 0.5 mg/ml BSA, 1 μg poly(dIdC), 12% glycerol, 20 000 cpm of radio-labeled double-stranded -120 bp ANF GATA probe, and increasing amount of the appropriate unlabeled competitor for 20 min at room temperature. Reactions were then loaded on a 4% polyacrylamide gel, and run at 200V at room temperature in 0.25X TBE. The gel was then dried and exposed to a PhosphorImager (Molecular Dynamics) cassette for quantitative analysis. Relative bindings were quantitated and plotted as a function of the unlabeled competitor amount. Probes used were, from 5` to 3` (only the coding strand is shown), rat ANF -120 (proximal) GATCTCGCTGGACTGATAACTTTAAAAGG, rat ANF -120mut TGACAAGCTTCGCTGGACT**CC**TAACTTTAAAAG, rat ANF -280 (distal) GATCTCCCAGGA AGATAACCAAGGACTCG, and rat □-MHC -265 bp GATCCTCCTCTATCTGCCCATCA, where the WGATAR consensus are underlined and the mutations are highlighted in bold.

For DNA-binding affinity measurement, EMSAs were performed in the conditions mentioned above, but using an increasing amount of radio-labeled ANF

proximal or distal GATA probe with a constant amount (3 μg) of nuclear extracts from L cells over-expressing GATA-4 or GATA-6. Scatchard analysis was then performed on the binding data by plotting the bound/free DNA ratio in function of the bound DNA. The resulting dissociation constant (Kd) was calculated using Microsoft Excel.

Western blots. Three days after infection, cardiomyocytes were harvested and nuclear extracts were prepared. 20 μg of cardiomyocyte nuclear extracts and 2 μg of GATA-4 or GATA-6 over-expressing L cell nuclear extracts were boiled in Laemmli buffer and resolved by SDS-PAGE. Proteins were transferred on Hybond-PVDF membrane and immunoblotted using the Renaissance chemiluminescence system (NEN Life Sciences, Boston, MA) as described by the manufacturer. Goat GATA-4 supershift antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a dilution of 1/1000 and was revealed with an anti-goat horseradish peroxydase antibody (Sigma, St-Louis, MO) at a dilution of 1/100 000. The GATA-6 antibody was made in rabbits by injection of a GATA-6 specific peptide linked to keyhole limpet hemocyanin (KLH) as described (2). The purified GATA-6 antibody was used at a dilution of 1/1000 and was revealed with an anti-rabbit horseradish peroxydase antibody (Sigma, St-Louis, MO) at a dilution of 1/100 000.

Radio-immunoassays (RIA). Immunoreactive ANF (irANF) concentration was determined in the cardiomyocyte culture medium by RIA as previously described (3).

Pull-down assays. Polyhistidine-tagged GATA-4 and wild-type GATA-6 were *in vitro* co-transcribed and co-translated in presence of radio-labeled methionine according to the manufacturer's protocol (TNT reticulocyte lysate kit; Promega Corp.). The proteins were then allowed to interact at 4°C with agitation in 400 μl of binding buffer [150 mM NaCl, 50 mM Tris-Cl (pH 7.5), 0.3% Nonidet P-40, 10 mM $ZnCl_2$, 1 mM dithiothreitol, 0.25% BSA] as described (9). After 2 hours, 50 μl of nickel resin (ProBond resin; Invitrogen Corp.) was added to the reaction and incubated further for 2 h at 4°C. The resin was then washed 3 times with binding buffer and twice with binding buffer minus BSA. Interacting proteins were resolved

by 15% SDS-PAGE. The gel was dried and exposed to a PhosphorImager cassette.

Cross-linking. Cardiomyocyte whole-cell extracts were prepared in RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and a cocktail of protease inhibitors) by repeated aspiration through a syringe needle, followed by centrifugation to pellet cellular debris. The supernatant was then incubated on ice for increasing amount of time in presence of 0.02% glutaraldehyde and the reaction was blocked by the addition of 1 M glycine (pH 7.6). To reduce non-specific binding, the cellular extracts were pre-incubated at 4°C for 30 min with 1 μg of normal goat serum and 20 μl of protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA). GATA-4/GATA-6 complexes were immunoprecipitated at 4°C overnight with agitation using 1 μl of anti-GATA-4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and 20 μl of protein A/G PLUS-Agarose. Subsequently, the immunoprecipitates were washed four times in RIPA, resolved by SDS-PAGE, and transferred to a PVDF membrane. The presence of the GATA-4/GATA-6 complexes was revealed by Western blotting using an anti-GATA-6 antibody.

Immunofluorescence. Paraformaldehyde-fixed heart sections from neonatal mice were processed for immunofluorescence as described (41). The GATA-4 antibody was used at a dilution of 1/500 and was revealed with a biotinylated anti-goat antibody (1/200; Vector Laboratories Inc., Burlingame, CA) followed by avidin-rhodamine (1/200; Vector Laboratories Inc.). The purified GATA-6 antibody was used at a dilution of 1/50 and was revealed with an anti-rabbit-FITC antibody (1/200; Sigma, St-Louis, MO).

RESULTS

***In vivo* target genes for GATA-4 and GATA-6 in postnatal atrial and ventricular cardiomyocytes.** In order to analyze the consequence of inhibiting GATA-4 or GATA-6 expression on endogenous myocardial gene expression, we used adenovirus-mediated gene delivery of antisense to cardiomyocytes in primary culture. For this, we initially determined the dose of adenovirus that would

efficiently infect ventricular cardiomyocytes isolated from 4-day neonatal rats using a replication-deficient adenovirus expressing a lacZ gene fused to a nuclear localization signal (Ctl adenovirus). At a multiplicity of infection (MOI) of 1, all the cardiomyocytes showed β-galactosidase activity (blue staining) in the nucleus with staining intensity increasing in a dose-dependent manner up to a MOI of 16 (Fig. 2.1B-D). No staining was observed in mock-infected cardiomyocytes (Fig. 2.1A). Similar results were obtained with atrial neonatal cardiomyocytes (data not shown). Higher dose of the Ctl adenovirus lead to cytotoxicity (data not shown). Thus, a MOI ranging from 1 to 16, which achieves efficient infection without any apparent cytotoxicity (Fig. 2.1A-D), was used in subsequent experiments.

In order to specifically inhibit GATA-4 or GATA-6 protein production, replication-deficient adenoviruses expressing an antisense directed specifically toward GATA-4 or GATA-6 were generated. A 358 bp fragment from the extreme N-terminal portion of GATA-4 was used for the antisense GATA-4 adenovirus (AS4) production (Fig. 2.2A). For the antisense GATA-6 adenovirus (AS6), a 359 bp fragment from the 5'-untranslated region (UTR) was used. These regions were chosen because of their low overall homology with other GATA factors and, as shown in Fig. 2.2B, the DNA fragments used to generate the AS4 and AS6 show no cross-hybridization with each other mRNAs.

Ventricular neonatal cardiomyocytes were infected at a MOI of 1, 4, and 16 with Ctl, AS4, and AS6 and RNA was isolated 3 days post-infection. Northern blot analysis showed that both antisense transgenes were efficiently expressed in a dose-dependent manner, to a level exceeding 100-fold that of endogenous GATA-4 or -6 (Fig. 2.2B). AS4 and AS6 were also specific and efficient at reducing GATA-4 and GATA-6 protein levels in a dose-dependent manner, as evidenced by Western blot analysis (Fig. 2.2B). As shown in Fig. 2.2C, AS4 reduced GATA-4 levels by 80% while AS6 reduced GATA-6 levels by 60% at a MOI of 4. The effect of AS4 and AS6 extended also to GATA-binding activity as assessed by EMSA; a 50% decrease in GATA-binding over the ANF -120 GATA probe was observed with AS4 or AS6 indicating that indeed both GATA-4 and -6 contribute to the GATA-binding activity in postnatal cardiomyocytes (data not shown). Moreover, AS4 and

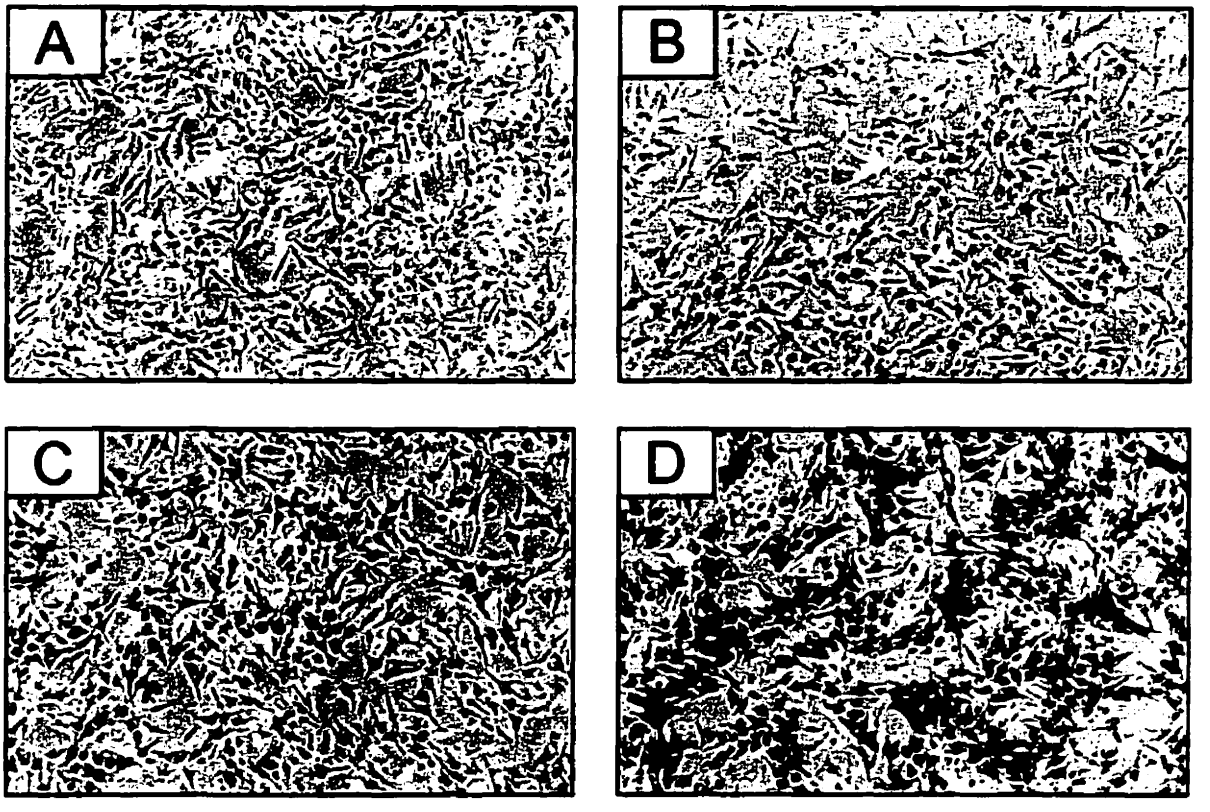
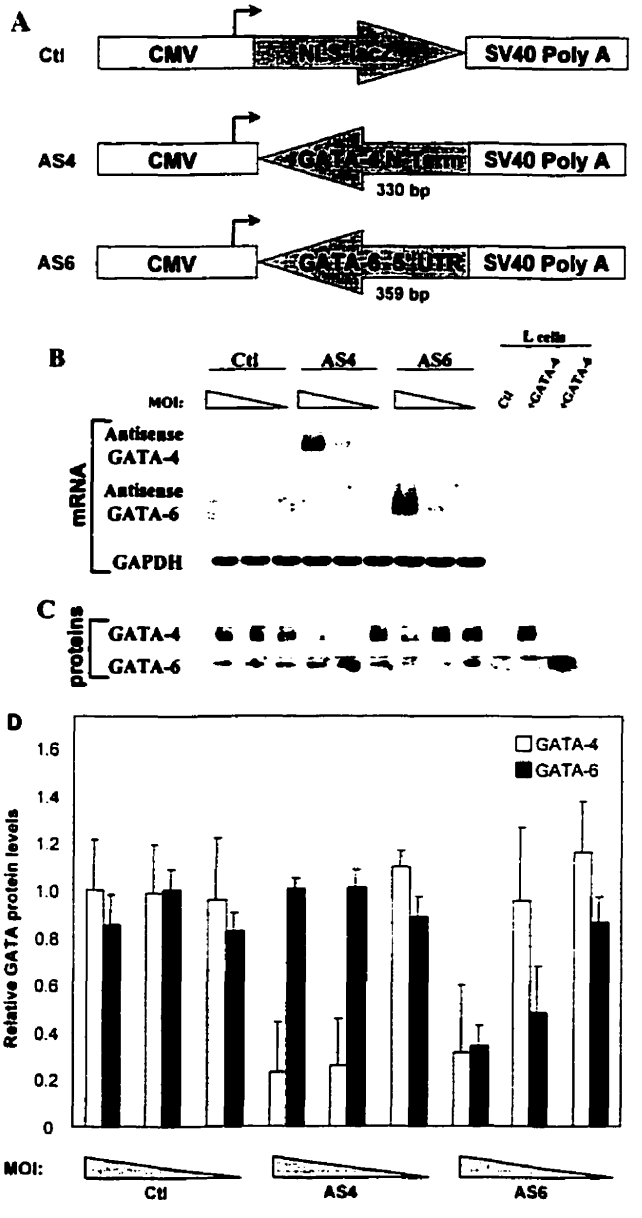


Figure 2.1. Cardiomyocytes are efficiently infected by adenovirus. Ventricular cardiomyocytes isolated from 4-day neonatal rats were mock-infected (**A**), infected at a MOI of 1 (**B**), MOI of 4 (**C**), and MOI of 16 (**D**) with the Ctl adenovirus which expresses the lacZ gene fused to a nuclear localization signal (NLS-lacZ). Twenty hours later, cells were assayed for β-galactosidase activity.

Figure 2.2. Characterization of the AS4 and AS6 adenoviruses. (**A**) Schematic representation of the constructs used to generate the recombinant adenoviruses. The NLS-lacZ, a 358 bp fragment from the extreme N-terminal of GATA-4, or a 359 bp fragment from the 5`-UTR of GATA-6 were cloned downstream of the CMV promoter and upstream of a SV40 polyA sequence in order to generate the recombinant adenoviruses. (**B**) Transgene expression is dose-dependent. Ventricular cardiomyocytes were infected at a MOI of 16, 4, and 1 (corresponding to the progressively narrowing triangle) with Ctl, AS4, and AS6. RNA was isolated 3 days post-infection. Northern blot analysis showed that both antisense transgenes were efficiently expressed in a dose-dependent manner. **(C)** AS4 and AS6 are specific and efficient at decreasing GATA-4 and GATA-6 protein levels. Ventricular cardiomyocytes were infected as mentioned above and nuclear extracts were isolated 3 days post-infection and analyzed by Western blot. L cells over-expressing GATA-4 or GATA-6 were used as controls for the specificity of the antibodies. (**D**) Quantification of the effect of AS4 and AS6 on GATA-4 and GATA-6 protein levels. The data represent the mean of two independent Western blots performed as described in (B) and quantified by densitometry. At a MOI of 4, GATA-4 levels were decreased specifically by AS4 (80% reduction), while GATA-6 levels were reduced specifically by AS6 (60% reduction).


A
Ctl
CMV
SV40 Poly A
AS4
CMV
SV40 Poly A
330 bp
AS6
CMV
SV40 Poly A
359 bp
B
Ctl
AS4
AS6
L cells
MOI:
Antisense GATA-4
Antisense GATA-6
GAPDH
mRNA
C
GATA-4
GATA-6
proteins
D
GATA-4
GATA-6
Relative GATA protein levels
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
MOI:
Ctl
AS4
AS6

AS6 had no effect on GATA-5 mRNA levels, suggesting that GATA-5 does not compensate for the lack of GATA-4 or GATA-6 in postnatal cardiomyocytes (data not shown).

In order to assess the effects of knocking-down GATA-4 or GATA-6 on endogenous gene expression, we initially analyzed changes in ANF mRNA and protein levels. The ANF promoter contains two conserved GATA elements (Fig. 2.4A) and was previously shown to be transactivated by GATA-4 and GATA-6 in heterologous cells (9;14). In GATA-4 or GATA-6 knocked-down ventricular neonatal cardiomyocytes, immuno-reactive ANF (irANF) secretion was decreased in a time-dependent manner (Fig. 2.3A). The effect was first observed at 3 days post-infection (50% decrease) and irANF secretion was decreased by 60 to 80% at 5 days post-infection. The decrease of secreted irANF was dose-dependent (Fig. 2.3B). Interestingly, the knock-down of both GATA-4 and GATA-6 (AS4 + AS6) had the same effect on irANF secretion as the knock-down of GATA-4 (AS4) or GATA-6 (AS6) alone (Fig. 2.3B), suggesting that either a maximal threshold is reached or that GATA-4 and GATA-6 are in the same transcriptional pathway. The effect of knocking-down GATA-4 or GATA-6 on ANF expression extended to the transcript level (Fig. 2.3C). Similar results were obtained with atrial cardiomyocytes (data not shown) and indicate that the ANF gene is an *in vivo* transcriptional target for GATA-4 and GATA-6 in postnatal cardiomyocytes.

We also verified if other cardiac genes were affected by the decrease in GATA-4 or GATA-6. BNP, α-MHC, β-MHC, cTnI, and PDGFRβ mRNA levels were down-regulated by AS4 and AS6 (Fig. 2.3D). Interestingly, α-MHC and β-MHC were preferentially down-regulated by AS4, suggesting that these genes are preferential targets for GATA-4. The lack of GATA factors did not affect all cardiac genes; for example, cardiac α-actin, myosin light chain 1 (MLC1), and GAPDH mRNA levels were not altered by AS4 or AS6 (Fig. 2.3C, 3D, and data not shown). Similar results were obtained by semi-quantitative RT-PCR analysis (data not shown). Thus, GATA-4 and GATA-6 are involved in regulating specific subsets of cardiac genes in postnatal cardiomyocytes.

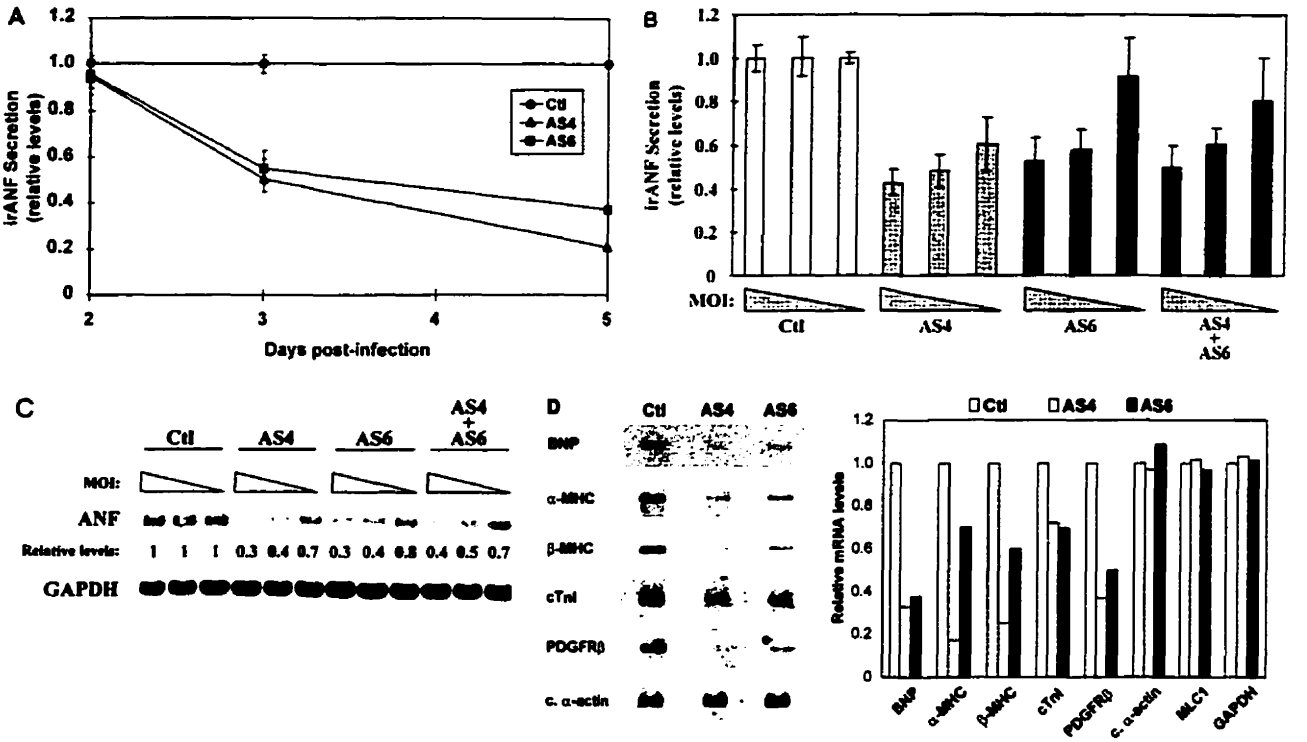


Figure 2.3. *In vivo* transcriptional targets for GATA-4 and GATA-6 in postnatal cardiomyocytes. (**A**) irANF secretion is decreased in a time-dependent manner by AS4 and AS6. Ventricular cardiomyocytes were infected at a MOI of 4 with Ctl, AS4, and AS6. Secreted irANF was assayed in the cardiomyocyte culture medium after a 24-hour accumulation period at 2, 3, and 5 days post-infection. Since the effect of the antisense on irANF secretion is clearly visible at 3 days post-infection, subsequent analyses were performed at this time point. (**B**) irANF secretion is decreased in a dose-dependent manner by AS4, AS6, and AS4+AS6. Ventricular cardiomyocytes were infected at a MOI of 16, 4, and 1 (corresponding to the progressively narrowing triangle) with Ctl, AS4, AS6, and AS4+AS6. (**C**) ANF mRNA levels are decreased in a dose-dependent manner by AS4, AS6, and AS4+AS6. Ventricular cardiomyocytes were infected as in (B) and RNA was analyzed by Northern blot. The ANF mRNA levels are expressed relative to the Ctl-infected cardiomyocytes. The GAPDH mRNA levels were unaffected. (**D**) Many cardiac genes are decreased by AS4 and AS6. Ventricular cardiomyocytes were infected at a MOI of 4 with Ctl, AS4, and AS6 and Northern blot analysis was performed (left panel). Relative mRNA levels were quantified using PhosphorImager (right panel). Note how α-MHC and β-MHC mRNA are preferentially down-regulated by AS4 whereas c. α-actin, MLC1, and GAPDH mRNA levels were not affected by AS4 or AS6.

ANF is a direct transcriptional target for both GATA-4 and GATA-6. To determine if ANF is a direct transcriptional target for both GATA-4 and GATA-6, a detailed analysis of the ANF promoter was performed. Comparison of the ANF promoter sequences available in the database (from human, rat, mouse, sheep, and bovine) shows that two consensus WGATAR elements, a proximal one at -120 bp and a distal one at -280 bp, are entirely conserved across species (Fig. 2.4A), suggesting that these GATA elements might play important evolutionay conserved functions in ANF gene regulation. Indeed, deletion or point mutation of the proximal or the distal GATA element decreased by about 50% ANF promoter activity in postnatal cardiomyocytes (Fig. 2.4B). A more drastic effect was observed when both elements were mutated, leaving only 30% of wild-type promoter activity. Similar results were observed for all constructs using 1-day ventricular or atrial cardiomyocytes. Thus, both proximal and distal GATA elements are major contributors of ANF promoter activity in postnatal cardiomyocytes.

Since both GATA-4 and GATA-6 are present in neonatal cardiomyocytes, we tested their relative efficiency at transactivating the ANF promoter. The results indicate that both GATA factors are potent activators of the ANF promoter, exhibiting about 25- to 30-fold activation (Fig. 2.4B). However, while mutation of the proximal or distal GATA element reduced GATA-4 transactivation by approximately 50%, mutation of the proximal GATA element fully abrogated GATA-6 transactivation.

To test whether this was due to differential affinity of GATA-4 and -6 for certain GATA elements, we analyzed the binding affinities of GATA-4 and GATA-6 for both ANF GATA elements. EMSAs were performed using increasing amount of radio-labeled probes and a constant amount of nuclear extracts from L cells over-expressing GATA-4 or GATA-6 (Fig. 2.5A). Scatchard analysis revealed that GATA-4 has a similar relative affinity for the proximal and for the distal GATA elements (relative Kd of 1,41 and 2,73 nM, respectively). However, the relative affinity of GATA-6 for the distal element was 8-fold lower than for the proximal GATA element (relative Kd of 6,4 and 0,81 nM, respectively). Similar Kds were

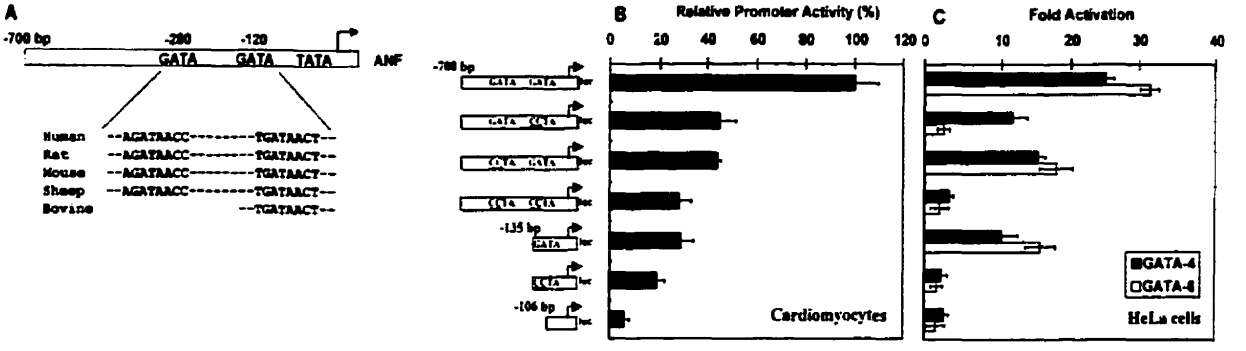


Figure 2.4. ANF is a direct transcriptional target for both GATA-4 and GATA-6. (**A**) Alignment of the ANF promoters from human, rat, mouse, sheep, and bovine (for which only a partial sequence is available). Note that two consensus WGATAR elements, a proximal one at -120 bp and a distal one at -280 bp, are entirely conserved across species. (**B**) *Left panel.* The proximal and the distal GATA elements are major contributors of ANF promoter activity in neonatal cardiomyocytes. Wild-type and mutated ANF promoters fused to the luciferase reporter gene were transiently transfected into ventricular cardiomyocytes isolated from 4-day neonatal rats. Luciferase activity was assayed 36 h post-transfection. The results are expressed relative to the -700 bp ANF promoter activity. *Right panel.* GATA-4 and GATA-6 transactivate the ANF promoter. HeLa cells were co-transfected with a GATA-4 or GATA-6 expression vector and wild-type or mutated ANF promoters fused to the luciferase reporter gene. Luciferase activity was assayed 36 h post-transfection. The results are expressed in fold activation by GATA-4 or GATA-6. In all cases, the data represent the mean ± SD of 2 to 3 independent experiments carried out in duplicate.

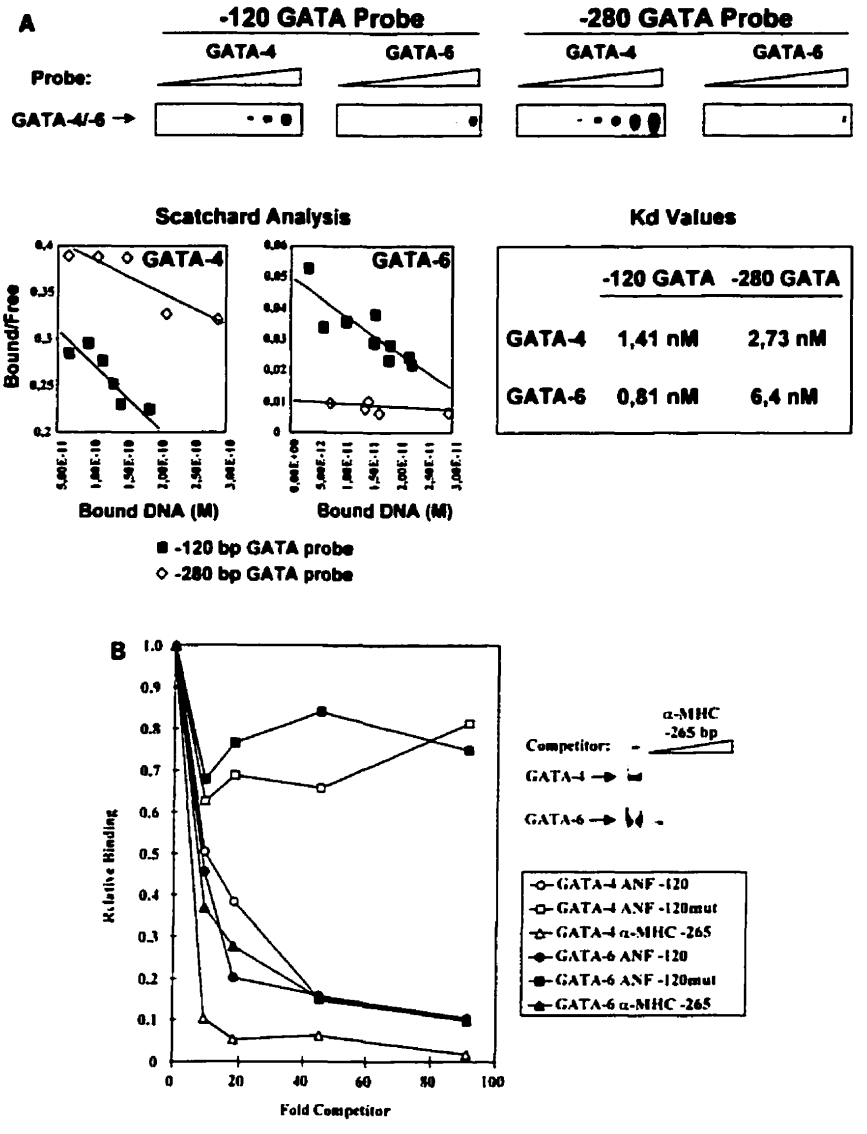


Figure 2.5. GATA-4 and GATA-6 bind GATA elements with different affinities. (**A**) EMSAs were performed using increasing amount of radio-labeled probes (-120 bp GATA or -280 bp GATA) and a constant amount of nuclear extracts from L cells over-expressing GATA-4 or GATA-6. The GATA binding is shown in the upper panel. Scatchard analysis were performed on the binding data and the relative affinities (Kd values) are shown. (**B**) GATA-4 has higher affinity than GATA-6 for the -265 bp a-MHC GATA element. EMSAs were performed using nuclear extracts from L cells over-expressing GATA-4 or GATA-6 incubated with radio-labeled -120 bp ANF GATA element and increasing amount of an unlabeled competitor (-120 bp ANF GATA, -120 bp ANF GATAmut, and -265 bp α-MHC GATA; top right panel). Relative bindings were quantitated and plotted as a function of the amount of unlabeled competitor (left panel).

obtained with bacterially-expressed proteins (data not shown). These results show for the first time that GATA-4 and GATA-6 possess differential affinities for naturally occurring sites.

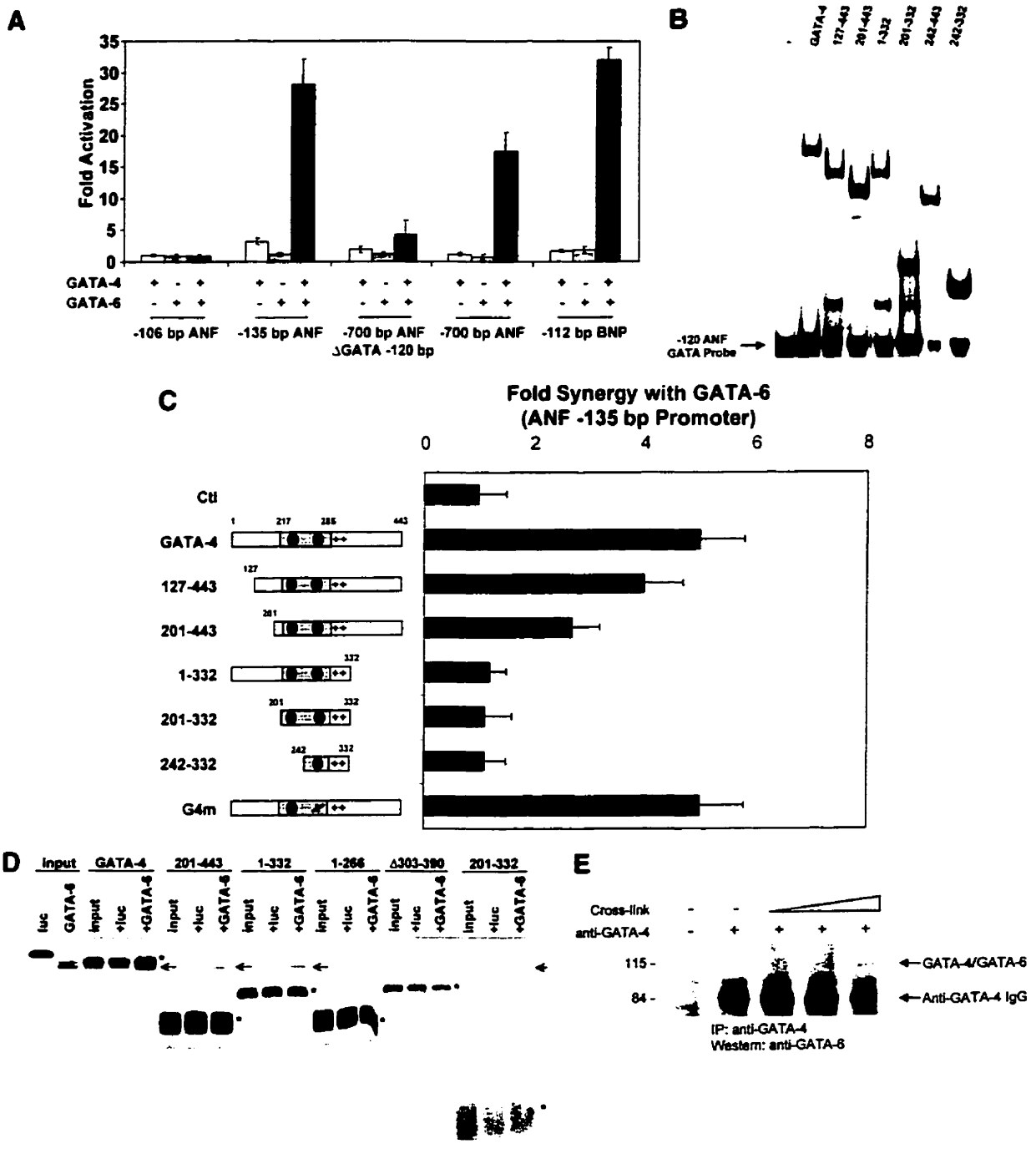
Since differential activation of the ANF promoter GATA elements by GATA-4 and -6 correlated perfectly well with the relative affinities for these sites, we tested whether the preferential regulation of α-MHC by GATA-4 was due to differential affinities of GATA factors for the α-MHC GATA element (30). As shown in Fig. 2.5B, the α-MHC GATA element was more efficient at competing GATA-4 than GATA-6 binding. Thus, the higher affinity of GATA-4 for this element may explain the preferential regulation of the α-MHC gene by GATA-4.

GATA-4 and GATA-6 functionally and physically interact to cooperatively activate cardiac promoters. The fact that the knock-down of both GATA-4 and GATA-6 had the same effect on ANF gene expression as the knock-down of either factor alone suggests that GATA-4 and GATA-6 are members of a single functional complex and that ablation of GATA-4 or GATA-6 in that complex is sufficient to disrupt its transcriptional activity.

To test whether GATA-4 and GATA-6 act cooperatively, the -700 bp ANF promoter fused to luciferase was co-transfected with various doses of GATA-4 and GATA-6 expression vectors. At low dose of expression vector where neither GATA-4 nor GATA-6 could activate transcription by themselves, synergistic activation of the ANF promoter was achieved when both GATA factors were added (Fig. 2.6A). Interestingly, cooperative activation by GATA-4 and -6 occured through a single GATA binding site as evidenced by the activation of the -135 bp ANF promoter construct. In fact, the proximal GATA element was necessary and sufficient for synergy and the distal GATA element [-700 bp ANF (ΔGATA -120 bp)] could not mediate cooperative activation. Synergistic activation by GATA-4 and GATA-6 was also observed on the BNP promoter, but not on a shorter ANF promoter lacking GATA elements (-106 bp ANF).

The domain(s) of GATA-4 required for synergy with GATA-6 were mapped by co-transfection in HeLa cells of GATA-6 and various GATA-4 mutant expression vectors (Fig. 2.6C and summarized in Fig. 2.8). All mutants were tested for

Figure 2.6. GATA-4 and GATA-6 functionally and physically interact to activate cardiac promoters. (**A**) GATA-4 and GATA-6 cooperatively activate cardiac promoters. ANF and BNP reporter vectors were co-transfected with 200 ng of GATA-4 and GATA-6 expression vectors in HeLa cells. Luciferase activity was assayed 36 h post-transfection. The results are expressed as fold activation by GATA-4 and/or GATA-6. (**B**) DNA-binding by the GATA-4 mutants. EMSAs were performed on the –120 bp ANF GATA element using various GATA-4 mutants over-expressed in L cells. The results are summarized in Fig. 8. (**C**) Mapping of the domain(s) required for synergy between GATA-4 and GATA-6. HeLa cells were co-transfected with GATA-6 and various GATA-4 mutant expression vectors. Luciferase activity was assayed 36 h post-transfection. The results are expressed as fold synergy of the -135 bp ANF promoter, which is defined by the activation of the -135 bp ANF promoter by both GATA-4 and GATA-6 divided by the sum of the activation by GATA-4 and GATA-6 alone. (**D**) The zinc fingers and the basic region of GATA-4 are sufficient for physical interaction with GATA-6. Polyhistidine-tagged GATA-4 and wild-type GATA-6 were *in vitro* co-transcribed and co-translated in presence of radio-labeled methionine. The reaction was then incubated with a nickel resin in order to pull-down his-tagged GATA-4 from the reaction. Interacting proteins were resolved by SDS-PAGE. Luciferase was used as a negative control for interaction. The various his-tagged GATA-4 proteins are indicated with an asterisk and the interacting GATA-6 proteins are evidenced by an arrow. Note that, due to the his-tag, GATA-4 has a slightly lower electrophoretic mobility than GATA-6. (**E**) GATA-4 and GATA-6 interact *in vivo* in postnatal cardiomyocytes. Cardiomyocyte whole-cell extracts were cross-linked using glutaraldehyde for 15, 30, and 150 min, followed by immunoprecipitation (IP) using an anti-GATA-4 antibody. The immunoprecipitates were resolved by SDS-PAGE and the GATA-4/GATA-6 complexes were revealed by Western blotting using an anti-GATA-6 antibody. Molecular weight standards (in kDa) are indicated on the left. The strong signal is due to the GATA-4 antibody IgG heavy chains. Note that the GATA-4/GATA-6 complex migrates at about 110 kDa, which corresponds to the sum of the molecular weight of GATA-4 and GATA-6.


A
Fold Activation
35
30
25
20
15
10
5
0
GATA-4
GATA-6
-106 bp ANF
-135 bp ANF
-700 bp ANF
ΔGATA -120 bp
-700 bp ANF
-112 bp BNP
B
GATA-4
127-443
201-443
1-332
201-332
242-443
242-332
-120 ANF
GATA Probe
C
Fold Synergy with GATA-6
(ANF -135 bp Promoter)
0
2
4
6
8
Ctl
GATA-4
127-443
201-443
1-332
201-332
242-332
G4m
D
Input
GATA-4
201-443
1-332
1-266
Δ303-390
201-332
luc
GATA-4
Input
+luc
+GATA-4
E
Cross-link
anti-GATA-4
115 -
84 -
←GATA-4/GATA-6
←Anti-GATA-4 IgG
IP: anti-GATA-4
Western: anti-GATA-6

expression and nuclear localisation. The mutants used in transfection assays were expressed at similar level as evidenced by EMSAs and Western blot analysis (Fig. 2.6B and data not shown). Progressive deletion of the N-terminal (127-443 and 201-443) activation domain of GATA-4 did not drastically affect synergy with GATA-6. However, deletion of the C-terminal activation domain of GATA-4 (1-332) or GATA-4 mutants harboring no transcriptional activation domain (201-332 and 242-332) did not exhibit synergy with GATA-6. DNA-binding by GATA-4 was not required since G4m, a GATA-4 mutant in the second zinc finger bearing no DNA-binding activity (but that still localizes to the nucleus), was able to provide synergistic activation with GATA-6. Thus, while the N-terminal activation domain of GATA-4 and GATA-4 DNA binding ability are dispensable, the C-terminal activation domain of GATA-4 is required for synergy with GATA-6.

To test whether this functional cooperation between GATA-4 and -6 involves direct interaction, polyhistidine-tagged GATA-4 and wild-type GATA-6 were *in vitro* co-transcribed and co-translated in presence of radio-labeled methionine. As shown in Fig. 2.6D and summarized in Fig. 2.7, GATA-4 interacted specifically with GATA-6 and this interaction required the zinc fingers and the basic region of GATA-4. The ability of GATA-4 and GATA-6 to contact each other was further confirmed *in vivo* by chemical cross-linking of cardiomyocyte extracts followed by immunoprecipitation with a GATA-4 antibody and Western blotting with a GATA-6 antibody. As shown in Fig. 2.6E, this resulted in immunoprecipitation of a heterodimer composed of endogenous GATA-4 and GATA-6 proteins.

GATA-4 and GATA-6 expression co-localize in postnatal cardiomyocytes. In order to ascertain that the immunoprecipitated GATA-4 and -6 complex reflects the ability of these proteins to associate with each other intracellularly, we verified the co-expression of GATA-4 and GATA-6 in cardiomyocytes. Immunofluorescence studies using specific anti-GATA-4 and anti-GATA-6 antibodies were performed on sections from neonatal mice heart and on primary rat cardiomyocyte cultures (Fig. 2.8 and data not shown). These studies revealed that GATA-4 and GATA-6 co-localize in most postnatal ventricular cardiomyocytes.

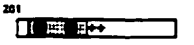
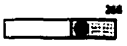
		Nuclear Localisation	DNA-Binding	Trans-activation	GATA-6 Synergy	GATA-6 Interaction
GATA-4		+	+	++	+	+
127-443		+	+	+	+	+
201-443		+	+	+	+	+
1-332		+	+	+	-	+
1-266		-	-	-	ND	-
Δ303-390		-	-	-	ND	-
201-332		+	+	-	-	+
242-332		+	+	-	-	ND
G4m		+	-	-	+	+

Figure 2.7. Summary of GATA-4 functional domains. The GATA-4 constructs used in this study are reported, along with some of their functional properties, including: nuclear localization, DNA-binding, transactivation, synergy with GATA-6, and interaction with GATA-6. ND = not determined.

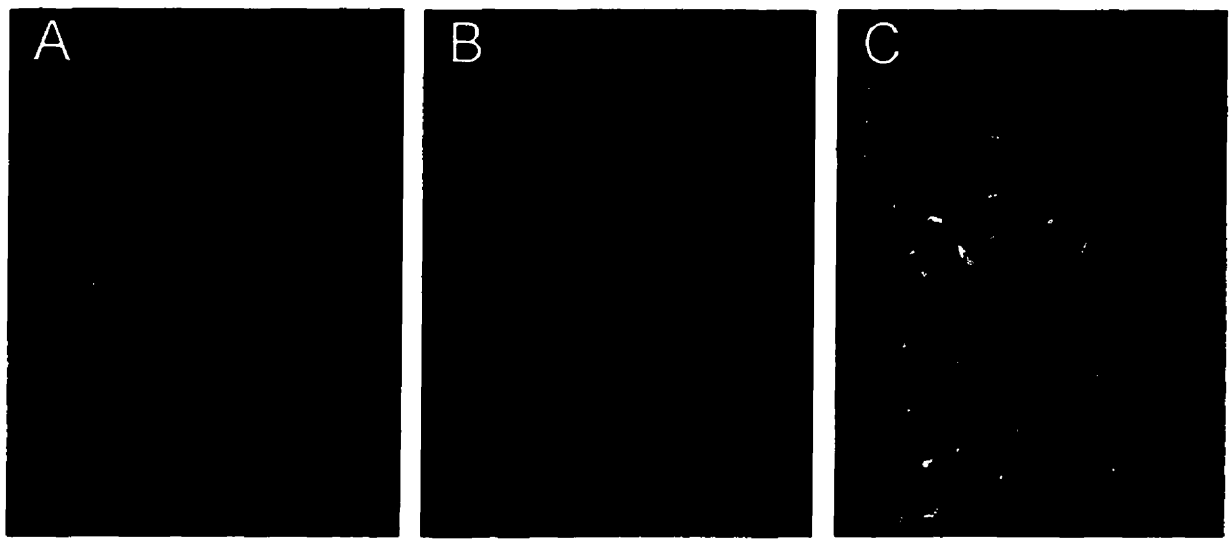


Figure 2.8. GATA-4 and GATA-6 co-localize in postnatal cardiomyocytes. Immunofluorescence studies were performed on heart sections from neonatal mice using (**A**) a specific anti-GATA-4 antibody and (**B**) a specific anti-GATA-6 antibody. (**C**) Superposition of (A) and (B) indicates that GATA-4 and GATA-6 co-localize in most postnatal cardiomyocytes.

DISCUSSION

Members of the GATA family of transcription factors play critical roles in diverse cellular processes. Although some members are co-expressed in specific cell types, each family member appears to fulfill essential, non-redundant functions during development. However, the mechanisms by which GATA factors control gene expression and cell fate as well as the molecular basis for their specificity remain poorly understood.

The data presented in this paper provide evidence for the existence of specific *in vivo* downstream targets for two members of the GATA family, GATA-4 and –6, which are co-expressed in myocardial cells. The results also show that GATA factors act in concert to regulate transcription of subsets of cellular genes, suggesting that combinatorial interactions among GATA factors may differentially control various cellular processes.

Identification of *in vivo* targets for GATA factors in cardiomyocytes. Regulatory elements containing GATA-binding sites have been identified in many cardiac promoters, including BNP, α-MHC, cTnI, and cardiac troponin C, and GATA-4 was shown to transactivate several of these promoters (8;14;20;28;30;35;38;42). However, several cardiac markers examined which were previously proposed to be GATA-4 targets were still expressed at high levels in GATA-4 null hearts, raising the possibility that these genes are either not *bona fide* GATA-4 targets or that other cardiac factors, including GATA-6 whose expression is up-regulated in GATA-4 null mice, provide compensatory pathways (25;31). Thus, determining the *in vivo* targets for GATA-4 and GATA-6 is crucial toward understanding their role in the heart. The data obtained in this study indicate that while some cardiac genes are regulated preferentially by GATA-4, others are targets for both GATA-4 and GATA–6. This is consistent with a specialized role for each factor in heart development and the requirement for both in normal heart formation. At present, GATA–6 has been linked to "cardioblast" proliferation while GATA-4 has been associated with terminal differentiation (12;15). In this respect, it is noteworthy that markers of later stages of cardiomyocyte differentiation – α- and

β-myosin heavy chain genes – appear to be preferential GATA-4 targets while genes expressed in pre-cardiomyocytes prior to the beating stage like the natriuretic peptide genes (ANF and BNP) and the PDGF receptors (16;24) are targeted by both GATA-4 and –6. Since the adenovirus-mediated antisense strategy revealed that GATA-4 and GATA-6 are required for the maintenance of the differentiated phenotype in postnatal cardiomyocytes, it could now be used to determine the role of these factors in embryonic myocytes. Finally, in light of recent reports suggesting a role for GATA-4 in mediating hypertrophic signals (17;19), it will be interesting to directly test the implication of GATA-4 or GATA-6 in cardiomyocyte hypertrophy using the adenovirus tools developed in this study.

Transcriptional mechanisms of GATA factors. The data presented show that subset of cellular genes may be *bona fide* targets for more than one GATA factors while others are under the control of a specific GATA factor. It may be significant to point out that this is the first time that specific *in vivo* targets for cardiac GATA factors are reported.

Differential affinity could be one of the mechanisms by which GATA factors target distinct downstream genes. In the case of the hematopoietic GATA-1, -2, and -3 proteins, *in vitro* binding site selection experiments have shown differences in DNA-binding specificity, although they have not been correlated yet with natural GATA elements present on hematopoietic promoters (23).

In this study we show that GATA-4 and GATA-6 bind to the two ANF GATA elements with different relative affinities that correlate perfectly well with their ability to transactivate the ANF promoter. The results also show that higher affinity of GATA-4 for the α-MHC GATA element correlate with the finding that the endogenous α-MHC gene is a preferential GATA-4 target. Interestingly, the ANF and α-MHC sequences that are preferential GATA-4 binding sites have an A residue at the W position of the consensus WGATAR. While ascertaining the generality of this observation has to await additional findings, it is noteworthy that at least one other natural AGATAA site present on the BNP promoter (at –30 bp) also appears to be a preferential GATA-4 binding site (F. Charron and M. Nemer,

unpublished data). The results raise the possibility of finding specific targets for GATA-4 or -6 based on differential affinity to their DNA sequence.

An important outcome of this study is the demonstration that some cardiac genes such as ANF and BNP are *bona fide* targets for both GATA-4 and GATA-6 and that the two GATA factors form transcriptionnally active complexes over a single GATA element. Several lines of evidence supporting the existence of functional interaction between GATA-4 and GATA-6 are presented. First, the knock-down of both GATA-4 and GATA-6 had the same effect on ANF expression as the knock-down of either factor alone, suggesting that GATA-4 and GATA-6 are members of a single functional complex and that the ablation of GATA-4 or GATA-6 in that complex is sufficient to disrupt its transcriptional activity. Second, when GATA-4 and GATA-6 are co-transfected in heterologous cells, they cooperatively activate ANF and BNP reporter genes. The presence of a functional complex between GATA-4 and GATA-6 is further supported by the finding that GATA-4 and GATA-6 physically interact *in vitro* and *in vivo* in postnatal cardiomyocytes. Finally, the co-localization of GATA-4 and GATA-6 in postnatal cardiomyocytes lends further credibility to the likelihood of *in vivo* relevance of a GATA-4 and GATA-6 interaction.

Homotypic (for GATA-1) and heterotypic interactions between hematopoietic GATA factors (GATA-1 and GATA-2 or GATA-3) via the DNA-binding domain of GATA-1 have been also reported (7;27;43). In the case of GATA-4/GATA–6, physical interaction also occurs via the DNA binding zinc finger domain; however, functional cooperativity requires the C-terminal activation domain of GATA-4 suggesting that the transcriptionally active complex includes additional co-factors that are involved in specific protein:protein interactions with one but not the other GATA member. In the case of ANF, such co-factor may be Nkx2-5 that was shown to specifically interact with GATA-4 but not GATA–6 (9;10). Thus, heterotypic interactions may be an intrinsic property of GATA factors and the combinatorial interaction of different GATA factors present in various cell types with each other and with other cellular co-factors might contribute to their cell-specific mode of action. The finding that GATA-4 and GATA-6 cooperatively target the ANF and

BNP genes in neonatal cardiomyocytes provides biological relevance for these heterotypic interactions.

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CHAPTER III. GATA-DEPENDENT RECRUITMENT OF MEF2 PROTEINS TO TARGET PROMOTERS

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FOREWORD

Our work established that GATA-4 and GATA-6 are required for the maintenance of cardiac gene expression and that GATA-4 and GATA-6 differentially regulate target genes (see Chapter II). We also showed that this specificity is due, at least in part, to their differential DNA-binding affinity.

Another way for GATA factors to acquire specificity is by differential interaction with cofactors. The only example reported yet is the cooperative interaction between Nkx2-5 and GATA-4, but not GATA-6 (Durocher et al., 1997). In Chapter III, we show that functional specificity is also imparted to GATA factors by their differential capacity to recruit and synergize with MEF2 cofactors: MEF2 cooperatively interacts with all GATA factors, except GATA-5. In addition, the recruitment of MEF2 proteins by GATA factors describes a novel pathway for transcriptional regulation by MEF2 factors and will be relevant to help to understand the mechanisms underlying cardiac gene transcription and development. More globally, this interaction provides a molecular paradigm for elucidating the mechanisms of action of MEF2 proteins in many tissues.

ABSTRACT

The myocyte enhancer factor-2 (MEF2) proteins are MADS-box transcription factors that are essential for differentiation of all muscle lineages but their mechanisms of action remain largely undefined. In mammals, the earliest site of MEF2 expression is the heart where the MEF2C isoform is detectable as early as embryonic day 7.5. Inactivation of the *MEF2C* gene causes cardiac developmental arrest and severe downregulation of a number of cardiac markers including atrial natriuretic factor (ANF). However, most of these promoters contain no or low affinity MEF2 binding sites and they are not significantly activated by any MEF2 proteins in heterologous cells suggesting a dependence on a cardiac-enriched cofactor for MEF2 action. We provide evidence that MEF2 proteins are recruited to target promoters by the cell-specific GATA transcription factors, and that MEF2 potentiates the transcriptional activity of this family of tissue-restricted zinc finger proteins. Functional MEF2/GATA-4 synergy involves physical interaction between the MEF2 DNA-binding domain and the carboxy zinc finger of GATA-4 and requires the activation domains of both proteins. However, neither MEF2 binding sites nor MEF2 DNA binding capacity are required for transcriptional synergy. The results unravel a novel pathway for transcriptional regulation by MEF2 and provide a molecular paradigm for elucidating the mechanisms of action of MEF2 in muscle and non-muscle cells.

INTRODUCTION

Members of the myocyte enhancer factor-2 (MEF2) family of MADS (MCM1, Agamous, Deficiens, Serum response factor)-box transcription factors are evolutionary conserved proteins that are expressed at high levels in all muscle cells. MEF2 proteins are also found in non-muscle cells including brain and lymphoid tissue [reviewed in (Black and Olson, 1998)]. In mammals, the MEF2 family is composed of four members, MEF2A, MEF2B, MEF2C, and MEF2D, which form homo- and heterodimers that bind the consensus DNA sequence (T/C)TA(A/T)$_4$TA(G/A) present in many muscle and non-muscle promoters. MEF2 proteins contain a conserved N-terminal 56-amino acid MADS domain and an adjacent 29-amino acid MEF2 domain, which together mediate DNA binding and dimerization.

Genetic studies have provided evidence for an essential role of MEF2 proteins in muscle-specific gene expression and differentiation of all three muscle lineages. In Drosophila, mutation of the D-*mef2* gene results in embryos lacking differentiated skeletal, cardiac, and visceral muscle cells (Lilly et al., 1995; Bour et al., 1995; Ranganayakulu et al., 1995). In mice, inactivation of the MEF2C gene, which is the first MEF2 isoform expressed during embryonic development, leads to cardiac morphogenetic defects, vascular abnormalities, and lethality by embryonic day 9.5 (Lin et al., 1997; Lin et al., 1998; Bi et al., 1999). The mechanisms by which MEF2 proteins regulate myogenesis of both striated and smooth muscle cells and the identity of their downstream targets in these various tissues is only starting to be elucidated. At present, the mechanisms of action of MEF2 have been analyzed mostly in skeletal muscle where MEF2 appears to act as cofactors for the myogenic basic helix-loop-helix (bHLH) proteins, MyoD, Myf5, myogenin, and MRF4 (Kaushal et al., 1994; Black et al., 1998; Molkentin et al., 1995). Thus, MEF2 proteins strongly potentiate the transcriptional activity of the bHLH myogenic factors and cooperate with them for inducing and maintaining the skeletal muscle phenotype. This cooperativity is mediated by direct interaction between the DNA-binding domains of MEF2 and myogenic proteins and necessitates a DNA-binding site for only one of the two factors. Therefore, in skeletal myocytes, MEF2 may

modulate transcription by two distinct pathways: one involving DNA binding to MEF2 sites and another one involving recruitment of MEF2 to E-boxes in target promoters via the myogenic bHLH factors. Whether similar mechanisms underlie the action of MEF2 in cardiac and visceral muscle cells where the MyoD family of transcription factors is not expressed remains unknown.

MEF2 binding sites have been reported in several cardiac promoters and their mutation was shown to decrease promoter activity in cardiomyocytes; they include the MEF2 sites in the ventricular myosin light chain (MLC2V), cardiac troponin T, cardiac troponin I, α-myosin heavy chain (α-MHC) and Desmin (Yu et al., 1992; Zhu et al., 1991; Iannello et al., 1991; Molkentin and Markham, 1993; Kuisk et al., 1996; Di Lisi et al., 1998). Analysis of cardiac gene expression in mice with targeted mutation of the MEF2C gene confirmed that some of these genes, like α-MHC, required MEF2C for optimum transcription (Lin et al., 1997). In addition to α-MHC, two other cardiac-specific genes, not previously associated with MEF2 proteins, atrial natriuretic factor (ANF) and α-cardiac actin (α-CA), were completely absent in the hearts of MEF2C-deficient embryos. How MEF2C regulates transcription of these target genes remains unclear; the two αMHC MEF2 sites are low affinity MEF2 binding sites (Yu et al., 1992; Molkentin and Markham, 1993), and MEF2 proteins are unable to activate αMHC-driven reporters in cotransfection assays although they can potentiate transactivation of the αMHC promoter by the thyroid hormone receptor (Lee et al., 1997). Moreover, ectopic expression of MEF2 proteins in explanted xenopus ectoderm failed to activate endogenous αMHC or α-CA genes (Chambers et al., 1994; Fu and Izumo, 1995). However, forced expression of MEF2 proteins in whole xenopus embryos results in precocious expression of endogenous αMHC and enlarged hearts (Fu and Izumo, 1995). Together, these studies suggest that MEF2 regulate transcription of αMHC and possibly other cardiac genes in conjunction with a cell-specific cofactor present in embryonic mesoderm (or endoderm) but not in ectoderm.

In the case of ANF, the cardiac promoter which is a known downstream target for the cardiac-specific transcription factors, GATA-4 and Nkx2-5, does not

contain MEF2 consensus binding sites (Durocher et al., 1996; Charron et al., 1999) (Durocher et al., 1997)suggesting an indirect action of MEF2 on ANF transcription possibly through modulation of GATA-4, Nkx2-5 or other ANF regulators; however, neither GATA–4 nor Nkx2-5 levels are altered in MEF2C$^{-/-}$ embryos (Lin et al., 1997).

In this study, we provide evidence that MEF2 proteins are recruited by the cardiac-specific transcription factor GATA-4 to synergistically activate ANF and several other MEF2C target promoters including αMHC and α-CA. The MEF2/GATA-4 synergy is mediated by physical interaction between the respective DNA-binding domains and requires the transactivation domains of both factors. GATA binding sites are necessary and sufficient for cooperativity with MEF2. Other GATA factors, including GATA-6, which is expressed in cardiac and smooth muscle cells, and GATA-2 and –3, which are present in hemopoietic and neuronal cells, are also able to cooperate with MEF2 proteins. Together the data suggest that in addition to cooperating with the myogenic bHLH proteins in skeletal muscle differentiation, MEF2 proteins act as co-factors for the tissue-restricted zinc finger GATA proteins in cardiac myogenesis and raise the possibility that GATA factors may be essential components of MEF2 action in several other cell types.

RESULTS

MEF2 proteins activate the ANF promoter via two distinct mechanisms. The absence of ANF transcripts in the heart of mice homozygous for a null mutation of the MEF2C gene (Lin et al., 1997) indicates that ANF is a downstream target for MEF2 proteins. We tested whether the effect of MEF2C was due to direct action on the ANF promoter be it via DNA binding or through recruitment by protein:protein interactions. The first 700 bp of the rat ANF promoter are sufficient to recapitulate cardiac specificity and spatio-temporal regulation of the endogenous gene in cultured cardiomyocytes (Argentin et al., 1994) and in transgenic mice (Durocher et al., 1998).

Sequence analysis of the entire 700 bp rat ANF promoter revealed no consensus MEF2 sites; the closest sequence homologies to MEF2 binding sites

mapped to an A/T-rich element sharing similarities with a MEF2 consensus (Fig. 3.1A). To verify if this A/T-rich element could be recognized by cardiac-derived or recombinant MEF2 proteins, it was used in gel shift assay to compete MEF2-binding on the well-characterized muscle creatine kinase (MCK) MEF2 site. As seen in Figure 3.1B, several A/T-rich ANF elements including the TATA-box, the SRE-like, and the CArG-box could not compete the MEF2 binding on the MCK probe even when used at a 500-fold molar excess. On the other hand, the distal A/T-rich element was able to displace the MEF2 binding although at a much lower efficiency than the MCK site. Identical results were obtained using MEF2C and MEF2D or cardiomyocyte nuclear extracts (data not shown). Consistent with its ability to recognize MEF2 proteins, the ANF A/T-rich probe was able to bind all three recombinant MEF2 proteins tested (MEF2A, MEF2C, and MEF2D) albeit with low affinity (Fig. 3.1B and data not shown). Finally, when cloned upstream of a minimal promoter, the ANF A/T-rich element could be transactivated 4-fold by cotransfection with MEF2 expression vectors in heterologous cells (Fig. 3.2B). These results suggest that the ANF promoter contains a very low affinity MEF2-binding site that could mediate MEF2 action.

Indeed, MEF2C and other MEF2 proteins activate the ANF promoter in a dose dependent manner in several non-cardiac cells (Fig. 3.2A). Interestingly, the magnitude of activation was much greater (4- to 6-fold higher) in HeLa cells than in most cell lines tested (including CV1, P19, and C2C12) with maximal ANF promoter induction of 15-fold. This difference in MEF2 responsiveness was also observed with the cardiac αMHC promoter which was induced by 10-fold in HeLa cells and was barely responsive in CV1 or P19 cells (Fig. 3.2B and data not shown); in contrast, a synthetic promoter harboring a multimerized MCK-MEF2 site upstream of a minimal ANF promoter was more similarly activated by MEF2 in HeLa (9-fold) and CV1 (6-fold) cells (Fig. 3.2B). The transfected MEF2 vectors produced similarly high levels of MEF2 proteins in all cell lines as assessed by gel shift assays (data not shown). Thus, the differences observed in the level of MEF2-dependent ANF and αMHC promoter activation may reflect cooperative

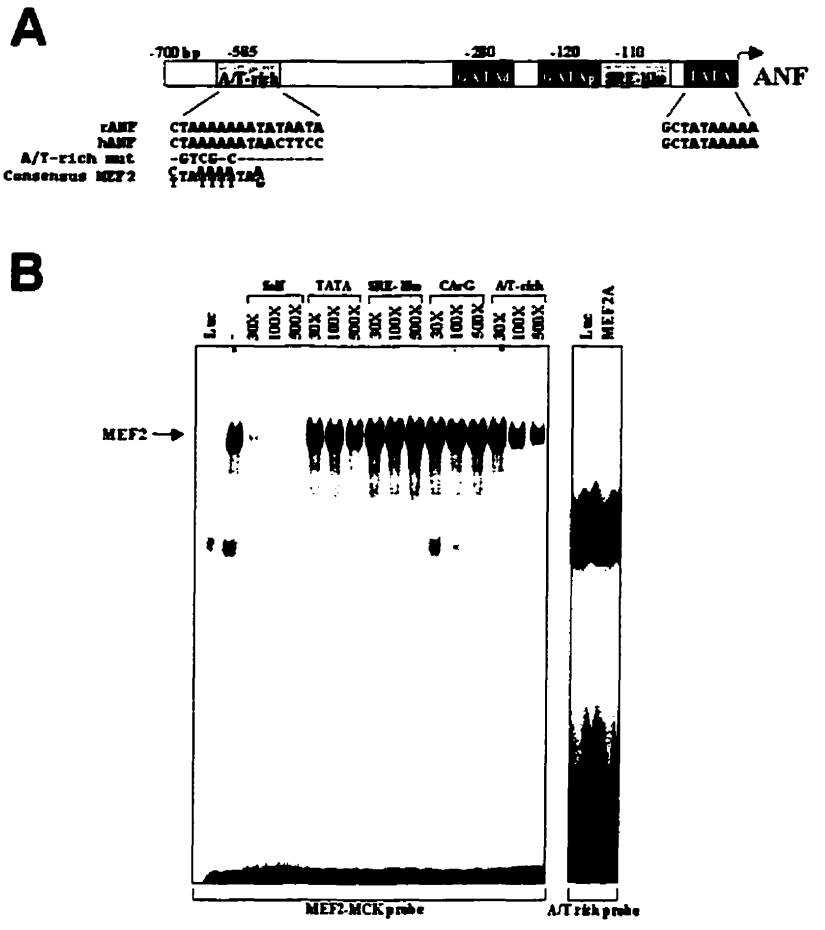


Figure 3.1. The ANF promoter harbors a low affinity MEF2-binding site. **A)** Schematic representation of the ANF promoter. Regulatory elements are boxed and their location relative to the transcription start site is indicated. All these elements are evolutionary conserved on the ANF promoter from many species. SRE-like is a low affinity serum response element, the GATAd and GATAp are the distal and proximal GATA-binding sites, respectively. The consensus MEF2-binding site is also shown. The A/T-rich mut sequence indicates the mutations introduced to abolish the A/T-rich element. rANF and hANF are the rat and human ANF promoter, respectively. **B)** The A/T-rich element is a low affinity MEF2-binding site. EMSAs were performed on the MEF2 element of the MCK promoter (MEF2-MCK, left panel) or the A/T-rich element of ANF (A/T-rich, right panel) using in vitro translated MEF2A. In the left panel, the MEF2A-binding was competed with different unlabelled ANF probes described in Materials and Methods. Only the A/T-rich element of the ANF promoter was able to compete the MEF2A binding. Similar results were obtained with in vitro translated MEF2C and MEF2D.

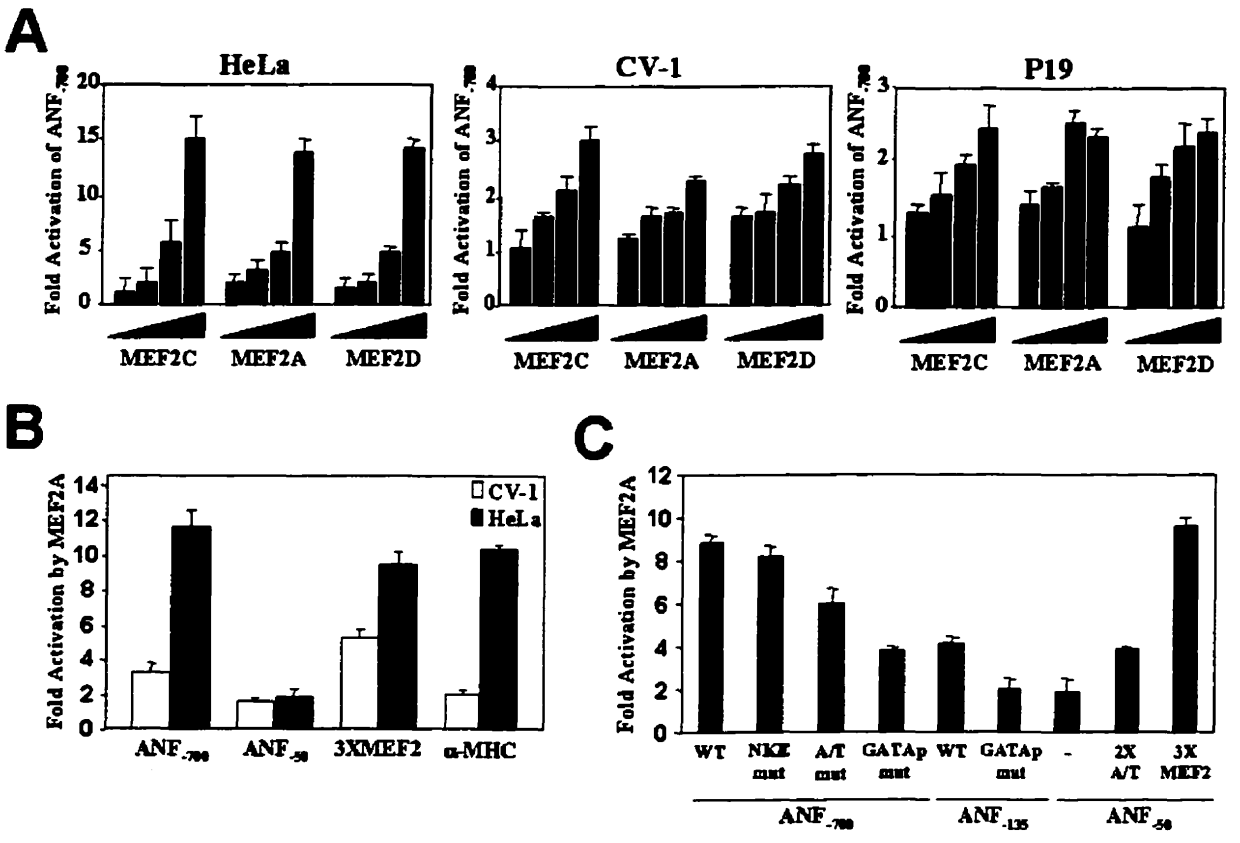


Figure 3.2. The low affinity A/T-rich and the proximal GATA elements contribute to MEF2-dependent ANF promoter activation. **A)** Dose-dependent ANF_{-700} promoter activation by MEF2A, MEF2C, and MEF2D in HeLa, CV-1, and P19 cell lines. Transient transfections were performed using 50 ng, 100 ng, 500 ng, and 1 μg of MEF2 expression vector. Note the fold-activation difference between HeLa and CV-1 or P19 cells. **B)** Preferential activation of the ANF_{-700} and α-MHC promoters, but not an artificial MEF2 reporter, in HeLa cells. Transfections were performed using 1 μg of MEF2A expression vector. Similar results were obtained using MEF2C and MEF2D. **C)** The low affinity A/T-rich and the proximal GATA elements contribute to MEF2-dependent ANF promoter activation. Transfections were performed in HeLa cells using 1 μg of MEF2A expression vector. Similar results were obtained using MEF2C and MEF2D. 3XMEF2 and 2XA/T are the MEF2-MCK and the ANF A/T-rich elements trimerized and dimerized, respectively, in front of the ANF_{-50} minimal promoter.

interaction between transfected MEF2 proteins and other cellular factors bound to the promoters.

Mutational analysis was used to test which DNA elements on the ANF promoter are required for activation by MEF2 (Fig. 3.2C). Consistent with its characterization as a weak affinity MEF2 site, the A/T-rich element was necessary for maximal MEF2 activation but its deletion or mutation reduced promoter activation by only 30%. Surprisingly, mutation of the proximal GATA element which can bind endogenous GATA-2 protein present in HeLa cells (Grépin et al., 1994), had a more drastic effect on MEF2 responsiveness suggesting that GATA factors may cooperate with MEF2. In fact, the proximal ANF promoter (ANF_{-135}), which lacks any MEF2 binding site was induced 4- to 5-fold by MEF2 proteins and mutation of the GATA site therein abrogated MEF2 responsiveness (Fig. 3.2C). This element is a high affinity binding site for GATA factors (Charron et al., 1999) but does not bind MEF2 proteins (Fig. 3.1B). Together, these results suggest that MEF2 could act as a cofactor of promoter-bound GATA proteins to activate the ANF and possibly other cardiac promoters.

Synergistic activation of the ANF promoter by MEF2 and GATA factors. Two members of the GATA family of zinc finger transcription factors, GATA-4 and GATA-6, are expressed in cardiac muscle cells and bind to and activate the ANF promoter (Charron et al., 1999). To test whether either factor could recruit MEF2 proteins to target promoters, the effect of co-expressing them with MEF2 in heterologous cells was assayed on ANF promoter activity. Co-transfection of GATA-4 with MEF2A, MEF2C or MEF2D leads to a synergistic 40- to 50-fold activation of the ANF promoter (Fig. 3.3A). MEF2 proteins were also able to cooperate to varying degrees with other GATA factors including the hemopoietic GATA-1, -2, and –3, and the other cardiac GATA factor, GATA-6, but not GATA-5 (Fig. 3.3B). The inability of MEF2C and MEF2A to synergize with GATA-5 and the more modest synergy achieved with GATA-2 and –3 are not due to different levels of GATA proteins produced as all expression vectors have been shown to produce similar protein levels (Viger et al., 1998; Nemer et al., 1999).

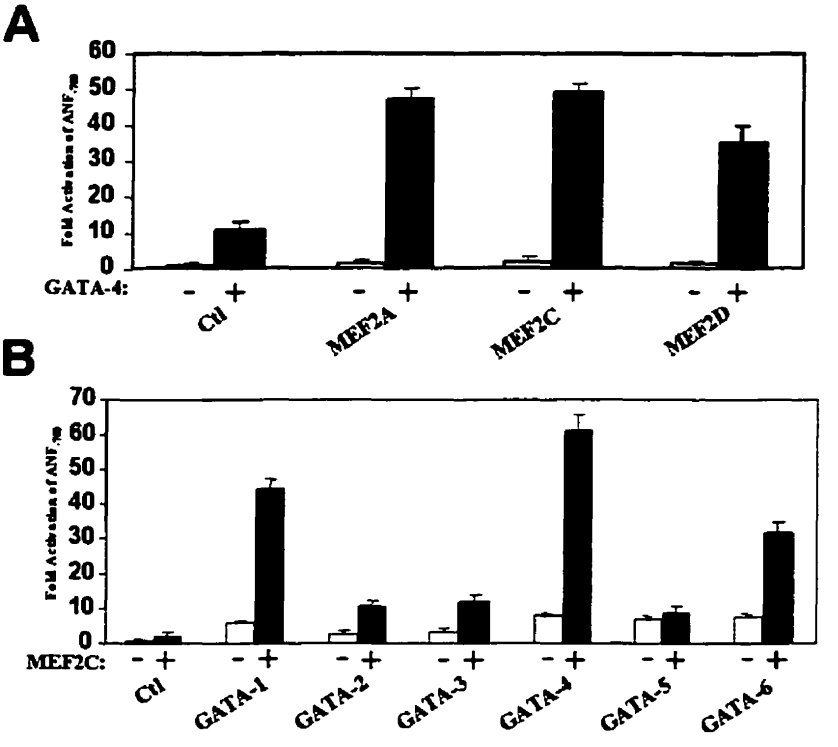


Figure 3.3. The MEF2 and GATA transcription factors cooperatively activate the ANF_{-700} promoter. **A)** MEF2A, MEF2C, and MEF2D functionally interact with GATA-4. Co-transfections were performed in HeLa cells using the $ANF\text{-}luc_{-700}$ construct and 1 μg of MEF2A, MEF2C, or MEF2D expression vector in absence (-) or presence (+) of 1.5 μg of GATA-4 expression vector. **B)** MEF2 proteins functionally interact with a subset of GATA proteins. Co-transfections were performed as in A) using 1.5 μg of various GATA expression vectors in absence (-) or presence (+) of 1 μg of MEF2C expression vector. Note that cooperative interaction between MEF2A and the different GATA factors was identical to the one shown here for MEF2A and GATA-1 through -6. Similar results were also obtained in the CV1 cell line.

In order to map the promoter element(s) required for the synergy between MEF2 and GATA-4, various ANF promoter mutations were tested. Mutation of the low affinity MEF2-binding element (A/T-rich) or the distal GATA element reduced maximal MEF2/GATA-4 synergy by 35 to 40% (Fig. 3.4A). Mutation of the proximal GATA element in the context of the –700 or the -135 bp promoter completely abolished synergy indicating that this element is essential for MEF2/GATA-4 cooperation. Interestingly, the -135 bp ANF promoter was sufficient to produce 50% of the maximal synergy obtained with the longer promoter (ANF_{-700}) and displayed the same response to the various MEF2/GATA combinations as the ANF_{-700} promoter (Fig. 3.3) suggesting that binding of GATA factors to the proximal GATA element may be sufficient to recruit MEF2 proteins to the promoter.

Indeed, an artificial reporter driven by multimerized GATA binding sites could be synergistically activated by GATA and MEF2 proteins; however, neither the high affinity MCK MEF2 binding site nor the lower affinity ANF MEF2 element were sufficient to support MEF2/GATA cooperativity (Fig. 3.4B).

MEF2 factors physically interact in vitro and in vivo with GATA-4. The observation that MEF2A/GATA-4 synergy required only the GATA binding site implied that GATA-4 recruits MEF2 proteins to the ANF promoter through protein:protein interaction. Indeed, MEF2A and GATA-4 could be co-immunoprecipitated in vivo (Fig. 3.5A) suggesting physical interaction between the two proteins.

To determine if this interaction was direct, we performed in vitro pull-down assays using immobilized MBP-GATA-4 and in vitro-translated ^{35}S-labeled MEF2 proteins. MBP-GATA-4 was able to retain specifically MEF2A, MEF2C, and MEF2D but not the control luciferase (Fig. 3.5B) confirming that GATA-4 and MEF2 directly interact.

In order to map the interaction domain between MEF2 and GATA proteins, different mutants of GATA-4 were in vitro co-translated with MEF2A and coimmunoprecipitated using an antibody directed against the extreme C-terminal of GATA-4 (present in all mutants tested). MEF2A was able to interact with the full-length GATA-4 and the N-terminal activation domain-deleted mutant (201-443)

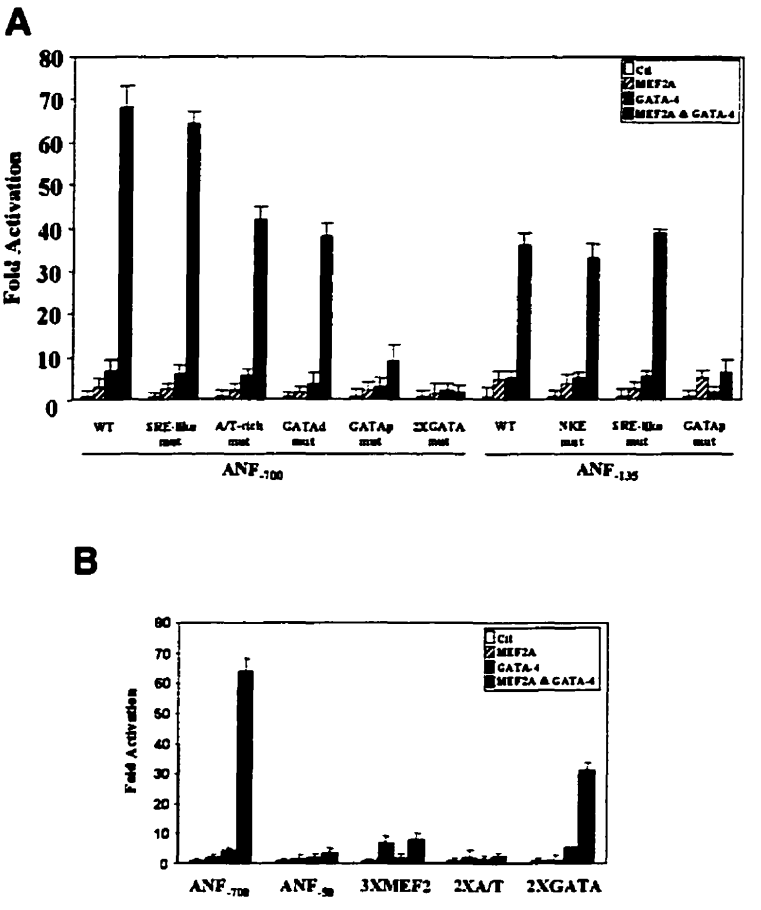
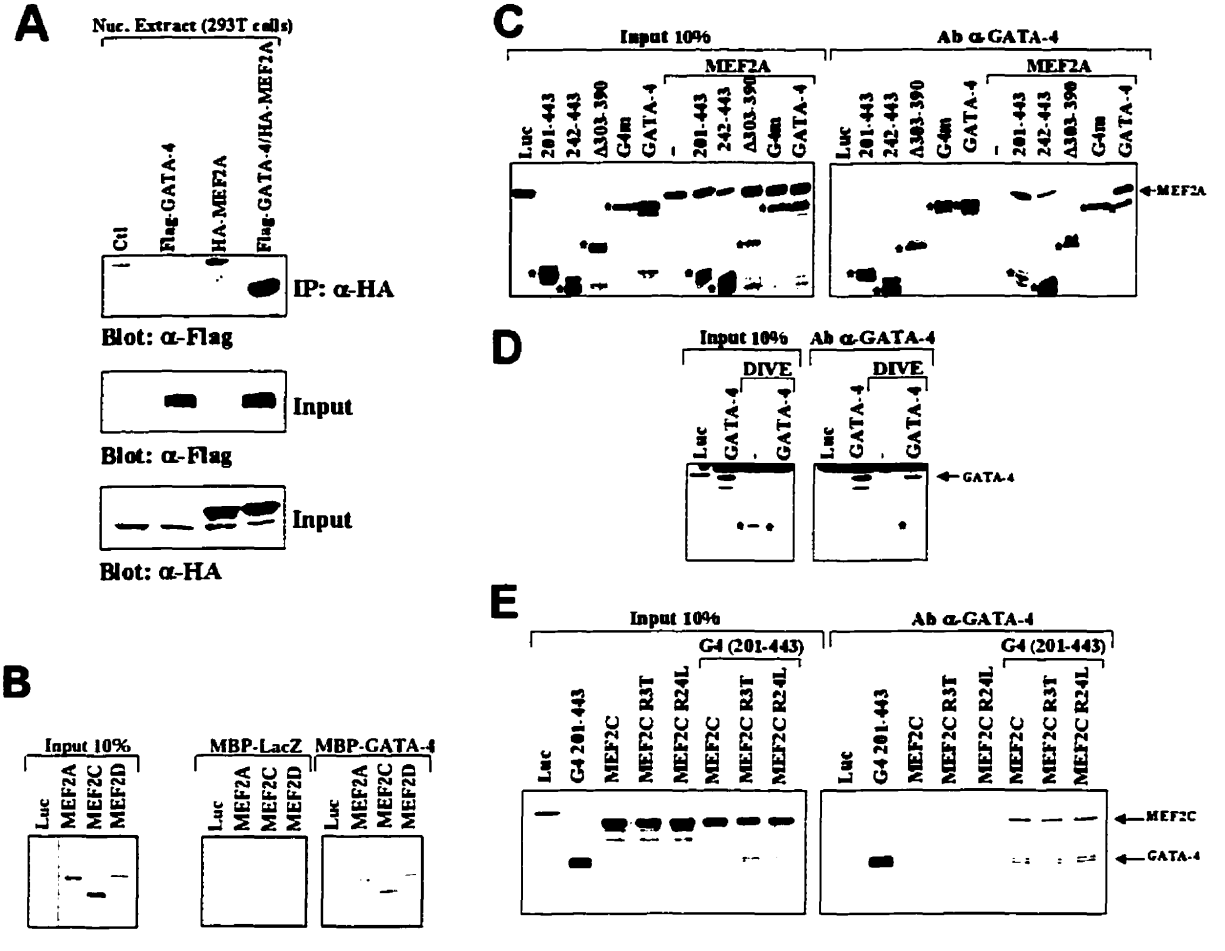


Figure 3.4. The proximal GATA element is necessary and sufficient for MEF2/GATA synergy. Co-transfections were performed in HeLa cells using various promoter constructs and 1μ g of MEF2A and/or 1.5 μg of GATA-4 expression vectors. The ANF promoter constructs used are described in material and methods. 3XMEF2 and 2XA/T are the MEF2-MCK and the ANF A/T-rich elements trimerized and dimerized, respectively, in front of the ANF_{-50} minimal promoter. 2XGATA is a dimer of the BNP GATA elements in front of the minimal BNP promoter.

Figure 3.5. MEF2 proteins physically interact with GATA-4. **A)** MEF2A interacts in vivo with GATA-4. Nuclear extracts from 293T cells transfected with empty vectors (ctl), flag-GATA-4 and/or HA-MEF2A were immunoprecipitated using an anti-HA antibody, separated on 10% SDS-PAGE electrophoresis, transferred to PVDF membranes, and subjected to immunoblotting using an anti-Flag antibody (top panel). The lower two panels are Western blots carried out on the same nuclear extracts using either HA (to reveal tagged MEF2A proteins) or Flag (to reveal tagged GATA-4 proteins) antibodies. **B)** MEF2A proteins interact in vitro with GATA-4. Pull-down assays were performed using immobilized, bacterially-produced MBP fusions (MBP-GATA-4 and MBP-LacZ as control) and in vitro translated, ^{35}S-labeled MEF2A, MEF2C, MEF2D, or luciferase (luc) protein. The protein complexes were resolved on a 10% SDS-PAGE. **C)** The physical interaction between GATA-4 and MEF2 requires the C-terminal zinc finger DNA-binding domain of GATA-4. Full-length GATA-4 and various GATA-4 mutants (depicted in Fig. 3.6A) were in vitro co-translated with MEF2A and co-immunoprecipitated using an antibody directed against the extreme C-terminal of GATA-4. The protein complexes were resolved on a 15% SDS-PAGE. The asterisks highlight GATA protein bands. **D)** The DNA-binding domain of MEF2 is sufficient for interaction with GATA-4. MEF2A DIVE (aa 1-86) retains the MADS and MEF2 domains. Co-immunoprecipitations were performed as described in (C). The asterisks highlight the MEF2A DIVE band. The protein complexes were resolved on a 20% SDS-PAGE. **E)** MEF2 DNA-binding defective mutants interact with GATA-4. MEF2C R3T and MEF2C R24L do not bind DNA but are still able to dimerize. A deleted GATA-4 construct [G4 (201-443)] was used to differentiate between GATA-4 and MEF2C, which have similar electrophoretic mobility. Co-immunoprecipitations were performed as described in (C). The protein complexes were resolved on a 10% SDS-PAGE.


A
Nuc. Extract (293T cells)
Ctl
Flag-GATA-4
HA-MEF2A
Flag-GATA-4/HA-MEF2A
IP: α-HA
Blot: α-Flag
Input
Blot: α-Flag
Input
Blot: α-HA
B
Input 10%
MBP-LacZ
MBP-GATA-4
Luc
MEF2A
MEF2C
MEF2D
C
Input 10%
Ab α-GATA-4
MEF2A
Luc
201-443
242-443
Δ303-390
G4m
GATA-4
MEF2A
D
Input 10%
Ab α-GATA-4
DIVE
Luc
GATA-4
GATA-4
E
Input 10%
Ab α-GATA-4
G4 (201-443)
Luc
G4 201-443
MEF2C
MEF2C R3T
MEF2C R24L
MEF2C
GATA-4

(Fig. 3.5C). Deletion of the N-terminal zinc finger of GATA-4 (242-443) reduced but did not abrogate interaction with MEF2A. However, MEF2A was unable to interact with the G4m [which harbors a point mutation in the C-terminal zinc finger abolishing DNA-binding (Charron et al., 1999)] or the 303-390 mutant, indicating that the C-terminal zinc finger structure and the basic region are essential for physical interaction with MEF2. The same approach was also used to map the GATA-4 interaction domain on MEF2 and revealed that the DNA-binding domain consisting of the MADS and MEF2 domains (MEF2A DIVE, aa 1-86) is sufficient for interaction with GATA-4 (Fig. 3.5D). Interestingly, within the MADS domain, interaction with GATA factors and binding to DNA could be segregated as two DNA binding defective mutants (MEF2C R3T and MEF2C R24L) retained the ability to bind GATA-4 (Fig. 3.5E). These results suggest that GATA-4 and MEF2 physically interact through their DNA-binding domains.

MEF2/GATA synergy does not require MEF2 DNA binding. To determine whether the activation domains of either or both GATA and MEF2 proteins are required, various GATA-4 mutants were tested for their capacity to synergistically activate the ANF promoter with MEF2A. The GATA-4 mutants which delete the N-terminal region (127-443 and 201-443) and the first zinc finger (242-443) were able to synergize with MEF2A (Fig. 3.6A). However, the GATA-4 mutants which delete the C-terminal transactivation domain (1-332, 201-332, and 242-332) were all unable to support MEF2 synergy. Consistent with a requirement for GATA-4 DNA binding (Fig. 3.4A) and the GATA-4 DNA binding domain for physical interaction with MEF2 (Fig. 3.5C), a point mutation in the second zinc finger that destroys DNA binding (G4m) abrogated MEF2 synergy (Fig. 3.6A).

Functional synergy also required the activation domain of MEF2 and deletion of the C-terminal activation domain (MEF2A DIVE) completely abolished the synergy with GATA-4 (Fig. 3.6B). However, and consistent with the requirement for GATA but not MEF2 binding sites, MEF2C mutants that are DNA binding defective retained the ability to synergize with GATA-4 (Fig. 3.6B). These results indicate that GATA-4 is able to recruit DNA-binding defective MEF2 proteins to transcriptionally active complexes.

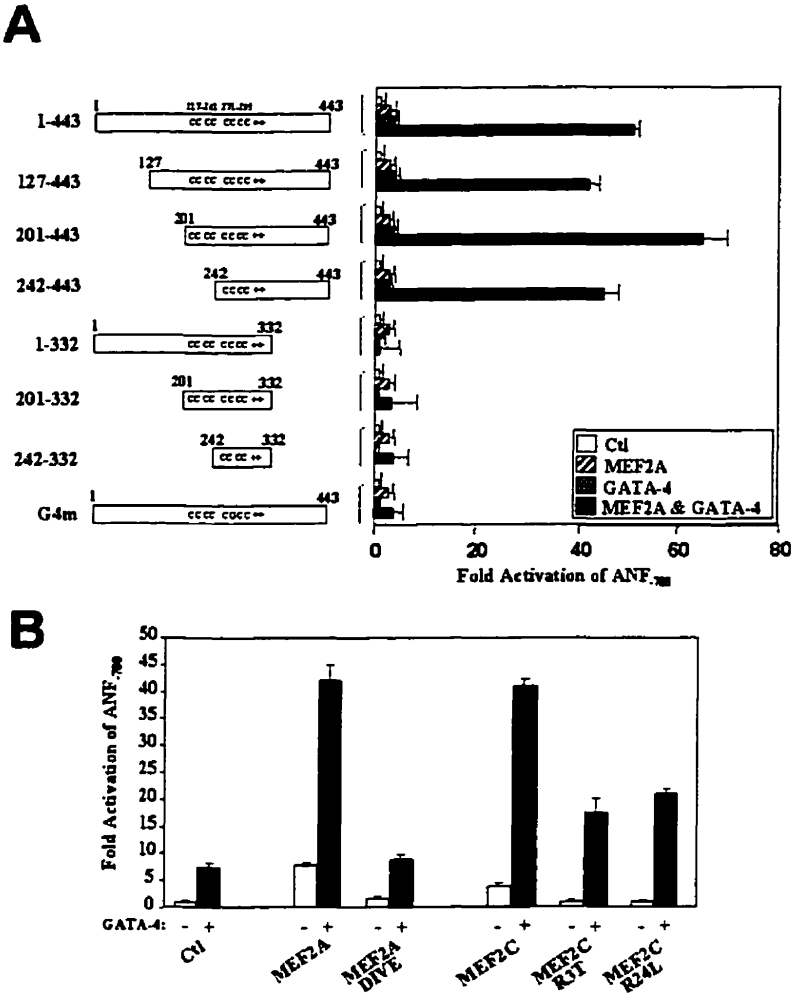


Figure 3.6. Mapping of the GATA-4 and MEF2 domains required for synergy. **A)** The C-terminal activation domain of GATA-4 is required for MEF2 synergy. Co-transfections were performed in HeLa cells on the ANF_{-700} promoter construct using 1 μg of MEF2 and 1.5 μg of GATA-4 expression vectors. **B)** The C-terminal activation domain of MEF2 but not its DNA-binding capacity, is required for synergy with GATA-4. As evidenced by the ability of MEF2C R3T and MEF2C R24L to synergize with GATA-4, note that the DNA binding domain (MEF2A DIVE) is not sufficient to support functional synergy although it interacts physically with GATA-4 as shown in the previous figure.

MEF2/GATA synergy : a mechanism for MEF2 action in the heart. We next tested whether transcription of other cardiac genes is cooperatively activated by MEF2 and GATA-4. As seen in Figure 3.7A, in addition to ANF, the αMHC, α-CA and B-type natriuretic peptide (BNP) promoters are also synergistically activated by MEF2 and GATA-4. Both αMHC and α-CA are downregulated in MEF2C null mice and neither contain a high affinity MEF2 binding site although both are GATA targets (Charron et al., 1999; Sepulveda et al., 1998). However, as evidenced by the βMHC promoter, not all GATA target promoters are synergistically activated by MEF2 suggesting that functional GATA/MEF2 synergy is promoter context dependent and may be targeted to a specific subset of cardiac genes.

Finally, to ascertain whether, in cardiac cells, MEF2 proteins are GATA cofactors, the effect of a dominant negative MEF2 protein on the activity of the proximal ANF promoter was determined. This promoter contains a GATA but no MEF2 binding site. Cotransfection of a MEF2 mutant which retains the ability to physically associate with GATA-4 but lacks the activation domain reduces by 50% the activity of the ANF promoter in primary cardiomyocyte cultures; in contrast, cotransfection with wild type MEF2A induces promoter activity by 80% (Fig. 3.7B). Both, activation by wild-type MEF2 and inhibition by its dominant negative form, are blunted by point mutation of the GATA binding site (Fig. 3.7B). These data are consistent with a role for MEF2 proteins as coactivators of GATA factors in cardiac muscle cells and point to a novel GATA-dependent pathway for transcriptional activation by MEF2.

DISCUSSION

The MEF2 transcription factors are key regulators of cardiac myogenesis and morphogenesis but the molecular basis for their actions are poorly understood. The data presented here provide evidence that, in cardiac myocytes, MEF2 proteins are recruited by the cardiac-specific GATA transcription factors to target promoters and functionally synergize with this family of tissue-restricted zinc finger proteins. This observation is reminiscent of the cooperative interaction between

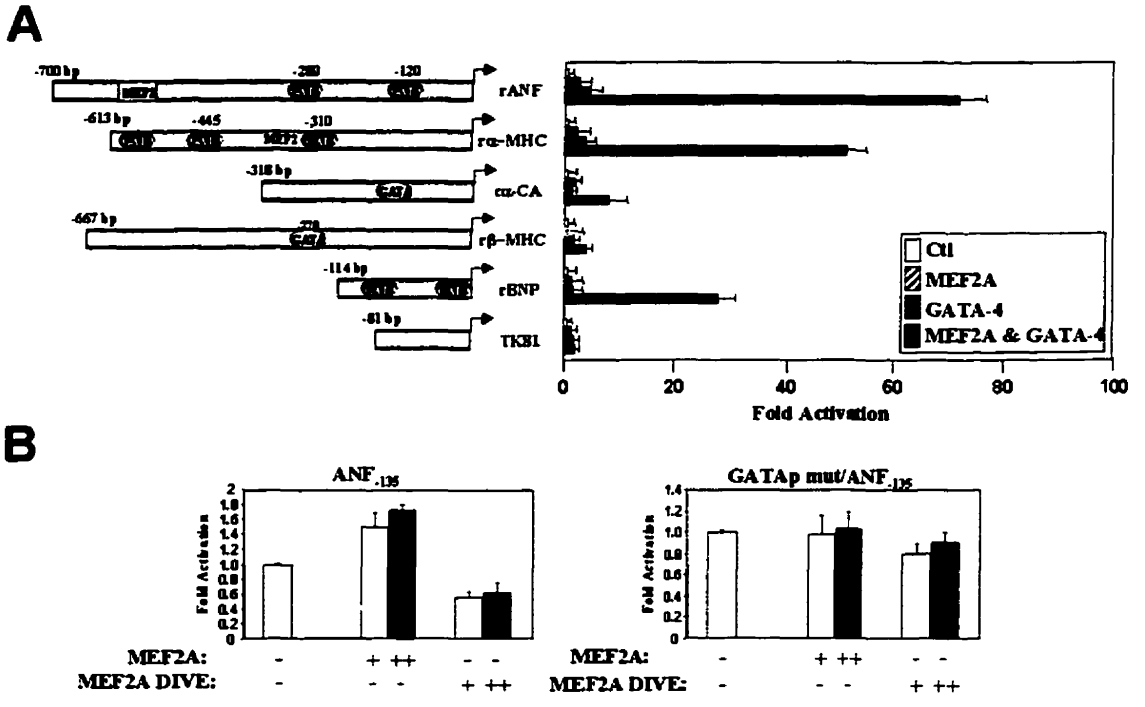


Figure 3.7. The MEF2/GATA-4 synergy: a mechanism for MEF2 action in the heart. **A)** The MEF2/GATA-4 synergy is not limited to the ANF promoter. HeLa cells were co-transfected with 1 μg of MEF2A and 1.5 μg of GATA-4 expression vectors together with various cardiac promoters. Except for the cardiac α-actin promoter that was from chicken, all other promoters used are from rat and are described in material and methods. TK81 is the thymidine kinase - 81 bp promoter. Elements shaded in black and grey are high and low affinity sites, respectively, as determined by DNA-binding assays. **B)** A dominant negative MEF2 protein decreases ANF promoter activity in cardiomyocytes. Primary culture of cardiomyocytes were transfected with the wild-type ANF_{-135} (left panel) or GATA-mutated ANF_{-135} promoter (GATAp mut/ANF_{-135}; right panel) and no (-), 50 ng (+), or 1 000 ng (++) of MEF2A or a dominant negative form of MEF2A (MEF2A DIVE). The results shown represent the mean ± SD of two independent experiments, each carried out in duplicate.

MEF2 proteins and the myogenic bHLH factors in skeletal muscles, and suggest that MEF2 proteins are able to interact with and potentiate the action of other classes of cell-specific transcription factors (Fig. 3.8). Given the coexpression of MEF2 and GATA factors in several cell types including smooth muscle, neuronal, and T cells, the GATA-dependent MEF2 pathway described in this work may provide a molecular paradigm for understanding the mechanisms of action of MEF2 in many target cells.

GATA proteins are evolutionary conserved cell-restricted transcription factors that play crucial roles in differentiation. In vertebrates, six GATA factors have been identified and they are all expressed in a lineage-restricted and developmentally-controlled manner. GATA-1, -2, and –3 are predominantly expressed in hemopoietic cells while GATA-4, -5, and –6 are largely restricted to the heart and gut. Genetic and biochemical studies have revealed crucial roles for specific family members in hemopoietic, cardiac, neuronal, and endodermal cells (Grépin et al., 1995; Molkentin and Olson, 1997; Morrisey et al., 1998; Tsai et al., 1994; Pandolfi et al., 1995; Pevny et al., 1991). In addition to their essential roles in development, GATA factors are also required for the proper function of adult organs. GATA binding sites are present on many hemopoietic and cardiac promoters, which are potently activated by GATA factors [reviewed in (Charron and Nemer, 1999)]. Moreover, GATA proteins act cooperatively with other classes of transcription factors including several zinc finger proteins, such as SP1 and FOG-1 (Friend of GATA-1) and homeodomain-containing factors like the cardiac-specific Nkx2-5 (Durocher et al., 1997; Tsang et al., 1997). The differential interactions of GATA proteins with other transcription factors are likely important for functional specificity of GATA proteins during embryonic development and in differentiated and adult cells.

In this study, we report for the first time that, in addition to interacting with homeodomain and zinc finger-containing proteins, GATA factors are also able to interact with members of the MADS-box family of transcription factors. This interaction involves physical contact between the C-terminal zinc finger DNA-binding domain and the adjacent basic region of GATA-4 and the MADS domain of

MEF2. This, in turn, leads to synergistic activation of the ANF and other MEF2 target promoters independently of the DNA-binding activity of MEF2. Synergy is observed with two of the three cardiac GATA factors: GATA-4 and –6 but not with GATA-5; MEF2 synergy was also detected with GATA-1, -2, and –3 though at varying levels. Thus, all GATA factors are not equally competent to synergize with MEF2. The observation that GATA-4 and –6 but not –5 synergize with MEF2 is interesting given that GATA-4 and –5 but not –6 synergize with the cardiac homeodomain protein Nkx2-5 (Durocher et al., 1997); this suggests that while MEF2 and Nkx2-5 interact with the same domain of GATA-4, they apparently do not recognize the same molecular determinants.

Although GATA factors physically contact MEF2 proteins through the DNA binding domain, DNA binding and GATA interaction are dissociable and neither physical nor functional interaction with GATA-4 on natural promoters requires MEF2 DNA binding. The ability of MEF2 to cooperate with MyoD in skeletal myogenesis and to synergize with MyoD in activating an artificial promoter driven by multimerized MyoD binding sites was also shown to be independent of MEF2 DNA binding capacity (Molkentin et al., 1995). DNA binding has been shown to be dispensable for some of the actions of two other sequence-specific DNA binding proteins, the glucocorticoid receptor (Reichardt et al., 1998) and the cell-specific homeodomain protein Pit1 (Dasen et al., 1999). However, in both cases, DNA-binding-independent activities involved transcriptional repression. In the present study, we show that transcriptional activation of several natural promoters by MEF2 proteins is independent of MEF2 binding sites and MEF2 DNA binding ability. Thus, both suppressive and activating functions of transcription factors may involve DNA-binding independent pathways.

In addition to the MADS domain, the activation domain of MEF2 is required for functional synergy with GATA-4 suggesting that MEF2 proteins potentiate transcriptional activity of GATA factors through recruitment and/or stabilization of coactivators in the GATA transcription complex. Such coactivators may include the CBP/p300 family as both MEF2 and GATA proteins have been shown to independently interact with these coactivators (Sartorelli et al., 1997; Kakita et al.,

1999; Blobel et al., 1998). Alternatively, MEF2/GATA interaction may displace or overcome a corepressor of either or both factors. For example, MEF2 interaction with GATA-4 or –6 in the heart may displace FOG-2, a GATA-associated cofactor that represses GATA-4 activity (Svensson et al., 1999; Lu et al., 1999). Conversely, recruitment of MEF2 by GATA factors may displace the MEF2-associated corepressors MITR or the HDAC4 deacetylase (Miska et al., 1999; Sparrow et al., 1999).

In addition to cardiomyocytes, MEF2 proteins are coexpressed with members of the GATA family in several other cell types. Most notably is the presence of MEF2 proteins with GATA-6 in smooth muscle cells (Suzuki et al., 1996; Narita et al., 1996), and with GATA-3 in T lymphocytes (Zheng and Flavell, 1997), somites (George et al., 1994), and brain (Pandolfi et al., 1995). Given the demonstrated role of GATA factors in cell differentiation, the GATA-MEF2 synergy provides a general paradigm for understanding the role of MEF2 proteins as determining factors in diverse cell lineages.

Finally, it is tempting to speculate on the role of the MEF2/GATA synergy as a nuclear target of several signaling cascades including calcineurin and p38 MAP kinase. Both pathways, which are highly relevant to many human disorders such as ischemia, heart failure, and inflammatory diseases, have been shown to activate MEF2 in cardiac (Kolodziejczy et al., 1999), skeletal muscle (Zetser et al., 1999), neuronal (Mao et al., 1999), and T cells (Han et al., 1997; Blaeser et al., 2000). GATA factors have also been suggested as downstream targets of calcineurin in cardiac and skeletal muscle hypertrophy in connection with the calcineurin-activated NFAT factor (Molkentin et al., 1998; Musaro et al., 1999; Semsarian et al., 1999). It would be interesting to test whether calcineurin or p38-dependent post-translational modifications of MEF2 or GATA proteins modulate the MEF2/GATA physical interaction and/or the resulting functional synergy.

MATERIALS AND METHODS

Cell cultures and transfections

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transfections were carried out using calcium phosphate 24 h after plating. At 36 h post-transfection, cells were harvested and luciferase activity was assayed with a Berthold LB953 luminometer. The amount of reporter was kept at 1.5 μg per 35 mm dish and the total amount of DNA was kept constant (usually 7 μg). The amount of expression vector used is indicated in the figure legends. Primary cardiomyocyte cultures were prepared from 4-day-old Sprague-Dawley rats as previously described (Charron et al., 1999). The results shown are the mean ± SD of at least two independent experiments carried out in duplicate.

Plasmids

ANF-luciferase promoter constructs were cloned in the PXP-2 vector as described previously (Argentin et al., 1994; Durocher et al., 1996). The BNP-luc constructs were described in (Grépin et al., 1994), the βMHC-luc and cardiac α-actin-luc reporters were described in (Abdellatif et al., 1994). The αMHC-luc vector was kindly provided by P.M. Buttrick (Buttrick et al., 1993). The construction of the various pCG-GATA-4 vectors was based on the original rat GATA-4 cDNA as previously described (Grépin et al., 1994). The various deletions or point mutations of the ANF promoter and the pRSET-GATA-4 derivatives were generated as described previously (Charron et al., 1999; Durocher et al., 1997). ANF constructs with mutations in the GATA elements or in the NKE were previously described (Charron et al., 1999; Durocher et al., 1996). The A/T-rich mutation is shown in Figure 3.1; the SRE-like mutation replaces the TTT of the ANF-SRE by GGG thus destroying SRF binding. Heterologous promoters were generated by multimerizing the relevant oligonucleotides flanked by *BamH*I and *BglII* sites upstream of the minimal (-50 bp) ANF-luciferase reporter. pcDNA-MEF2A DIVE was constructed by insertion of the XbaI-BamHI fragment of the corresponding pCGN-MEF2A DIVE construct into the XbaI-BamHI sites of the pcDNA-3 vector. MEF2 plasmids were kindly provided by E. N. Olson (Molkentin et al., 1996a) and K. Walsh (Andres et al., 1995). The MBP-GATA-4 plasmid was prepared by subcloning a XbaI-BamHI rat GATA-4 cDNA fragment containing the

entire open reading frame and 1.2 kb of 3' untranslated sequences (Grépin et al., 1994) into the MBP-expressing pMalc-2 vector (New England Biolabs, Beverly MA, USA) cut with Nhe1-BamHI.

Recombinant protein production

Recombinant MBP-GATA-4 was obtained according to our previously described protocol (Durocher et al., 1997). Essentially, individual colonies were picked and grown in 500 ml LB up to an OD of 0.6 at 600 nm. Induction of the recombinant protein was carried out by adding Isopropyl thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM during 2 hours at 37°C. The cultures were centrifuged and the bacteria were resuspended and lysed by sonication. Purification on amylose columns (New England Biolabs, Beverly MA) was performed according to the manufacturer's instructions.

In vitro transcribed/translated ^{35}S-labeled MEF2 and GATA proteins were produced in rabbit reticulocyte lysates using the TNT-coupled in vitro transcription/translation system (Promega Corp., Madison, WI) from pcDNA-MEF2 derivatives using either T7 or Sp6 RNA polymerase.

In vitro protein-protein interactions

In vitro binding studies were performed using purified MBP-GATA-4 immobilized on an amylose-Sepharose resin (New England Biolabs) and in vitro transcribed / translated MEF2 proteins. Typically 2-6 µl of ^{35}S-labeled MEF2 proteins were incubated in the presence of 300 ng of immobilized GATA-4 fusion protein in 500 µl of binding buffer (150 mM NaCl, 50 mM Tris-Cl pH 7.5, 0.3% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.25% BSA) for 2 h at 4°C with agitation and then centrifuged for 2 min at 15 000 r.p.m. at room temperature. The resin was washed three times by vortexing in 500 µl of binding buffer at room temperature and three other times by vortexing in 500 µl of binding buffer without BSA. The protein complexes were released from the resin after boiling in Laemmli buffer and resolved by SDS-PAGE. Labeled proteins were visualized and quantified by autoradiography on phospho storage plates (PhosphorImager, Molecular Dynamics).

To determine the domains of GATA-4 and MEF2 required for physical interaction, full-length GATA-4 or mutated GATA-4 plasmids were used for in vitro co-transcription/co-translation with wild type or mutant MEF2A and MEF2C. The co-translated proteins were incubated in 500 μL of binding buffer with 1 μL of GATA-4 antibody (Santa-Cruz Biotechnology) for 2 h at 4°C with agitation and for an additional 2 h with 20 μL of protein A/G Plus-Agarose added (Santa Cruz Biotechnology). Bound immunocomplexes were washed and visualized as mentioned above.

Electrophoretic mobility shift assays

Three μl of the in vitro translated MEF2A, MEF2C, and MEF2D proteins were used for the binding reactions performed essentially as previously described for GATA binding (Charron et al., 1999) except that 100 ng of dI-dC were included in the binding reaction. Reactions were loaded on a 4% polyacrylamide gel and run at 200 V at room temperature in 0.25X Tris-borate-EDTA. The MEF2-MCK probe is as described in (Molkentin et al., 1996b). The rat ANF probes used were as follow:

TATA-box $^{-46}$TCAGGGAGCTGGGGGCTATAAAAACGGGAGACGCC^{-11},

SRE-like $^{-124}$GATCCACTGATAACTTTAAAAGGGCATCTTCA^{-99},

CArG $^{-417}$GATCCTCCCGCCCTTATTTGGAGCCCCTGA^{-390},

A/T-rich $^{-597}$GATCCATACTCTAAAAAAATATAATAGCTCTTTCA^{-567}.

Immunoprecipitations and immunoblots

Co-immunoprecipitations of Flag-GATA-4 and HA-MEF2A were carried out using nuclear extracts of 293T cells over-expressing the relevant proteins. Nuclear extracts were prepared as follows : 5 million 293T cells transfected with 15 μg of expression vectors were harvested 48 h post-transfection in ice-cold phosphate-buffered saline (PBS) containing 1 mM Na Orthovanadate and scraped in 1 ml of ice-cold PBS containing 1 mM EDTA. The cells were resuspended in hypotonic buffer (20 mM HEPES pH7.9, 20 mM sodium fluoride, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, 0.25 mM sodium molybdate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 2 mM DTT, 0.5 mM PMSF, and 100 nM okadaic acid) and swell on ice for 15 min.

Twenty five μl of 10% NP-40 were added and the microtubes were vortexed vigorously. The nuclei were then pelleted by centrifugation at 7000 r.p.m. at 4°C. The nuclear pellet was resuspended in 50-100 μl of high salt buffer (hypotonic buffer containing 20% glycerol and 0.4% NaCl) and shaken vigorously at 4°C for 1h. The nuclear extracts were cleared by centrifugation at 15 000 r.p.m. for 15 min at 4°C and the protein concentration was determined by the Bradford assay. Co-immunoprecipitation reactions were carried out on 50 μg of nuclear extracts using 1 μl of 12CA5 antibody in 500 μl of binding buffer without BSA, and bound immunocomplexes were washed and subjected to SDS-PAGE, as described previously (Durocher et al., 1997). Proteins were transferred on Hybond-PVDF membrane and subjected to immunoblotting. Anti-Flag M5 (Sigma) and 12CA5 (anti-HA) monoclonal antibodies were used at a dilution of 1/8000, revealed with an anti-mouse-HRP (Sigma) at a dilution of 1/50 000, and visualized using ECL Plus (Amersham Pharmacia Biotechnology).

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CHAPTER IV. THE CARDIAC TRANSCRIPTION FACTOR GATA-4 IS AN EFFECTOR OF THE SMALL GTPASE RHOA AND MEDIATES SARCOMERE REORGANIZATION

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FOREWORD

Cardiomyocytes are composed of a highly specialized cytoskeleton partitioned into contractile units named sarcomeres, which consist of highly organized actin and myosin filaments. Many studies, including ours, have established a crucial role for GATA-4 in the regulation of the expression of the contractile actin and myosin protein genes in cardiomyocytes, suggesting that GATA-4 might play a role in cardiomyocyte sarcomere formation. Indeed, we found that overexpression of GATA-4 in cardiomyocytes induces sarcomere formation. Conversely, inhibiting GATA-4 expression blocked the sarcomere-inducing activity of hypertrophic stimuli, such as endothelin-1 (Et-1) and phenylephrine (Phe). These results are very similar to the effect of the small GTPase RhoA in cardiomyocytes and suggested that RhoA and GATA-4 may function in the same signaling pathway. Accordingly, we find that RhoA potentiates GATA-4 transcriptional activity. These results identify for the first time a signaling pathway converging on a transcription factor to control sarcomere reorganization and suggest that RhoA, in addition to its direct effect on myofibrillar assembly, also regulates the expression of sarcomeric proteins and sarcomere reorganization by inducing the transcriptional activity of GATA-4, thereby linking the regulation of transcription by RhoA and its effects on the cytoskeleton.

ABSTRACT

Sarcomeres are the contractile units of the heart and their organization has a direct and profound influence on cardiac function. However, the mechanism governing the normal and abnormal regulation of cardiomyocyte sarcomere organization remains unknown. Members of the Rho family of small GTPase proteins govern the assembly of cytoskeletal actin fibers in many cell types, possibly including cardiomyocytes. In addition to their effect on the cytoskeleton, Rho family members also regulate transcriptional activation of cardiac genes. However, the transcriptional and cytoskeletal effectors of the Rho family proteins in cardiomyocytes remain undefined. The cardiac-enriched transcription factor GATA-4 regulates the expression of many contractile protein genes in cardiomyocytes, suggesting that GATA-4 could be important for sarcomere formation in cardiomyocytes. In this paper, we report that GATA-4 and the small GTPase RhoA are essential for sarcomeric reorganization during cardiomyocyte hypertrophy. Consistent with GATA-4 being a transcriptional effector of RhoA in cardiomyocytes, we show that RhoA potentiates GATA-4 transcriptional activity both on isolated GATA elements and on cardiac promoters known to be GATA-4 targets. Moreover, the N- and C-terminal transactivation domains of GATA-4 are necessary and sufficient to support potentiation by RhoA, indicating that RhoA potentiates GATA-4 activity by stimulating the transcriptional activity of its activation domains. Together, these results identify for the first time a signaling pathway converging on a transcription factor to control sarcomere reorganization and suggest that RhoA, in addition to its direct effect on myofibrillar assembly, also regulates the expression of sarcomeric proteins and sarcomere reorganization by inducing the transcriptional activity of GATA-4, a key regulator of contractile protein gene expression.

INTRODUCTION

Sarcomeres are the contractile units of the heart. They are composed of highly organized actin and myosin filaments, stabilized by α-actinin. Other proteins such as troponin and tropomyosin also compose the contractile filaments and are involved in regulating contraction. Sarcomere organization has a direct and profound influence on cardiac function; importantly, abnormal sarcomere organization is commonly observed in cardiac hypertrophy and failure (Braunwald, 1997). However, the mechanism governing the normal and abnormal regulation of cardiomyocyte sarcomere organization remains unknown.

Although members of the Rho family of small GTPase proteins govern the assembly of cytoskeletal actin fibers and focal adhesion complex in fibroblasts and in other cell types (Hall, 1998), conflicting results have been obtained regarding their role in cardiomyocyte sarcomere reorganization, particularly in response to hypertrophic stimuli (Hoshijima et al., 1998; Thorburn et al., 1997). Nonetheless, two members of the Rho family, RhoA and Rac1, induce the formation of sarcomeres in cardiomyocyte cultures (Hoshijima et al., 1998; Pracyk et al., 1998). Moreover, cardiac-specific overexpresssion of Rac1 induces focal adhesion reorganization and cardiomyopathy in mice (Sussman et al., 2000). Although these results implicate RhoA and Rac1 in cardiomyocyte sarcomere organization, the effectors of the Rho family proteins in cardiomyocytes remain undefined (Clerk and Sugden, 2000).

In addition to their effect on the cytoskeleton, Rho family members also regulate transcriptional activation by serum response factor (SRF), NF-κB, and STAT3 (Hill et al., 1995a; Perona et al., 1997; Simon et al., 2000). Although the changes in cytoskeleton and the transcriptional regulation properties of Rho proteins are considered to be mediated by different effectors (Sahai et al., 1998; Zohar et al., 1998), the cell type-dependent effects of Rho family members on the cytoskeleton suggest that their transcriptional regulation properties and their effect on the cytoskeleton are intimately linked. However, ubiquitously or widely expressed transcription factors such as SRF, NF-κB, and STAT3 cannot account for these tissue-specific effects. Given the unique identities of the contractile proteins

composing cardiomyocyte sarcomeres, cardiac-specific transcriptional effectors are likely involved in their highly regulated expression and assembly process (Epstein and Fischman, 1991).

The cardiac-enriched transcription factor GATA-4 transactivates the promoter and regulates the expression of many contractile protein genes in cardiomyocytes ((Charron et al., 1999), reviewed in (Charron and Nemer, 1999)). Moreover, GATA-4 has been implicated in the cardiomyocyte response to hypertrophic stimuli (Morin et al., 2001). Together, these results suggest that GATA-4 could be important for sarcomere formation in response to hypertrophic stimuli in cardiomyocytes.

In this paper, we report that GATA-4 and the small GTPase RhoA are essential for sarcomeric reorganization during cardiomyocyte hypertrophy. Consistent with GATA-4 being a transcriptional effector of RhoA in cardiomyocytes, we show that RhoA potentiates GATA-4 transcriptional activity both on isolated GATA elements and on cardiac promoters known to be GATA-4 targets. This potentiation was dependent on the presence of an intact GATA element in the promoter, since mutation of the GATA element completely abolished the effect of RhoA on GATA-4 transactivation. Moreover, deletion and Gal4 DNA-binding domain fusion analyses revealed that the N- and C-terminal transactivation domains of GATA-4 are necessary and sufficient to support potentiation by RhoA, indicating that RhoA potentiates GATA-4 activity by stimulating the transcriptional activity of its activation domains, consistent with RhoA inducing post-translational modifications and/or the recruitment of a cofactor which affect GATA-4 transactivation properties. Finally, this effect is not limited to GATA-4 since RhoA was also able to potentiate the activity of GATA-5 and GATA-6. Together, these results identify for the first time a signaling pathway converging on a transcription factor to control sarcomere reorganization and suggest that RhoA, in addition to its direct effect on myofibrillar assembly, also regulates the expression of sarcomeric proteins and sarcomere reorganization by inducing the transcriptional activity of GATA-4, a key regulator of contractile protein gene expression.

RESULTS

GATA-4 is a mediator of Et-1 and Phe-induced cardiomyocyte sarcomere reorganization.

It has been previously shown that GATA-4 regulates the expression of many contractile proteins, such as α-myosin heavy-chain, β-myosin heavy-chain, and cardiac troponin I in cardiomyocytes (Charron et al., 1999) and activates the promoter of many other contractile protein genes, such as cardiac troponin C, slow myosin heavy-chain, and cardiac α-actin, alone or in combination with other transcription factors (Morin et al., 2000; Sepulveda et al., 1998; Di Lisi et al., 1998; Ip et al., 1994; Molkentin et al., 1994; Murphy et al., 1997; Wang et al., 1998). This suggested that GATA-4 could be important for sarcomere formation in cardiomyocytes. To test this possibility, we have used neonatal cardiomyocyte cultures, where sarcomere reorganization induced by endothelin-1 (Et-1) and phenylephrine (Phe; an α-adrenergic agonist) can be readily observed by the appearance of striated acto-myosin fibers upon phalloidin staining (Figure 4.1a). Since GATA-4 null mice are embryonic lethal (Kuo et al., 1997; Molkentin et al., 1997) and thus cannot be used to test the role of GATA-4 in cardiomyocyte sarcomere reorganization, we used an adenovirus expressing an antisense GATA-4 cDNA (AS GATA-4; which we have previously shown to be efficient and specific) to downregulate GATA-4 in postnatal cardiomyocyte cultures (Charron et al., 1999). Downregulation of GATA-4 in cardiomyocytes blocked sarcomere reorganization induced by Et-1 and Phe, while a control adenovirus expressing a nls-lacZ cDNA (lacZ) had no effect on sarcomere reorganization (Figure 4.1a). Quantification of reorganized cardiomyocytes showed that while Et-1 and Phe induced respectively a 3.2 and 4.0 fold increase in the number of cardiomyocytes which underwent sarcomere reorganization, this effect was almost abolished (1.3 and 1.6 fold, respectively) in cardiomyocytes where GATA-4 was downregulated (Figure 4.1b).

Conversely, the effect of upregulating GATA-4 activity in cardiomyocytes was also tested. An adenovirus harboring the GATA-4 cDNA under the control of a CMV promoter was generated. Infection of cardiomyocytes with the GATA-4 adenovirus

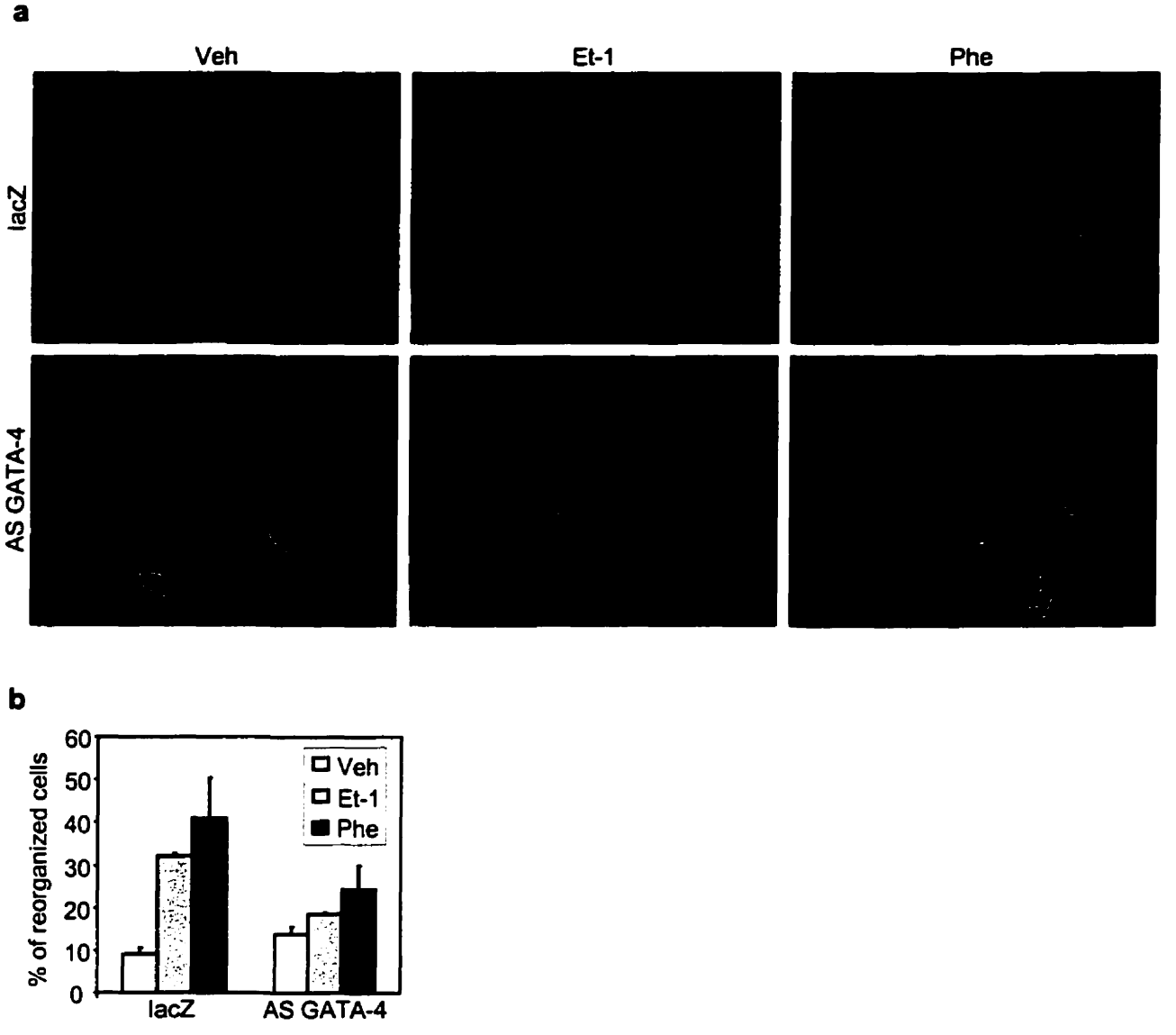


Figure 4.1. GATA-4 is a mediator of Et-1 and Phe-induced cardiomyocyte sarcomere reorganization. **(a)** The antisense GATA-4 adenovirus (AS GATA-4) inhibits Et-1 and Phe-induced cardiomyocyte sarcomere reorganization. Cardiomyocytes infected with a lacZ control adenovirus or with AS GATA-4 were stimulated 24 h later with vehicle (Veh), Et-1, or Phe for 48 h. Cardiomyocytes were fixed and actin filaments were revealed using phalloidin-FITC. **(b)** Quantification of the percentage of reorganized cardiomyocytes. Cells were scored as described in Material and Methods.

induced nuclear GATA-4 protein level by 45-fold, as revealed by Western blot and immunofluorescence analysis (Figure 4.2a and 2d). This increase in protein level correlated with a 10-fold induction of GATA-4 DNA binding activity in cardiomyocytes (Figure 4.2b). Analysis of gene expression following GATA-4 over-expression revealed a 2-fold and 1.5-fold increase in B-type natriuretic peptide (BNP) and α-skeletal actin (α-SA) transcripts, respectively (Figure 4.2c). This increase in α-SA transcripts is consistent with a role for GATA-4 in contractile protein gene expression in cardiomyocytes. Consistent with this role, the over-expression of GATA-4 induced sarcomere reorganization in cardiomyocytes (Figure 4.2d). Interestingly, GATA-4 was as potent as Et-1 or Phe to induce cardiomyocyte sarcomere reorganization (Figure 4.2e). Together, these results indicate that GATA-4 is an essential mediator of Et-1 and Phe-induced cardiomyocyte sarcomere reorganization and that upregulation of GATA-4 activity leads to contractile protein gene induction and cardiomyocyte sarcomere reorganization.

RhoA is also a mediator of Et-1 and Phe-induced cardiomyocyte sarcomere reorganization.

Although the small GTPase family member RhoA is known to govern the assembly of cytoskeletal actin fibers and focal adhesion complex in fibroblasts and in other cell types (Hall, 1998), conflicting results have been obtained regarding its role in cardiomyocyte sarcomere reorganization in response to hypertrophic stimuli (Hoshijima et al., 1998; Thorburn et al., 1997). Therefore, we decided to investigate in further details whether RhoA plays a role in cardiomyocyte sarcomere reorganization.

Using a recently developed assay specific for GTP-bound cellular Rho (Ren et al., 1999), we determined that cardiomyocyte stimulation with Phe induces GTP loading of Rho in a time-dependent manner (Figure 4.3a). These results indicate that Phe induces Rho activity and that Rho may mediate at least part of the Phe-induced sarcomeric reorganization. To test whether RhoA is able to induce sarcomere reorganization in cardiomyocytes, cardiomyocytes were transfected with a constitutively active form of RhoA (RhoA V14) and stained with phalloidin.

Figure 4.2. Upregulation of GATA-4 activity induces contractile protein gene expression and sarcomere reorganization in cardiomyocytes. **(a)** Western blot analysis of nuclear extracts from cardiomyocytes and 293 cells infected with a lacZ control or with GATA-4 adenovirus. **(b)** The GATA-4 adenovirus induces GATA DNA-binding activity in cardiomyocytes. EMSA were performed using nuclear extracts from cardiomyocytes infected with lacZ or GATA-4 adenovirus and the ANF –120bp GATA probe. **(c)** GATA-4 induces BNP and α-skeletal actin gene expression. Total RNA was extracted from cardiomyocytes infected with lacZ or GATA-4 adenovirus and 20 μg of RNA was analyzed by Northern blot, as described in Materials and Methods. **(d)** Upregulation of GATA-4 activity induces cardiomyocyte sarcomere reorganization. Cardiomyocytes infected with lacZ or GATA-4 adenovirus were fixed and co-stained using phalloidin-FITC (green) and anti-GATA-4 antibody (red). **(e)** Quantification of the percentage of reorganized cardiomyocytes. Cells were scored as described in Material and Methods.

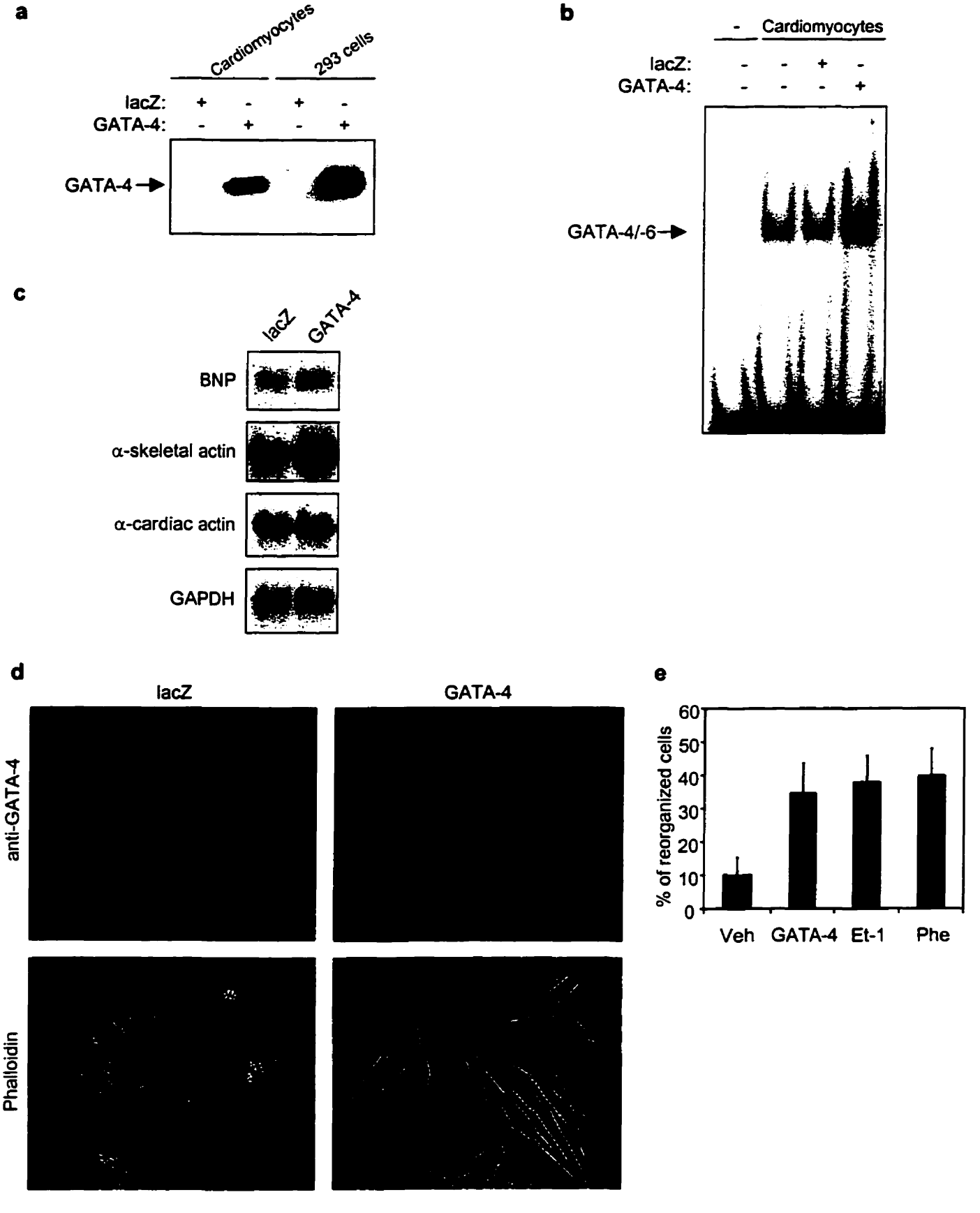
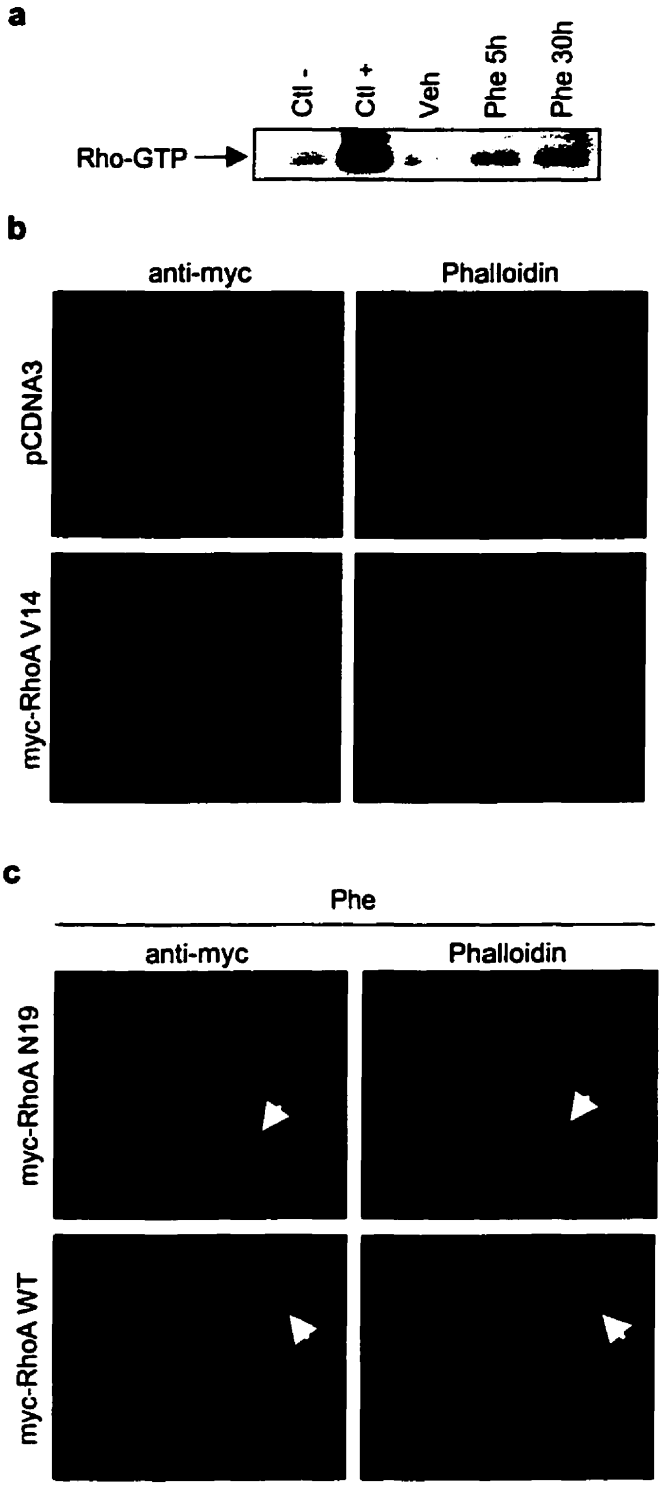

a
Cardiomyocytes
293 cells
lacZ: + - + -
GATA-4: - + - +
GATA-4
b
- Cardiomyocytes
lacZ: - - + -
GATA-4: - - - +
GATA-4/-6
c
lacZ
GATA-4
BNP
α-skeletal actin
α-cardiac actin
GAPDH
d
lacZ
GATA-4
anti-GATA-4
Phalloidin
e
% of reorganized cells
60
50
40
30
20
10
0
Veh
GATA-4
Et-1
Phe

Figure 4.3. RhoA is a mediator of Et-1 and Phe-induced cardiomyocyte sarcomere reorganization. **(a)** Phe induces Rho activity in a time-dependent manner. Whole-cell extracts were prepared from cardiomyocytes stimulated with Phe for 5 and 30 h. The active form of Rho (Rho-GTP) was selectively affinity-precipitated using a GST-Rhotekin protein and revealed using anti-Rho antibody. Extracts were incubated in presence of an exces of GDP (Ctl-) or GTP-γ-S (Ctl+) as negative and positive controls, respectively. **(b)** RhoA V14 induces sarcomere reorganization in cardiomyocytes. Cardiomyocytes transfected with pCDNA3 or pCDNA3-myc-RhoA V14 were fixed and co-stained using phalloidin-FITC (green) and anti-myc antibody (red). **(c)** RhoA N19, but not RhoA WT, inhibits Phe-induced cardiomyocyte sarcomere reorganization. Cardiomyocytes transfected with pCDNA3-myc-RhoA WT or pCDNA3-myc-RhoA N19 were stimulated for 48 h with Phe, fixed, and co-stained using phalloidin-FITC (green) and anti-myc antibody (red). The arrow indicates myc-positives cardiomyocytes.


a
Ctl -
Ctl +
Veh
Phe 5h
Phe 30h
Rho-GTP
b
anti-myc
Phalloidin
pCDNA3
myc-RhoA V14
c
Phe
anti-myc
Phalloidin
myc-RhoA N19
myc-RhoA WT

Interestingly, most of the cardiomyocytes expressing RhoA V14 underwent sarcomere reorganization (Figure 4.3b), suggesting that upregulation of RhoA activity might be involved in sarcomere reorganization in cardiomyocytes. To directly test the involvement of RhoA in Phe-induced cardiomyocyte sarcomere reorganization, a dominant negative form of RhoA (RhoA N19) was transfected in cardiomyocytes stimulated with Phe. Interestingly, RhoA N19 inhibited Phe-induced sarcomere reorganization, while the wild-type form of RhoA (RhoA WT) did not (Figure 4.3c). Similarly to GATA-4, these results strongly support an essential role for RhoA as a mediator of Et-1 and Phe-induced cardiomyocyte sarcomere reorganization and lead us to hypothesize that GATA-4 and RhoA could act in the same signaling pathway in response to Et-1 and Phe.

RhoA potentiates GATA-4 transcriptional activity.

To test whether GATA-4 could modulate RhoA activity, the amount of active (GTP-loaded) RhoA was determined in cardiomyocytes where GATA-4 activity was down- or up-regulated. Altering the levels of GATA-4 had no effect on RhoA activity (Figure 4.4), even though the expression of other genes was affected in these conditions [Figure 4.2c and (Charron et al., 1999)]. These results suggest that GATA-4 does not regulate RhoA activity, and indicate that, if RhoA and GATA-4 are in the same signaling pathway, GATA-4 rather lies downstream of RhoA.

To determine if this is indeed the case, the effect of RhoA on GATA-4 was investigated. Cardiomyocyte transfection with RhoA V14 did not affect endogenous GATA-4 protein level or subcellular localization (Figure 4.5a). We thus assessed whether RhoA was able to regulate GATA-4 transcriptional activity. For this, we co-transfected GATA-4 with RhoA V14 and tested whether RhoA V14 is able to potentiate GATA-4 transcriptional activity in serum-starved NIH 3T3 cells, which do not express GATA factors (FC, GN, and MN, unpublished data) and have low endogenous RhoA activity (Hill et al., 1995b). For these transactivation assays, we have used a GATA-responsive promoter composed of two GATA elements cloned upstream of the brain natriuretic peptide -50 bp minimal promoter [$(GATA)_2$-BNP_{-50bp}; (Grépin et al., 1994)]. Although RhoA V14 had no effect on its own on this promoter, it strongly potentiated GATA-4 transcriptional activity (Figure 4.5b). This

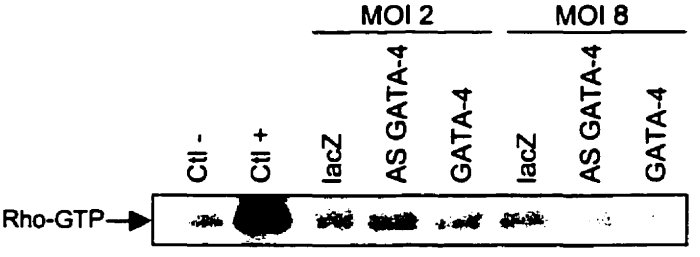
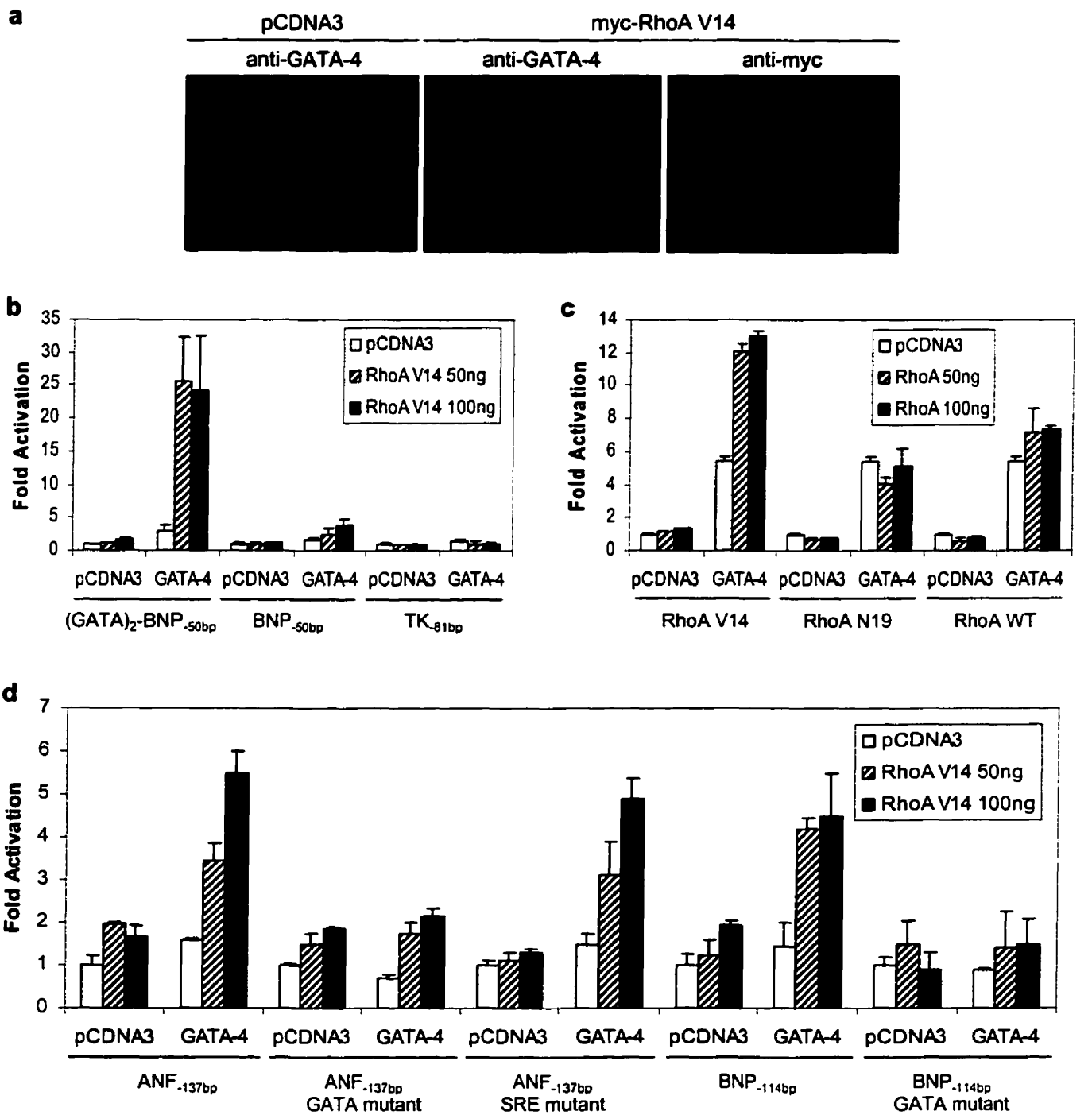


Figure 4.4. Overexpression or downregulation of GATA-4 does not affect RhoA activity in cardiomyocytes. Cardiomyocytes were infected with lacZ, AS GATA-4, or GATA-4 adenovirus, at MOIs of 2 or 8, for 36h. Rho activity was assessed as described in Fig. 4.3a. Extracts were incubated in presence of an exces of GDP (Ctl-) or GTP-γ-S (Ctl+) as negative and positive controls, respectively.

Figure 4.5. RhoA potentiates GATA-4 transcriptional activity. **(a)** RhoA V14 does not affect GATA-4 protein level or subcellular localization. Cardiomyocytes transfected with pCDNA3 or pCDNA3-myc-RhoA V14 were fixed and co-stained using an anti-myc antibody (red) and an anti-GATA-4 antibody (green). **(b)** RhoA potentiates GATA-4 transcriptional activity. Serum-starved NIH 3T3 cells were transfected with pCDNA3 or pCDNA3-GATA-4 and increasing amounts of pCDNA3-myc-RhoA V14 (0, 50, and 100 ng), together with the indicated reporter plasmid. BNP_{-50bp} and TK_{-81bp} are the minimal BNP and thymidine kinase promoters cloned upstream of a luciferase reporter gene, respectively. $(GATA)_2$-BNP_{-50bp} contains two GATA elements from the BNP promoter in front of BNP_{-50bp}. Reporter activity was assayed 72 h after transfection. **(c)** RhoA-mediated potentiation of GATA-4 transcriptional activation requires RhoA activity. Serum-starved NIH 3T3 cells were transfected with pCDNA3 or pCDNA3-GATA-4 and increasing amounts of pCDNA3-myc-RhoA V14, pCDNA3-myc-RhoA N19, or pCDNA3-myc-RhoA WT, together with the $(GATA)_2$-BNP_{-50bp} reporter plasmid. **(d)** RhoA potentiates GATA-4 activity on cardiac gene promoters. Serum-starved NIH 3T3 cells were transfected with pCDNA3 or pCDNA3-GATA-4 and increasing amounts of pCDNA3-myc-RhoA V14, together with the indicated reporter plasmid.

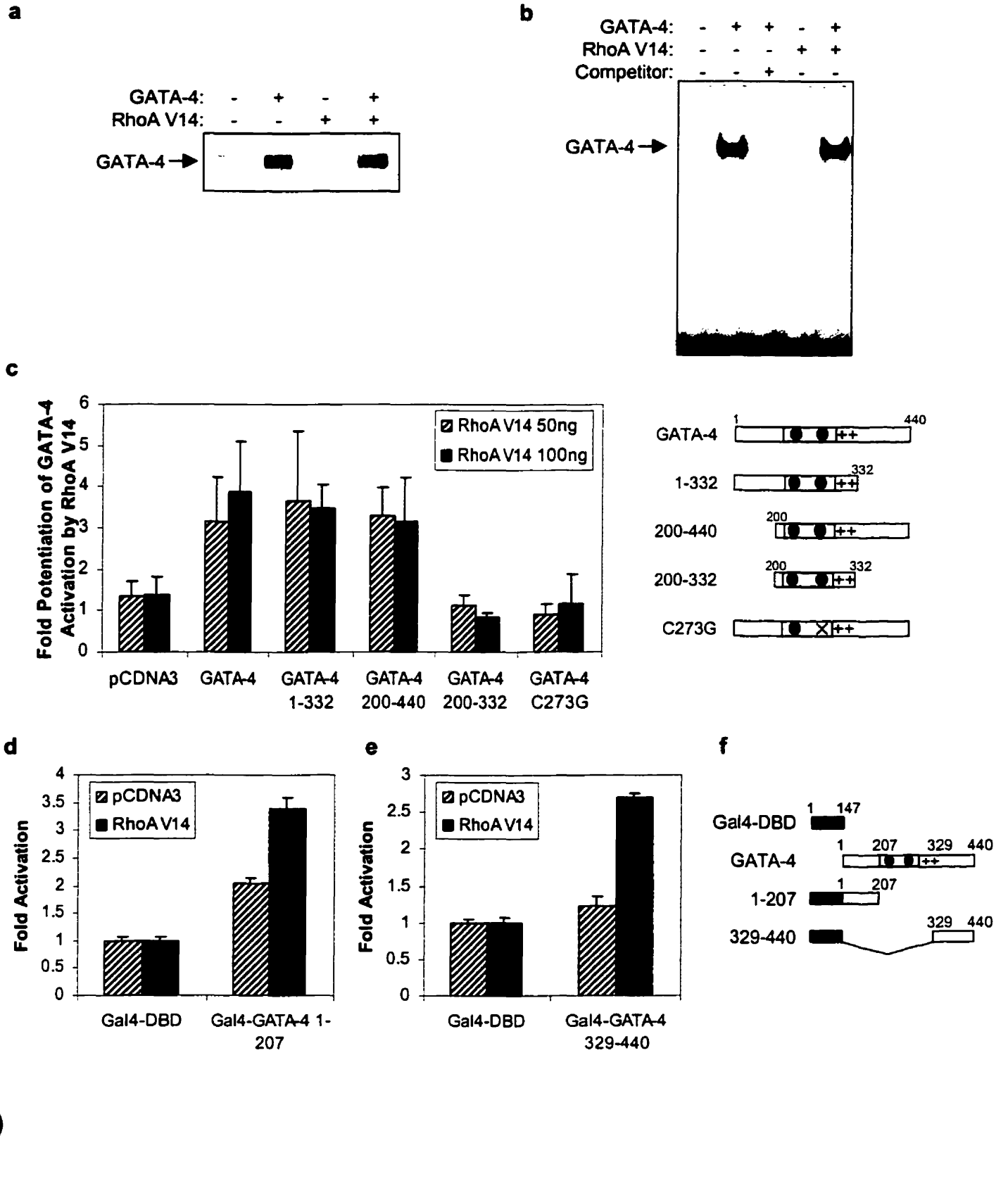

a
pCDNA3
myc-RhoA V14
anti-GATA-4
anti-GATA-4
anti-myc
b
Fold Activation
pCDNA3
RhoA V14 50ng
RhoA V14 100ng
pCDNA3 GATA-4 pCDNA3 GATA-4 pCDNA3 GATA-4
(GATA)2-BNP-50bp
BNP-50bp
TK-81bp
c
Fold Activation
pCDNA3
RhoA 50ng
RhoA 100ng
pCDNA3 GATA-4 pCDNA3 GATA-4 pCDNA3 GATA-4
RhoA V14
RhoA N19
RhoA WT
d
Fold Activation
pCDNA3
RhoA V14 50ng
RhoA V14 100ng
pCDNA3 GATA-4 pCDNA3 GATA-4 pCDNA3 GATA-4 pCDNA3 GATA-4 pCDNA3 GATA-4
ANF-137bp
ANF-137bp GATA mutant
ANF-137bp SRE mutant
BNP-114bp
BNP-114bp GATA mutant

effect was specific since RhoA V14 had no effect on the BNP minimal promoter (BNP_{-50bp}) and on the thymidine kinase promoter (TK_{-81bp}). Moreover, this effect of RhoA on GATA-4 requires RhoA activity, since the inactivated form of RhoA (RhoA N19) or the wild-type form of RhoA (RhoA WT; which is not active in serum-starved conditions) were not able to potentiate GATA-4 activity (Figure 4.5c). Interestingly, RhoA V14 was not only able to potentiate GATA-4 activity on isolated GATA elements, but was also able to potentiate GATA-4 activity on cardiac promoters known to be GATA-4 targets (Charron et al., 1999), such as the atrial natriuretic factor (ANF_{-137bp}) and BNP (BNP_{-114bp}) promoters (Figure 4.5d). Of note, this potentiation was dependent on the presence of an intact GATA element in the promoter, since mutation of the GATA element in the ANF or BNP promoter (ANF_{-137bp} GATA mutant and BNP_{-114bp} GATA mutant) completely abolished the effect of RhoA V14 on GATA-4 transactivation. Mutation of the serum response element (SRE; ANF_{-137bp} SRE mutant) abolished the GATA-independent effect of RhoA V14 on the ANF promoter, likely reflecting the potentiation of the already described RhoA target SRF (Hill et al., 1995b). However, the SRE mutation had no effect on the potentiation of GATA-4 activity by RhoA V14 (Figure 4.5d), suggesting that this effect is independent of SRF (see discussion).

RhoA potentiates GATA-4 activity by stimulating the transcriptional activity of its activation domains.

We next sought to determine the mechanism by which RhoA potentiates GATA-4 activity. The effect of RhoA on GATA-4 was not due to change in GATA-4 protein levels or DNA-binding activity, as determined by Western blotting and EMSA (Figure 4.6a and 6b), suggesting that RhoA might alter GATA-4 activity by inducing post-translational modifications and/or recruitment of a cofactor which affect its transactivation properties. To determine the domain(s) of GATA-4 required for potentiation by RhoA, various GATA-4 deletions and point mutants were tested. Deletion of the C-terminal (GATA-4 1-332) or N-terminal (GATA-4 200-440) transactivation domain of GATA-4 had no effect on the capacity of RhoA to potentiate GATA-4 activity (Figure 4.6c). However, deletion of both activation domains of GATA-4 (GATA-4 200-332) completely abolished the effect of RhoA on

Figure 4.6. RhoA potentiates GATA-4 activity by stimulating the transcriptional activity of its activation domains. RhoA V14 does not affect **(a)** GATA-4 protein level or **(b)** GATA-4 DNA-binding activity, as assessed by Western blot and EMSA, respectively. **(c)** The N- or C-terminal transactivation domain of GATA-4 is required for potentiation by RhoA. Serum-starved NIH 3T3 cells were transfected with pCDNA3 or pCDNA3-GATA-4 mutants and increasing amounts of pCDNA3-myc-RhoA V14, together with the $(GATA)_2$-BNP_{-50bp} reporter plasmid. The GATA-4 mutants are depicted in the right panel. **(d)** The N- and **(e)** C-terminal transactivation domains of GATA-4 are sufficient to support potentiation by RhoA. Serum-starved NIH 3T3 cells were transfected with pCMX-Gal4-DBD, pCMX-Gal4-GATA-4 1-207, or pCMX-Gal4-GATA-4 329-440 and pCDNA3 or pCDNA3-myc-RhoA V14, together with the $(UAS)_5$-TK_{-81bp} reporter plasmid. The Gal4-GATA-4 constructs are depicted in **(f).**


a
GATA-4: - + - +
RhoA V14: - - + +
GATA-4
b
GATA-4: - + + - +
RhoA V14: - - - + +
Competitor: - - + - -
GATA-4
c
Fold Potentiation of GATA-4 Activation by RhoA V14
RhoA V14 50ng
RhoA V14 100ng
pCDNA3
GATA-4
GATA-4 1-332
GATA-4 200-440
GATA-4 200-332
GATA-4 C273G
GATA-4
1-332
200-440
200-332
C273G
d
Fold Activation
pCDNA3
RhoA V14
Gal4-DBD
Gal4-GATA-4 1-207
e
Fold Activation
pCDNA3
RhoA V14
Gal4-DBD
Gal4-GATA-4 329-440
f
Gal4-DBD
GATA-4
1-207
329-440

GATA-4, suggesting that at least one the transactivation domain of GATA-4 is required for potentiation by RhoA. As expected, RhoA had no effect on a GATA-4 point mutant in which DNA-binding capacity is abolished [GATA-4 C273G; (Charron et al., 1999)], further supporting the idea that GATA-4 needs to bind to its cognate DNA-binding element for RhoA-mediated potentiation.

To determine if the N- and C-terminal transactivation domains of GATA-4 are sufficient to support potentiation by RhoA, these domains were fused to the DNA-binding domain of Gal4 (Gal4-DBD) and tested in reporter gene transactivation assays. While the N-terminal activation domain of GATA-4 (Gal4-GATA-4 1-207) activated transcription by 2-fold on its own, RhoA V14 further potentiated this activation by 1.7-fold (Figure 4.6d). The C-terminal activation domain of GATA-4 (Gal4-GATA-4 329-440) did not induce transcription by itself; however, in presence of RhoA V14, it activated transcription by 2.7-fold (Figure 4.6e). Taken together, these results indicate that RhoA potentiates GATA-4 activity by stimulating the transcriptional activity of its activation domains.

RhoA potentiates the transcriptional activity of other cardiac GATA factors.

The ability of RhoA to potentiate the activity of the other cardiac GATA transcription factors GATA-5 and GATA-6 was tested. Interestingly, the effect of RhoA appears not to be limited to GATA-4 since RhoA V14 was able to potentiate the activity of GATA-5 and GATA-6 to the same extent as GATA-4 (Figure 4.7).

DISCUSSION

Rho-like GTPases play a pivotal role in the orchestration of changes in the actin cytoskeleton in response to receptor stimulation, but have also been shown to be involved in other cellular processes, such as transcriptional activation, cell cycle regulation, and oncogenic transformation (Hall, 1998). Although the changes in cytoskeleton and the transcriptional regulation properties of Rho proteins are considered to be mediated by different effectors based on RhoA effector loop mutants (Sahai et al., 1998; Zohar et al., 1998), the cell type-dependent effects of Rho family members on the cytoskeleton suggest that their transcriptional regulation properties and their effect on the cytoskeleton are intimately linked. The

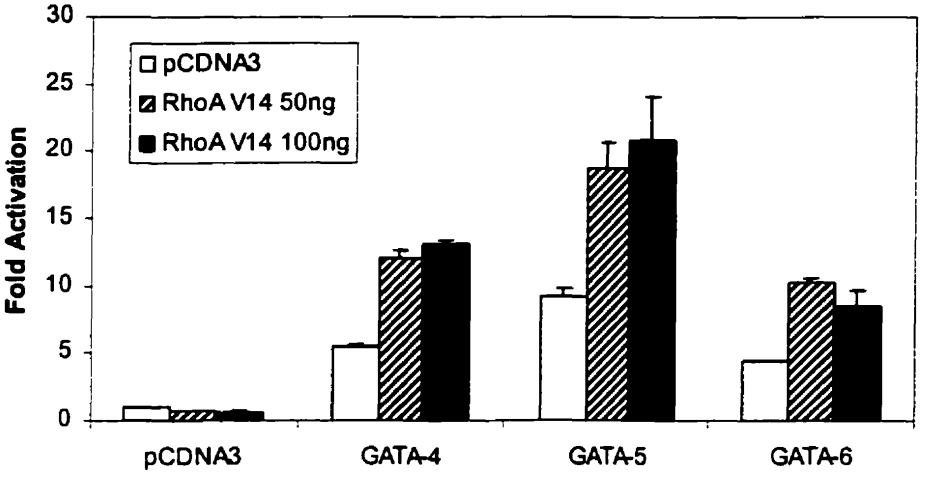


Figure 4.7. RhoA potentiates GATA-4, GATA-5, and GATA-6 transcriptional activity. Serum-starved NIH 3T3 cells were transfected with pCDNA3, pCDNA3-GATA-4, pCDNA3-GATA-5, or pCDNA3-GATA-6 and increasing amounts of pCDNA3-myc-RhoA V14, together with the $(GATA)_2$-BNP_{-50bp} reporter plasmid.

results presented in this paper support the idea that, in cardiomyocytes, the cardiac-enriched transcription factor GATA-4 acts as an effector of RhoA to regulate a cell-specific pathway of contractile protein gene expression and sarcomere assembly: First, GATA-4 regulates the expression of many genes encoding sarcomeric proteins (Charron et al., 1999). Second, the overexpression of GATA-4 is sufficient to induce sarcomeric reorganization. Third, GATA-4 is required for sarcomeric reorganization induced by Et-1 and Phe. Fourth, the transcriptional activity of GATA-4 is regulated by RhoA. These results identify for the first time a signaling pathway converging on a tissue-specific transcription factor to control sarcomere reorganization. Thus, in addition to its direct effect on myofibrillar assembly, RhoA also regulates the expression of sarcomeric proteins and sarcomere reorganization by inducing the transcriptional activity of GATA-4, thereby linking the transcriptional effects of RhoA with its regulatory effects on the cytoskeleton (Figure 4.8).

Consistent with these results, RhoA was shown to regulate the expression of genes encoding contractile proteins in various cell types, such as skeletal α-actin in skeletal muscle cells (Wei et al., 1998) and smooth muscle α-actin in smooth muscle cells (Mack et al., 2000). In these cells, RhoA was proposed to mediate transcriptional activation through SRF. However, in the present work, we found that the GATA, but not the SRE element, on the cardiac ANF promoter is the major target of RhoA activation. Given that SRF and GATA factors were recently reported to act combinatorially to activate gene expression (Morin et al., 2001; Belaguli et al., 2000) and that GATA factors are present in skeletal and smooth muscle cells (Musaro et al., 1999; Suzuki et al., 1996), we propose that SRF may regulate the expression of these RhoA targets in concert with GATA factors.

Mechanism of GATA-4-induced sarcomere formation

GATA-4 regulates the expression of many contractile proteins, such as α-myosin heavy-chain, β-myosin heavy-chain (β-MHC), and cardiac troponin I in cardiomyocytes (Charron et al., 1999) and activates the promoter of many other contractile proteins, such as cardiac troponin C, slow myosin heavy-chain, and cardiac α-actin, alone or in combination with other transcription factors (Morin et

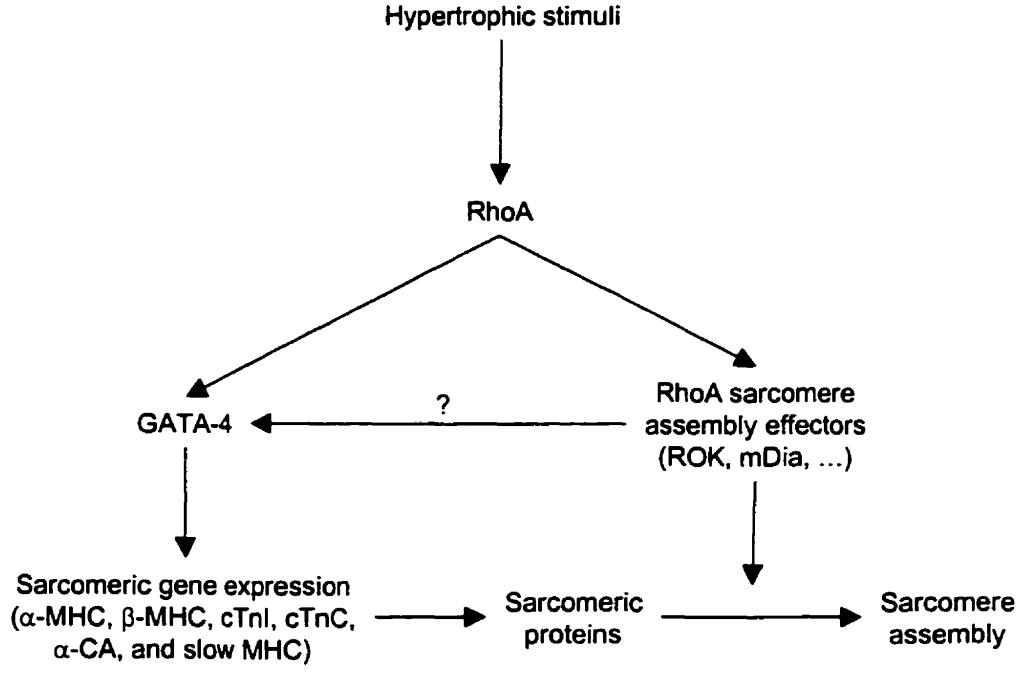


Figure 4.8. GATA-4 is an effector of RhoA and mediates cardiomyocyte sarcomere reorganization. Hypertrophic stimuli induce RhoA activity, which responds in two ways. First, RhoA potentiates GATA-4 transcriptional activity, leading to the induction of sarcomeric gene expression. Second, RhoA activates its effectors involved in sarcomere assembly, such as mDia and ROK, to polymerize already present and newly synthesized sarcomeric proteins into contractile filaments, leading to sarcomere formation. RhoA-induced sarcomere assembly effectors may also modulate GATA-4 transcriptional activity.

al., 2000; Sepulveda et al., 1998; Di Lisi et al., 1998; Ip et al., 1994; Molkentin et al., 1994; Murphy et al., 1997; Wang et al., 1998). This suggests that the role of GATA-4 in sarcomere reorganization is to induce the expression of genes encoding sarcomeric proteins. In such a model, upregulation of RhoA activity in response to trophic stimulation leads to an upregulation of GATA-4 transcriptional activity which would cause an increase in contractile gene expression. RhoA, via its cytoskeletal remodelling effectors, would then organize the polymerization and myofibrillar assembly of the newly synthetized contractile proteins (Figure 4.8).

In support of such a model, GATA DNA binding sites have been found to be essential for the induction of many cardiac promoters in response to trophic stimuli known to activate RhoA: they include the promoters of ANF, BNP, angiotensin receptor type 1a (AT1aR), adenylosuccinate synthetase 1 (Adss1), Et-1, and β-MHC (Xia et al., 2000; Hasegawa et al., 1997; Herzig et al., 1997; Marttila et al., 2000; Morimoto et al., 2000; Morin et al., 2001). It is noteworthy that the upregulation of β-MHC gene expression by GATA-4 could directly contribute to sarcomere formation.

RhoA-modulation of transcription factor activity

In addition to GATA-4, RhoA activates two other transcription factors : SRF and NF-κB (Hill et al., 1995a; Perona et al., 1997). RhoA-mediated SRF activation requires Diaphanous family proteins and is mediated through the Src tyrosine kinase (Tominaga et al., 2000). Since depletion of the free cellular actin pool activates SRF (Sotiropoulos et al., 1999), the Diaphanous family proteins may mediate their effect on SRF through their actin polymerization-inducing activity (Watanabe et al., 1999). The mechanism of NF-κB activation by RhoA involves phosphorylation of the NF-κB inhibitor IκB, causing its degradation and permitting NF-κB to translocate to the nucleus to activate transcription (Perona et al., 1997). However, the mechanism by which RhoA triggers IκB phosphorylation remains to be elucidated.

In the case of GATA-4, RhoA stimulates the transcriptional activity of GATA-4 through potentiation of its transcription activation domains. This may occur through RhoA induction of GATA-4 phosphorylation and/or regulation of GATA-4 interaction

with co-factors such as SRF, MEF2, or Nkx2-5 (Durocher et al., 1997; Morin et al., 2000; Morin et al., 2001; Belaguli et al., 2000; Lee et al., 1998; Sepulveda et al., 1998). Since Rho GTPases are potent activators of JNK, p38, and ERK mitogen activated protein kinases (MAPKs) (Blackwood and Kadonaga, 1998; Lemon and Tjian, 2000; Hirose and Manley, 2000; Brewer et al., 1999), we tested whether these kinase are involved in the potentiation of GATA-4 by RhoA. However, specific inhibitors of p38 and ERK kinases were not able to inhibit the potentiation of GATA-4 by RhoA (data not shown), suggesting that these MAPK are not involved in this process. Due to the unavailability of a JNK inhibitor, we were not able to directly test the role of this kinase in GATA-4 potentiation by RhoA; however, in vitro kinase assays showed that JNK is able to phosphorylate GATA-4 (FC, M. Arcand, S. Meloche, and MN, manuscript in preparation). We are currently mapping the JNK phosphorylation site(s) on GATA-4 and we are directly testing the involvement of JNK, as well as other RhoA effectors, in RhoA-mediated potentiation of GATA-4. Although the precise mechanisms underlying RhoA effects on these transcription factors are still uncertain, it is clear that RhoA is able to regulate transcription via different pathways.

Genetic evidence supports a role for GATA-4 in Rho signaling

In GATA-4 null mice, the cardiac tube does not form due to a migration defect of the heart precursors to the midline, a condition known as *cardia bifida* (Kuo et al., 1997; Molkentin et al., 1997). Since the Rho GTPase signaling pathway has been shown to be involved in cell motility during development (Hall, 1998), it is tempting to suggest that the cell migration defect of GATA-4 null mice might be attributed to defective Rho/GATA-4 signaling. Consistent with a role for GATA factors in cell motility during development, the GATAc/*grain* GATA transcription factor has been shown to regulate cell movement in *Drosophila* (Brown and Castelli-Gair, 2000). Whether the activity of GATAc is regulated by Rho family members during this process would be interesting to determine.

Mice harboring a mutation in another gene that has been linked to the Rho signaling pathway present a phenotype similar to *Gata4* null mice. Fibronectin, a component of the extracellular matrix and a ligand for integrin adhesion receptors,

is known to signal to the actin cytoskeleton through Rho GTPase proteins in order to effect cytoskeletal changes necessary for cell motility during development (Schwartz and Shattil, 2000). Although cardiomyocytes appear normally specified in *fibronectin* null mice, these animals present a migration defect of the heart precursors similar to the *Gata4* null phenotype (George et al., 1993; George et al., 1997). Thus, it is tempting to speculate that this phenotype could be due to the lack of Rho/GATA-4 signaling activation by fibronectin. These results raise the possibility that defective Rho/GATA-4 signaling (either due to the lack of GATA-4 or to the lack of Rho signaling activation by fibronectin) may be the unifying theme that could explain why mutations in such different gene types (a transcription factor and an extracellular matrix component) cause a similar phenotype.

The Rho/GATA signaling pathway is evolutionary conserved

The role of GATA transcription factors in Rho-induced cytoskeletal reorganization may also be important in other muscle and non-muscle cell types. RhoA and two of its effectors, ROKα and ROKβ, have been shown to be involved in smooth muscle contraction (Gong et al., 1996; Hirata et al., 1992; Kimura et al., 1996; Kureishi et al., 1997; Uehata et al., 1997). Given that the GATA transcription factor family member GATA-6 is expressed in smooth muscle cells (Suzuki et al., 1996) and that our results suggest that RhoA also modulates GATA-6 transcriptional activity, it is tempting to speculate that a RhoA/GATA-6 pathway might be a mediator of smooth muscle contraction. In addition, it is well established that Rho family GTPases act as unique molecular switches at several critical checkpoints in lymphocyte development and function and more particularly in the organization, via the actin cytoskeleton, of a specialized zone required for sustained signaling between T cells and antigen presenting cells (Acuto and Cantrell, 2000). Given the essential function of GATA-3 in T cell differentiation and activation (Kuo and Leiden, 1999), it would be worthwhile to test the role of GATA-3 as a mediator of Rho signaling in these cells.

Finally, during cell-fate determination in yeast, the GATA transcription factor Ash1p is regulated by the Cdc42p/Rho signaling pathway effector Bni1p (a Diaphanous family protein) (Takizawa et al., 1997; Evangelista et al., 1997). In addition, Ash1p

is also regulated by the Cdc42p effector Ste20p (a Ser/Thr kinase homologous to the mouse Cdc42p effector p65/PAK) during regulation of filamentous pseudohyphal growth in response to nitrogen starvation (Chandarlapaty and Errede, 1998). Despite the fact that the molecular mechanism underlying Ash1p regulation by Rho proteins remains unknown, it is nonetheless interesting to note that the functional interaction between GATA transcription factors and Rho GTPases is evolutionary conserved from yeast to mammals.

MATERIALS AND METHODS

Plasmids and adenoviral vectors. The recombinant replication-deficient adenoviruses type 5 (Ad5) expressing a nuclear localization signal (nls)-lacZ and antisenses directed specifically toward GATA-4 or GATA-6 were described previously (Charron et al., 1999; French et al., 1994). The GATA-4 overexpressing adenovirus was generated using the cloning system developed and generously provided by F.L. Graham (McGrory et al., 1988). Briefly, the *Xba*I fragment from the rat GATA-4 cDNA (Grépin et al., 1994) was subcloned into the *Xba*I site of the Ad5 shuttle vector pΔE1sp1B/CMV/BGH, a plasmid generously provided by B.A. French (Agah et al., 1997; Charron et al., 1999). The shuttle vector was co-transfected into 293 embryonic kidney cell line with pJM17 that contains a circularized *dl*309 adenoviral genome to generate replication deficient viruses with substitution of the Ad5 E1 genes for the GATA-4 cDNA. Putative Ad5 clones were plaque-purified, screened for inserts, propagated, isolated, and tittered according to the protocol of Graham and Prevec (Graham and Prevec, 1991), to produce viral stocks with titers > 2×10^9 PFU/ml.

ANF and BNP reporter plasmids and the various GATA-4, GATA-5, and GATA-6 constructs have been described previously (Grépin et al., 1994; Nemer et al., 1999; Charron et al., 1999; Morin et al., 2000; Argentin et al., 1994; Durocher et al., 1996). The Gal4-DNA-binding domain (DBD)-GATA-4 fusions were generated by subcloning the PCR-amplified N-terminal (codons 1 to 207) and C-terminal (codons 329 to 440) fragments of the rat GATA-4 cDNA into pCMX-Gal4-DBD. All constructs were confirmed by sequencing. pCDNA3-myc-RhoA wild type,

pCDNA3-myc-RhoA V14, and pCDNA3-myc-RhoA N19 expression vectors were kind gifts from R. Béliveau.

Cell culture, infections, and transfections. Neonatal cardiomyocytes were prepared from 4-day old Sprague-Dawley rats as described previously (Charron et al., 1999). The following day, the media was changed for serum-free hormonally defined medium (SFHF)(Argentin et al., 1994). Four hours later, cardiomyocytes were either infected or transfected. Infections were performed at a multiplicity of infection (MOI) of 4 by adding the appropriate recombinant adenovirus to the culture media overnight. Transfections were done using calcium phosphate precipitation with 1.5 µg of luciferase reporter plasmid and various amounts of RhoA and GATA expression vectors (see figure legends) per 9.5 cm^2 culture dish. The amount of DNA was kept constant using the empty expression vector. Sixteen to 20 h later, cardiomyocytes were washed twice with Dulbecco's modified Eagle's medium (DMEM, Canadian Life Technologies Inc.) and SFHF medium was added. Cardiomyocytes were fixed or harvested 72 h after infection or transfection. When required, SFHF was supplemented with vehicle, Et-1 (100 nM), or Phe (100 µM) for a period of 48 h before fixation or harvesting. Luciferase activity was assayed using a Berthold LB 953 luminometer. The results shown are the mean ± SD of at least two independent experiments carried out in duplicate.

NIH 3T3 cells were plated at a density of 100 000 cells per 9.5 cm^2 in 6-well plates (Falcon) in DMEM supplemented with 10% FBS (Qualified grade, Canadian Life Technologies Inc.). Transfections were carried out as in cardiomyocytes, except that after the DMEM washes, DMEM supplemented with 0.5% FBS was added.

RNA extraction and Northern blots. Total RNA was isolated from cardiomyocytes by the guanidium thiocyanate-phenol-chloroform method and blotted as previously described (Charron et al., 1999). Blots were hybridized with random prime-labeled cDNA probes for BNP, α-SA, α-CA, and GAPDH, exposed in a PhosphorImager cassette, and analyzed with ImageQuant (Molecular Dynamics).

Nuclear extracts and electrophoretic mobility shift assays (EMSAs). Nuclear extracts were prepared as previously described (Morin et al., 2000). Binding

reactions were performed in 20-µl reaction mixtures with 3 µg of nuclear extracts in a buffer containing 12 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 60 mM KCl, 4 mM Tris-HCl (pH 7.9), 0.6 mM EDTA, 0.6 mM DTT, 0.5 mg/ml BSA, 1 µg poly(dIdC), 12% glycerol, and 20 000 cpm of radio-labeled double-stranded -120 bp ANF GATA probe (Charron et al., 1999) for 20 min at room temperature . Reactions were loaded on a 4% polyacrylamide gel and run at 200V at room temperature in 0.25X TBE. The gel was dried and exposed to a PhosphorImager cassette (Molecular Dynamics).

Western blots. 10 µg of nuclear extracts were boiled in Laemmli buffer and resolved by SDS-PAGE. Proteins were transferred on Hybond-PVDF membrane, immunoblotted, and revealed using ECL Plus (Amersham Pharmacia Biotechnology) as described by the manufacturer. Goat GATA-4 supershift antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a dilution of 1/1000 and was revealed with an anti-goat horseradish peroxydase antibody (Sigma, St-Louis, MO) at a dilution of 1/100 000.

Immunofluorescence. Cardiomyocytes were washed twice with PBS, fixed with 3% paraformaldehyde in PBS for 15 min at RT, permeabilized with 0.3% Triton X-100 and 3% paraformaldehyde in PBS for 1 min at RT, and washed three times with PBS. Cardiomyocytes were then processed for immunofluorescence as described (Viger et al., 1998). The GATA-4 antibody was used at a dilution of 1/500 and was revealed with a biotinylated anti-goat antibody (1/200; Vector Laboratories Inc., Burlingame, CA) followed by avidin-rhodamine or avidin-FITC antibody (1/200; Vector Laboratories Inc.). The anti-myc monoclonal antibody (Santa Cruz Biotechnology) was used at a dilution of 1/1000 and was revealed with an anti-mouse-FITC or anti-mouse-rhodamine antibody (1/200; Vector Laboratories Inc.). Phalloidin-FITC (Sigma, St-Louis, MO) was used at 1 µg/ml. To score for reorganized cells, random fields were taken and the number of cardiomyocytes harboring striated acto-myosin fibers extending from one extremity of the cell to the other was counted and divided by the total number of cardiomyocytes in the same field. These observations were performed blindly. Identical results were obtained

using a similar CMV promoter-driven rat GATA-4 adenovirus, a kind gift of C.E. Murray.

Rho-GTP loading assays. Whole-cell extracts were prepared from cardiomyocytes and the active form of Rho (Rho-GTP) was selectively affinity-precipitated from these extracts using a GST-Rhotekin fragment coupled to glutathione-Sepharose, according to the manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY). The affinity-precipitated complexes were resolved by SDS-PAGE, transferred to PVDF, and revealed using an anti-Rho antibody.

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CHAPTER V. DISCUSSION

1 MECHANISMS FOR GATA FACTOR SPECIFICITY

Members of the GATA transcription factor family play critical roles in diverse cellular processes. Although some members are coexpressed in specific cell types, each family member appears to fulfill essential, nonredundant functions during development. The molecular basis for the differential roles of GATA factors remains poorly understood, but could occur via at least three nonexclusive mechanisms: differential expression of GATA factors in subsets of cells or tissues, differential affinity of GATA factors for GATA elements, or differential interaction of GATA proteins with cofactors.

1.1 Differential interaction with cofactors

While the differential expression of GATA factors has been discussed in details in the *Tissue distribution and roles of the GATA transcription factors* section, support for the capacity of GATA factors to differentially interact with cofactors has only been provided recently. Nonetheless, two types of interaction clearly show that this mechanism imparts functional specificity to GATA factors. The first example comes from the study of Durocher et al., who showed functional and physical interaction between GATA-4, but not GATA-6, and Nkx2-5 (Durocher et al., 1997; Durocher and Nemer, 1998). This differential interaction may explain why GATA-6 is not able to compensate for GATA-4 in *Gata4* null mice, with respect to cardiogenesis (Kuo et al., 1997; Molkentin et al., 1997). The second example comes from the study of Morin et al., who showed functional and physical interactions between MEF2 and all GATA family members, except GATA-5 (Morin et al., 2000). These observations suggest that, while MEF2 and Nkx2-5 interact with the same C-terminal zinc finger domain of GATA-4, they apparently do not recognize the same molecular determinants. It would be interesting to determine the C-terminal zinc finger residues involved in these differential interactions by using GATA-4, GATA-5, and GATA-6 non-conserved amino acid swapping mutants (microchimera analysis).

While two GATA factors may interact with the same cofactor, another level of specificity may arise from the differential regulation of this interaction for certain GATA factors, but not for others. For example, post-translational modifications,

such as phosphorylation or acetylation, may modulate the capacity of certain GATA factors to interact with a cofactor, but may not affect the interaction of other GATA family members with this same cofactor. Such a mechanism would impart functional specificity to GATA factors in response to signaling cascades.

Differential regulation may also be regulated at the transcriptional level by the production of GATA factor isoforms that gain or lose a cofactor interaction domain. An example for this type of regulation occurs with GATA-5, where the use of an alternative first exon produces a GATA-5 isoform which is composed only of the C-terminal zinc finger and the C-terminal transactivation domain (MacNeill et al., 1997). Thus, this isoform lacks the N-terminal zinc finger, a domain essential for GATA-5 interaction with FOG-1 and/or FOG-2. Although the physiological role of this isoform remains to be determined, this type of regulation may be important in spleen and heart, where GATA-5 is coexpressed with FOG-1 and FOG-2, respectively (Tsang et al., 1997; Lu et al., 1999; Svensson et al., 1999; Tevosian et al., 1999; Holmes et al., 1999).

There is increasing evidence for the differential capacity of GATA factors to regulate target genes *in vivo* (Morrisey et al., 2000). Dab2, a mitogen-responsive protein involved in signaling, is expressed in the visceral endoderm of wild type and GATA-4-deficient embryos, but not in the visceral endoderm of GATA-6-deficient embryos. Forced expression of GATA-6, but not GATA-4, transactivates the Dab2 promoter in heterologous cells. This specificity is not due to DNA-binding affinity differences, as the DNA-binding domain of GATA-4 or GATA-6 fused to the VP16 transcriptional activation domain transactivated the Dab2 promoter equally. Although further experiments are required to understand how GATA-4 and GATA-6 differentially regulate gene expression in visceral endoderm, these results suggest that the N- and/or C-terminal activation domains of GATA-4 and GATA-6 interact differentially with a cofactor to activate the Dab2 promoter.

1.2 Differential DNA-binding site affinity

Differential DNA-binding affinity is another mechanism by which GATA factors target distinct downstream genes. In the case of GATA-1, GATA-2, GATA-3, and GATA-6, *in vitro* binding site selection experiments have shown differences in

DNA-binding specificity, although these differences have not been correlated with natural GATA elements present in GATA-target promoters (Ko and Engel, 1993; Merika and Orkin, 1993; Sakai et al., 1998).

The data presented in this thesis show that a subset of cellular genes may be *bona fide* targets for more than one GATA factor, whereas others are under the control of a specific GATA factor (Charron et al., 1999). For example, the higher affinity of GATA-4 for the α-MHC GATA element correlates with the finding that the endogenous α-MHC gene is a preferential GATA-4 target. This was the first time that specific *in vivo* targets for a GATA factor were reported.

Interestingly, the ANF and α-MHC sequences that are preferential GATA-4 binding sites have an A at the underlined position of the (A/T)GATA(A/G) consensus. Given that the GATA-6 binding site selection experiments did not reveal any exclusion for an A at this site (Sakai et al., 1998), it would be interesting to determine whether GATA-4 site selection experiments would reveal a preference for an A at this position.

Additional results from our laboratory further support differential affinity of GATA factors for specific DNA-binding sites. For example, GATA-5 has a higher affinity than GATA-4 for the Et-1 promoter GATA element (Nemer et al., 1999); accordingly, the Et-1 promoter is more strongly activated by GATA-5 than GATA-4, suggesting a specific role for GATA-5 in Et-1 gene expression.

Further work from our laboratory suggests that GATA-4 and GATA-6 differentially regulate target genes. Differential display-PCR experiments comparing the effect of inhibiting GATA-4 and inhibiting GATA-6 expression in postnatal cardiomyocytes (using adenovirus-mediated transfer of antisense cDNAs) identified a new gene, designated 1-15-2a, which is specifically regulated by GATA-6, but not by GATA-4 (F. Charron and M. Nemer, unpublished data). Although 1-15-2a has not been characterized previously, homologous sequences exist in the expressed sequence tag (EST) databases of many vertebrate species, indicating that it is conserved during vertebrate evolution. Whole-mount *in situ* hybridization and RT-PCR analyses revealed that it is expressed in embryonic cardiac and brain tissues, two sites of GATA-6 expression (S. Caron, F. Charron, and M. Nemer, unpublished

data). Further characterization of this and other GATA target genes identified by differential display is ongoing in our lab and will help to understand the respective roles of GATA factors.

2 REGULATION OF CARDIAC GENE EXPRESSION BY GATA FACTORS: COMBINATORIAL MECHANISMS

2.1 Regulation of gene expression by combinatorial mechanisms

Human development requires the differential transcription of about 30 000 genes in a precise spatial and temporal pattern. A key problem is understanding how an organism can achieve such cellular diversity, while maintaining the capacity to respond to its environment. One solution is to employ a limited set of activators to integrate diverse regulatory signals in a combinatorial manner. In agreement with this view, earlier studies of gene regulation in sea urchin embryos showed the possibility of modeling gene regulation as logic circuits, and revealed the computational power of enhancers and promoters (Yuh et al., 1998). Recent studies in *Drosophila* further support the idea that regulatory regions of genes control development by acting as molecular integrators, establishing cell fate according to the combined effects of different transcription factors (Halfon et al., 2000; Flores et al., 2000; Xu et al., 2000).

One way the transcriptional effect of these transcription factors may be integrated together is by the use of cooperativity or transcriptional synergy, where the final effect is greater than the sum of the individual effects. In such a model, a unique combination of transcription factors would be organized at the enhancer-promoter into a nucleoprotein complex (the enhanceosome) that promotes their interaction and cooperative binding to DNA, leading to synergistic activation of transcription (reviewed in (Carey, 1998)). Two layers of stereo-specificity are necessary for gene activation. The first layer arises from the contextual activator-activator interactions to promote cooperative assembly of the enhanceosome on naked DNA or chromatin. The second layer arises from the specific activation surface displayed by the enhanceosome, which is chemically and spatially complementary to target surfaces on coactivators and the basal transcription machinery (Bruhn et al., 1997; Kim and Maniatis, 1997; Merika et al., 1998).

The two best-described enhanceosomes are the ones formed over the interferon β (IFNβ) and the T cell receptor α (TCRα) gene enhancers (Giese et al., 1995; Mayall et al., 1997; Kim and Maniatis, 1997; Merika et al., 1998). The IFNβ enhanceosome is formed on a 57 bp enhancer and contains the p50 and p65 subunits of NF-κB, IRF-1, ATF-2, c-Jun, and HMG I (high mobility group I). The TCRα enhanceosome is formed on a 75 bp enhancer and contains Ets-1, AML-1 (CBFα2, PEB2αB), ATF (or CREB), and LEF-1. Thus, both enhanceosome are composed of ubiquitous and tissue-specific transcription factors, in addition to architectural proteins (HMG I and LEF-1) that serve to bend DNA, thereby allowing protein-protein interactions normally proscribed by the energetic cost of DNA bending. In both enhanceosomes, the CBP coactivator also plays a crucial role in mediating synergy.

The next sections will discuss novel GATA factor interactions identified during the course of my work. These and the other GATA interactions presented will lead us to propose that GATA-4 and GATA-6 are essential components of an ANF enhanceosome.

2.2 Heterotypic interactions between GATA-4 and GATA-6 and the maintenance of cardiac gene expression in the postnatal heart

Because mice lacking GATA-4 or GATA-6 die prior to formation of the primitive heart tube, they are not useful for assessing the role of GATA-4 and GATA-6 in postnatal heart development. Thus, an adenovirus-mediated antisense transfer strategy that specifically inhibits GATA-4 or GATA-6 protein production in cardiomyocytes was developed and used to assess the role of these factors in postnatal cardiomyocytes (Charron et al., 1999). The results indicate that several endogenous cardiac genes, including ANF, BNP, cTnI, α-MHC, β-MHC, and PDGFRβ, are downregulated in cardiomyocytes lacking either GATA-4 or GATA-6, suggesting that these genes are transcriptional targets for both GATA-4 and GATA-6. Interestingly, the promoter of all these genes contains at least one GATA element, suggesting that they are direct downstream targets for GATA-4 and GATA-6 in postnatal cardiomyocytes. Moreover, this approach revealed that a subset of genes is targeted by both GATA-4 and GATA-6; remarkably, removal of

both GATA proteins had the same effect as removing either one by itself, suggesting that GATA-4 and GATA-6 might be part of the same active transcription complex. Indeed, experiments using the ANF and BNP promoters revealed that GATA-4 and GATA-6 form a heterotypic complex that binds a single GATA element and synergistically activates transcription of cardiac promoters. Taken together, these results suggest that GATA-4 and GATA-6 act in concert to regulate expression of cardiac genes.

As discussed in the *Interaction with cofactors* section, a possible role for these homo- and heterotypic GATA factor interactions could be the formation of higher-order structures among distant regulatory elements that share GATA sites. Such GATA factor higher-order structures could be involved in the interaction between the globin LCRs and downstream globin enhancers and promoters, they could mediate the formation of chromatin loops and the establishment of regions of active chromatin, and/or they could allow for the formation of an enhanceosome at the enhancer-promoter, as described below. These hypotheses are in agreement with the role of GATA factors in chromatin remodeling, as discussed in the *Introduction*.

Our laboratory has also used the adenovirus-mediated antisense transfer strategy to screen cDNA macroarrays for GATA-4 and GATA-6 targets in postnatal cardiomyocytes (F. Charron, S. Caron, and M. Nemer, unpublished data). The results indicate that GATA-4 and GATA-6 regulate the expression of several endogenous cardiomyocyte genes that are involved in many cellular processes, including metabolism (e.g. arachidonate 12-lipoxygenase), signal transduction (e.g. Ras-GTPase-activating protein), stress response (e.g. BiP; steroidogenesis-activator polypeptide), cell cycle (e.g. cyclin D2), cell adhesion (e.g. E-selectin), and apoptosis (e.g. growth arrest and DNA-damage-inducible protein; GADD45). Together, these results suggest that GATA-4 and GATA-6 are essential for the maintenance of the differentiated phenotype of cardiomyocytes and that, in addition, they control a variety of cellular processes in these cells. The technology available at the time allowed us to screen for about 600 cDNAs per array; however, with the refinements in array technologies, which now allow to screen more than 15

000 genes simultaneously, and the recent sequencing of the human genome, it is possible to envisage that such techniques could be used to screen exhaustively the genes of an organism for GATA transcriptional targets. Such experiments would help the creation of tissue-specific "GATA-target repertoires", allowing to associate GATA factors with distinct cellular processes in various cell types.

2.3 GATA-dependent recruitment of MEF2 to target promoters

MEF2 transcription factors are key regulators of cardiac myogenesis and morphogenesis, but the molecular basis for their actions was poorly understood. The work described in Chapter III provides evidence that, in cardiomyocytes, MEF2 proteins are recruited by GATA-4 to target promoters, such as α-MHC, α-CA, and BNP (Morin et al., 2000). The observation that all GATA factors, except GATA-5, are able to synergize with MEF2 suggests that GATA factors are not equally competent to interact with MEF2 proteins and that this combinatorial interaction may impart functional specificity to GATA factors (reviewed in (Charron et al., 2001)).

In addition to cardiomyocytes, MEF2 proteins are coexpressed with members of the GATA family in several other cell types. Most notable is the presence of MEF2 proteins with GATA-3 in T lymphocytes, somites, and brain (George et al., 1994; Joulin et al., 1991; Lim et al., 2000; Nardelli et al., 1999; Pandolfi et al., 1995; Swanson et al., 1998; Zheng and Flavell, 1997); with GATA-6 in smooth muscle cells (Narita et al., 1996; Suzuki et al., 1996); and with GATA-2 in skeletal muscle (Musaro et al., 1999). Given the role of GATA factors in cell differentiation, the GATA-MEF2 combinatorial interactions might provide a general paradigm for understanding the role of MEF2 proteins as determining factors in diverse cell lineages.

The recruitment of MEF2 to its target promoters by GATA factors is reminiscent of the cooperative interaction between MEF2 proteins and the myogenic bHLH factors in skeletal muscle differentiation, where bHLH protein activity is potentiated by MEF2 proteins independently of their DNA-binding activity (Molkentin et al., 1995). However, the transcriptional mechanism by which GATA and bHLH interactions with MEF2 factors lead to synergistic promoter activation remains

largely unknown. The capacity of p300 and CBP to act as coactivators of myogenic bHLH, GATA, and MEF2 proteins might reveal such a mechanism: it is possible that the synergy is due to the cooperative binding of MEF2 and bHLH or MEF2 and GATA proteins to CBP/p300 (Sartorelli et al., 1997; Blobel et al., 1998). In addition, the steroid receptor coactivator (SRC) GRIP-1 acts as a cofactor of myogenic bHLH-MEF2-mediated transcription (Chen et al., 2000b). Given that GRIP-1 directly interacts with MEF2 and serves to recruit the p300 and PCAF coactivators, potentiation of MEF2 binding to GRIP-1 might be another mechanism that helps to recruit coactivators to MEF2/bHLH and MEF2/GATA complexes. Thus, the recruitment of p300, CBP, and/or PCAF by MEF2 may represent the general mechanism by which MEF2 potentiates transcription of its cofactors.

Alternatively, MEF2-GATA or MEF2-bHLH interactions may displace or overcome a corepressor. For example, MEF2 interaction may displace FOG-2-mediated GATA repression (Lu et al., 1999; Svensson et al., 1999; Tevosian et al., 1999; Holmes et al., 1999). Conversely, recruitment of MEF2 by GATA factors may displace the MEF2-associated corepressor MITR or the HDAC4 or HDAC5 deacetylases (Miska et al., 1999; Sparrow et al., 1999; Lu et al., 2000).

Interestingly, the interaction between GATA and MADS-box family proteins is not limited to members of the MEF2 family as the MADS-box superfamily member SRF is also a GATA cofactor (Morin et al., 2001; Belaguli et al., 2000). This interaction appears to be important to mediate cardiomyocyte nuclear signaling induced by Et-1, suggesting that GATA-MADS complexes may be involved in tissue-specific nuclear signaling by extracellular stimuli. The possibility that GATA-4-MEF2 complexes mediate nuclear signaling will be discussed in the next section.

2.4 GATA-4, GATA-6, and the ANF enhanceosome

Studies from our laboratory suggest that the ANF promoter is organized into three modular domains, which each possess different cardiac activities (Argentin et al., 1994; Durocher et al., 1996). The A domain (-700 to -380 bp) is required for adult, ventricular activity; the B domain (-380 to -137 bp) is required for embryonic activity; whereas the C domain (-137 to -78 bp) is required for basal cardiac

activity. Interestingly, the C domain is essential for A and B domain activity in the context of the promoter (Durocher and Nemer, 1998).

Although GATA factors have not been associated with enhanceosome formation yet, many evidence lead us to propose that the cardiac GATA-4 and GATA-6 transcription factors are essential components of an enhanceosome formed over the C domain of the ANF promoter. First, a GATA-4-GATA-6 complex is required for ANF expression in cardiomyocytes. Second, many transcription factors, including MEF2, SRF, and Nkx2-5, synergize and interact physically with GATA-4 and/or GATA-6 to regulate ANF expression. Third, DNA-binding sites for these GATA cofactors are localized within a short region of the proximal ANF promoter (the C domain) and their phasing is conserved throughout evolution. Fourth, mutation of any of these DNA-binding sites, including GATA elements, significantly reduces the proximal ANF promoter activity in cardiomyocytes.

These characteristics are reminiscent of the IFNβ and TCRα enhanceosomes (Carey, 1998), and lead us to propose the following model (Figure 5.1): (i) GATA-4 or GATA-6 is bound to the -120 bp GATA element of the ANF promoter (Charron et al., 1999), (ii) heterotypic GATA-4-GATA-6 interaction occurs with the DNA-bound GATA factor (Charron et al., 1999), (iii) a SRF dimer is bound to the -106 bp SRE-like and interacts with GATA-4 and/or GATA-6 (Morin et al., 2001; Belaguli et al., 2000), (iv) Nkx2-5 is bound to the -90 bp NKE and interact with GATA-4 and SRF (Durocher et al., 1997; Chen and Schwartz, 1996), (v) MEF2 interacts with GATA-4 and/or GATA-6 (Morin et al., 2000), (vi) FOG-2 interacts with GATA-4 and/or GATA-6 (Lu et al., 1999; Svensson et al., 1999; Tevosian et al., 1999; Holmes et al., 1999), and (vii) CBP and/or p300 coactivators are likely to be in this complex since they have been shown to interact with GATA, MEF2, and SRF (Sartorelli et al., 1997; Blobel et al., 1998; Ramirez et al., 1997).

Although our model involves DNA bending to allow contacts between distant proteins, no "classic" architectural proteins of the HMG family have been identified yet that binds to the ANF promoter. It is likely that GATA and SRF, which both possess intrinsic DNA bending properties (Omichinski et al., 1993; West et al., 1997), contribute to DNA looping of the ANF promoter.

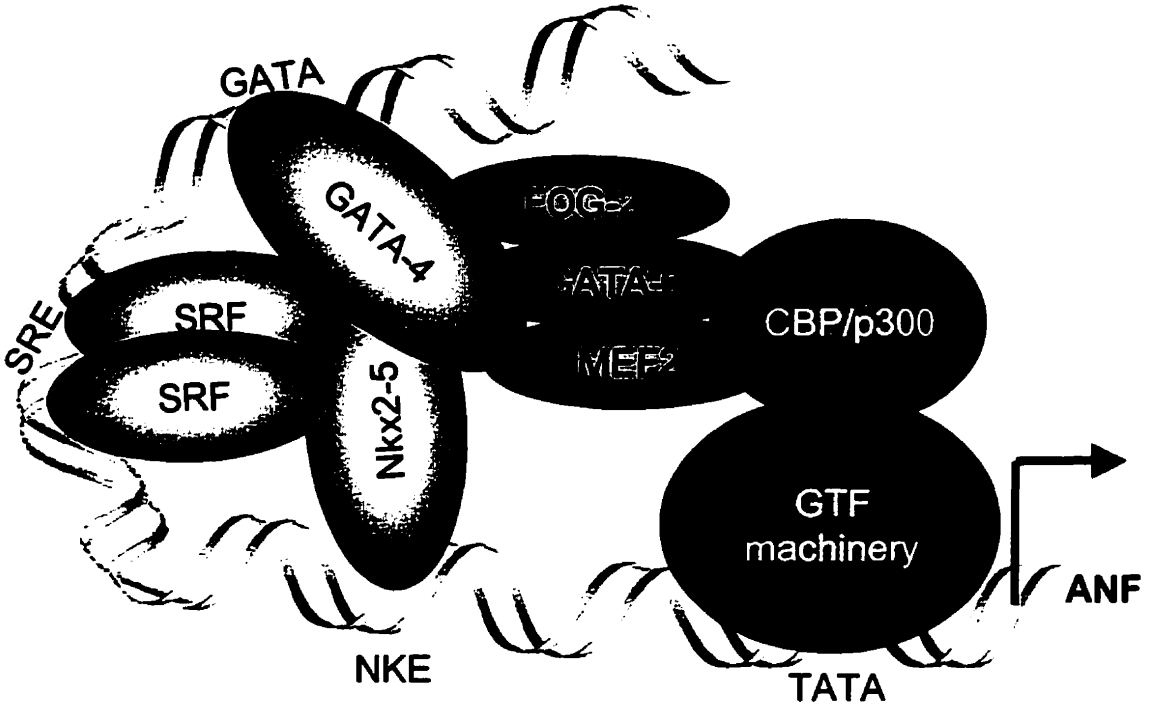

Figure 5.1 Model of the ANF enhanceosome. The C domain (-137 to -78 bp) and the minimal ANF promoter (-78 to +1 bp) are represented. GATA, SRE, NKE, and TATA are GATA-4 and/or GATA-6, SRF, Nkx2-5, and general transcription factor (GTF) machinery binding sites, respectively. Although all the proteins shown to interact with the cardiac GATA factors on the ANF promoter are shown here, all interactions may not occur at the same time. In addition, post-translational modifications or recruitment of certain cofactors may affect the presence of other proteins, such as coactivators or corepressors, in the complex.

Although further experiments will be needed to prove the formation of an enhanceosome over the ANF promoter, new techniques are now available to facilitate the characterization of an enhanceosome. Kim and Maniatis showed that synergistic transcriptional activation can be observed from an *in vitro*-assembled IFNβ enhanceosome: after depleting nuclear extracts of endogenous IFNβ-binding proteins, it is possible to supplement the extract with limiting concentrations of the individual recombinant proteins and recapitulate the synergistic response observed *in vivo* (Kim and Maniatis, 1997). Such experiments using the basal ANF promoter would allow to determine the transcriptional activators, the protein domains, and the post-translational modifications necessary for enhanceosome formation and for synergy. In addition, these experiments would also allow to determine the coactivators and the general transcription factors interacting with the ANF enhanceosome.

Given that the activity of many ANF enhanceosome components, including SRF, MEF2, and GATA-4, have been shown to be induced by signal transduction pathways (Morin et al., 2001; Han et al., 1997; Treisman, 1995; Zhao et al., 1999)(F. Charron, M. Arcand, G. Tsimiklis, L. Robitaille, K. McBride, S. Meloche, and M. Nemer, manuscript in preparation) and that the proximal ANF promoter is involved in induced ANF expression in response to growth factor stimulation (Morin et al., 2001), it is tempting to speculate that this induction is mediated by a change in the stereo-specific arrangement of GATA, SRF, MEF2, and/or Nkx2-5 activation domains, leading to the release of a corepressor, such as FOG-2, MITR, HDAC4, or HDAC5, or alternatively, to the recruitment of a coactivator, such as CBP, p300, or GRIP-1, or the basal transcription machinery. Biochemical experiments on purified ANF enhanceosome complexes will help to determine whether stimulation induces recruitment or release of a cofactor.

3 GATA-4 MEDIATES RHOA SIGNALING AND SARCOMERE REORGANIZATION IN CARDIOMYOCYTES

3.1 Rho GTPases and cardiomyocyte sarcomere formation

Cardiomyocytes are composed of a highly specialized cytoskeleton partitioned into contractile units named sarcomeres. Sarcomeres consist of highly organized actin

and myosin filaments, stabilized by α-actinin. Despite their crucial role in contraction, the mechanism governing the regulation of cardiomyocyte sarcomere organization remains unknown.

Members of the Rho family of small GTPase proteins govern the assembly of cytoskeletal actin fibers and focal adhesion complex in fibroblasts and in other cell types (Hall, 1998). In their inactive state, members of the Rho family are bound to GDP. They are activated by the exchange of GDP for GTP, a reaction catalyzed by guanine nucleotide exchange factors (GEFs). The endogenous GTPase activity of Rho proteins hydrolyzes bound GTP to GDP, returning them to their inactive state. This GTPase activity can also be stimulated by GTPase-activating proteins (GAPs). When activated, the Rho proteins undergo a conformational change, allowing them to couple to their effectors (Bishop and Hall, 2000).

The work presented in Chapter IV supports the independent findings of Hoshijima et al. and establishes that (i) activated RhoA is sufficient to induce cardiomyocyte sarcomere reorganization and that (ii) the RhoA signaling pathway is required for Phe and Et-1-induced sarcomere reorganization in cardiomyocytes [(F. Charron, G. Tsimiklis, L. Robitaille, and M. Nemer, submitted, Chapter IV)(Hoshijima et al., 1998)]. Given that other Rho family members, such as Rac1 and Cdc42, have been shown to cross talk with RhoA and to be involved in cytoskeleton remodeling (Bar-Sagi and Hall, 2000; Hall, 1998), it will be interesting to determine the contribution of these proteins to the effect of RhoA.

3.2 GATA-4 mediates sarcomere reorganization

In this work, we also report that GATA-4 overexpression in cardiomyocytes induces sarcomeric reorganization (F. Charron, G. Tsimiklis, L. Robitaille, and M. Nemer, submitted, Chapter IV). Moreover, GATA-4, similarly to RhoA, is essential for cardiomyocyte sarcomeric reorganization induced by Phe and Et-1, suggesting that GATA-4 is a mediator of sarcomeric reorganization.

In addition to GATA-4, GATA-6 is also required for Phe and Et-1-induced sarcomere reorganization (F. Charron and M. Nemer, unpublished data). Although this finding is consistent with a role for heterotypic GATA-4-GATA-6 complexes in sarcomere formation, the fact that the overexpression of GATA-4 alone (without

increasing GATA-6 levels) in cardiomyocytes is sufficient to induce sarcomere reorganization suggests that a certain threshold level of GATA factors is required to support sarcomere reorganization. The overexpression of GATA-6 in cardiomyocytes will help to verify this hypothesis: if sarcomeres are not induced, it would mean that an increase in total GATA levels is not sufficient to mediate cytoskeletal remodeling; conversely, if sarcomeres are induced, these results would suggest that increasing GATA levels is sufficient to mediate cytoskeletal remodeling.

Given the similarities between the gain- and loss-of-function phenotypes of GATA-4 and RhoA in cardiomyocytes, we proposed that these two proteins act in the same signaling pathway to regulate cardiomyocyte sarcomere formation. In agreement with this hypothesis, we show that RhoA acts upstream of GATA-4 to regulate its transcriptional activity.

3.3 GATA-4 is a transcriptional target for RhoA

In addition to their effect on the cytoskeleton, Rho family members regulate transcriptional activation (Hill et al., 1995; Perona et al., 1997; Simon et al., 2000). Consistent with GATA-4 being a transcriptional effector of RhoA, we show that RhoA potentiates GATA-4 transcriptional activity on cardiac promoters known to be GATA-4 targets (F. Charron, G. Tsimiklis, L. Robitaille, and M. Nemer, submitted, Chapter IV). These results identify for the first time a signaling pathway converging on a transcription factor to control sarcomere reorganization and suggest that RhoA, in addition to its direct effect on myofibrillar assembly, also regulates the expression of sarcomeric proteins and sarcomere reorganization by inducing the transcriptional activity of GATA-4, thereby linking the regulation of transcription by RhoA and its effects on the cytoskeleton.

In addition to GATA-4, RhoA activates two other transcription factors: SRF and NF-κB (Hill et al., 1995; Perona et al., 1997). RhoA-mediated SRF activation requires Diaphanous family proteins and is mediated through the Src tyrosine kinase (Tominaga et al., 2000; Sotiropoulos et al., 1999). The mechanism of NF-κB activation by RhoA involves phosphorylation of the NF-κB inhibitor IκB, causing its degradation and permitting NF-κB to translocate to the nucleus to activate

transcription (Perona et al., 1997). However, the mechanism by which RhoA triggers IκB phosphorylation remains to be elucidated.

In the case of GATA-4, RhoA stimulates the transcriptional activity of GATA-4 through potentiation of its transcription activation domains. This may occur through RhoA induction of a GATA-4 post-translational modification, such as phosphorylation or acetylation, and/or regulation of GATA-4 interaction with cofactors such as SRF, MEF2, or Nkx2-5 (Durocher et al., 1997; Morin et al., 2000; Morin et al., 2001; Belaguli et al., 2000; Lee et al., 1998; Sepulveda et al., 1998). Consistent with this hypothesis, results from our laboratory indicate that GATA-4 is a phosphoprotein in cardiomyocytes and that its phosphorylation is induced by RhoA-inducing agonists, such as Phe and Et-1 (F. Charron, M. Arcand, G. Tsimiklis, L. Robitaille, K. McBride, S. Meloche, and M. Nemer, manuscript in preparation). Since Rho GTPases are potent activators of JNK, p38, and ERK mitogen activated protein kinases (MAPKs) (Blackwood and Kadonaga, 1998; Lemon and Tjian, 2000; Hirose and Manley, 2000; Brewer et al., 1999), we tested whether these kinases are involved in the potentiation of GATA-4 by RhoA. However, our results suggest that the ERK and p38 MAPKs are not involved in this process (F. Charron, G. Tsimiklis, L. Robitaille, and M. Nemer, submitted, Chapter IV). Due to the unavailability of a JNK inhibitor, we were not able to directly test the role of this kinase in GATA-4 potentiation by RhoA; however, *in vitro* kinase assays showed that JNK is able to phosphorylate GATA-4 (F. Charron, M. Arcand, G. Tsimiklis, L. Robitaille, K. McBride, S. Meloche, and M. Nemer, manuscript in preparation). Interestingly, GATA-4 harbors two potential JNK docking sites. We are currently mapping the JNK phosphorylation and docking sites on GATA-4 and we are directly testing the involvement of JNK, as well as other RhoA effectors, such as Rho kinase α (ROKα) and ROKβ, in RhoA-mediated potentiation of GATA-4.

3.4 Genetic evidence support a role for GATA-4 in Rho signaling

Although the early embryonic lethality of *Gata4* null mice limits their use in analyzing the role of GATA-4 in sarcomere reorganization (Kuo et al., 1997; Molkentin et al., 1997), the defect in cardiomyocyte migration to form a primitive

heart tube is reminiscent of documented roles of the Rho GTPase signaling pathway in cell motility during development (Hall, 1998). Thus, the cell migration defect of *Gata4* null mice might reflect defective Rho/GATA-4 signaling. Consistent with a role for GATA factors in cell motility during development, the GATAc/*grain* GATA transcription factor has been shown to regulate cell movement during organ morphogenesis in *Drosophila* (Brown and Castelli-Gair, 2000). By crossing heterozygous *Drosophila* mutants of Rho and GATAc to generate compound heterozygotes and analyze cell movement in these animals, it would be interesting to determine whether GATAc interacts genetically with Rho during this process.

Mice harboring a mutation in another gene that has been linked to the Rho signaling pathway present a phenotype similar to *Gata4* null mice. Fibronectin, a component of the extracellular matrix and a ligand for integrin adhesion receptors, is known to signal to the actin cytoskeleton through Rho GTPase proteins in order to effect cytoskeletal changes necessary for cell motility during development (Schwartz and Shattil, 2000). Although cardiomyocytes appear normally specified in *fibronectin* null mice, these animals present a migration defect of the heart precursors similar to the *Gata4* null phenotype (George et al., 1993; George et al., 1997). Thus, it is tempting to speculate that this phenotype could be due to the lack of Rho/GATA-4 signaling activation by fibronectin. These results raise the possibility that defective Rho/GATA-4 signaling (either due to the lack of GATA-4 or to the lack of Rho signaling activation by fibronectin) may be the unifying theme that could explain why mutations in such different gene types (a transcription factor and an extracellular matrix component) cause a similar phenotype. It would be interesting to cross heterozygous *fibronectin*$^{+/-}$ and *Gata4*$^{+/-}$ mice and verify whether compound heterozygous mice present a heart precursor migration defect. These experiments should also be tried with heterozygous mice deficient in other genes involved in fibronectin/integrin/Rho signaling and would confirm genetically whether the fibronectin signaling pathway is mediating its signal through GATA-4.

3.5 The Rho/GATA signaling pathway is evolutionary conserved

The role of GATA transcription factors in Rho-induced cytoskeletal reorganization may also be important in other muscle and non-muscle cell types. RhoA and two of

its effectors, ROKα and ROKβ, have been shown to be involved in smooth muscle contraction (Gong et al., 1996; Hirata et al., 1992; Kimura et al., 1996; Kureishi et al., 1997; Uehata et al., 1997). Given that the GATA transcription factor family member GATA-6 is expressed in smooth muscle cells (Suzuki et al., 1996) and that our results suggest that RhoA also modulates GATA-6 transcriptional activity, it is tempting to speculate that a RhoA/GATA-6 pathway might be a mediator of smooth muscle contraction. In addition, it is well established that Rho family GTPases act as unique molecular switches at several critical checkpoints in lymphocyte development and function, and more particularly in the organization, via the actin cytoskeleton, of a specialized zone required for sustained signaling between T cells and antigen presenting cells (Acuto and Cantrell, 2000). Given the essential function of GATA-3 in T cell differentiation and activation (Kuo and Leiden, 1999), it would be worthwhile to test the role of GATA-3 as a mediator of Rho signaling in these cells.

Finally, during cell-fate determination in yeast, the GATA transcription factor Ash1p is regulated by the Cdc42p/Rho signaling pathway effector Bni1p (a Diaphanous family protein) (Takizawa et al., 1997; Evangelista et al., 1997). In addition, Ash1p is also regulated by the Cdc42p effector Ste20p (a Ser/Thr kinase homologous to the mouse Cdc42p effector p65/PAK) during regulation of filamentous pseudohyphal growth in response to nitrogen starvation (Chandarlapaty and Errede, 1998). Despite the fact that the molecular mechanism underlying Ash1p regulation by Rho proteins remains unknown, it is nonetheless interesting to note that the functional interaction between GATA transcription factors and Rho GTPases is evolutionary conserved from yeast to mammals.

3.6 GATA factors in stimulated transcription and hypertrophic development

In addition to its functional and physical interaction with the transcription factor NF-AT3 that mediates calcineurin-dependent cardiac hypertrophy (see the *Introduction*) (Molkentin et al., 1998), other lines of evidence are consistent with a role for GATA factors in cardiac hypertrophy. First, using direct injection of DNA into the myocardium, three groups have found that GATA elements present on the promoters of the angiotensin type 1A receptor ($AT1_AR$), β-MHC, and BNP genes

are required for activation of these promoters in response to hypertrophic stimuli (Herzig et al., 1997; Hasegawa et al., 1997; Marttila et al., 1999). Second, analysis of genetic and experimental models of hypertrophy, such as the SHR and the one kidney one clip rat models, revealed increased GATA-4 transcripts in association with ventricular hypertrophy (G. Nemer and M. Nemer, unpublished data). Third, a GATA-4-SRF complex mediates the effect of the hypertrophic agonist Et-1, leading to induction of hypertrophic markers such as ANF, α-SkA, and c-fos (Morin et al., 2001). Together with our results showing that (i) RhoA potentiates GATA-4 transcriptional activity to activate hypertrophic markers such as ANF and BNP and (ii) GATA-4 is a mediator of Et-1 and Phe-induced sarcomeric reorganization - two hallmarks of cardiomyocyte hypertrophy – the data strongly support a role for GATA-4 in mediating cardiomyocyte hypertrophy. We propose that GATA-4 plays a central role in the genetic reprogramming of the hypertrophied heart. This role likely involves post-translational changes of GATA-4 that influence its combinatorial interactions with cofactors, such as SRF, MEF2, Nkx2-5, and NF-AT3, thereby modulating the capacity of GATA-4-containing enhanceosomes to recruit coactivators and/or the basal transcription machinery.

4 SUMMARY AND FUTURE PROSPECTS

The cardiac GATA-4 and GATA-6 transcription factors play non-redundant roles during embryonic development (Kuo et al., 1997; Molkentin et al., 1997; Morrisey et al., 1998) and in postnatal cardiomyocytes, where they are required for the maintenance of cardiac gene expression (Charron et al., 1999). The specificity of GATA-4 and GATA-6 is due, at least in part, to their differential DNA-binding affinity, allowing them to differentially regulate target genes (Charron et al., 1999). This work also shows that functional specificity is imparted to GATA factors by their differential capacity to interact with cofactors (Durocher et al., 1997; Morin et al., 2000). In addition, several other GATA target genes are regulated by both GATA-4 and GATA-6 and require the combinatorial action of a GATA-4-GATA-6 heterotypic complex at their promoter (Charron et al., 1999). A possible role for these homo- and heterotypic GATA factor interactions could be the formation of higher-order structures among distant regulatory elements that share GATA sites, the

establishment of regions of active chromatin, and/or the formation of an enhanceosome at the enhancer-promoter.

In this regard, the current paradigm on transcriptional regulation supports the notion that a promoter, through its topology, its *cis*-regulatory element disposition and its chromatin structure, is able to integrate a complex network of transcriptional inputs, carried by the *trans*-acting factors, to generate a unique transcriptional output (Carey, 1998). In this mode of regulation, only a small number of transcription factors can generate complex expression patterns. Thus, the identification and the characterization of the transcription factors and their interactions with cofactors responsible for ANF gene expression will be relevant to help to understand the mechanisms underlying cardiac gene transcription in general (Charron et al., 1999; Durocher et al., 1997; Morin et al., 2000; Morin et al., 2001). Moreover, based on the knowledge gained from the ongoing characterization of the IFNβ and TCRα enhanceosomes, it is expected that a detailed biochemical and genetic understanding of the ANF enhanceosome will be crucial to fully understand the complex spatio-temporal expression pattern of ANF during development and in adult cardiac pathologies. In the long term, this work should identify common sets of molecular interactions leading to discrete patterns of gene expression and might perhaps establish a molecular code for regional and temporal cardiac transcriptional activities.

In addition to be essential for the maintenance of cardiac gene expression (Charron et al., 1999), GATA-4 also plays a role in stimulated transcription in response to hypertrophic signals (Morin et al., 2000; Morin et al., 2001). Relevant to these results, the hypertrophic agonist-induced RhoA GTPase stimulates GATA-4 transcriptional activity, which may lead to an increase in contractile protein gene expression and sarcomere reorganization, two hallmarks of cardiomyocyte hypertrophy (F. Charron, G. Tsimiklis, L. Robitaille, and M. Nemer, submitted, Chapter IV). Together, these results strongly support a role for GATA-4 in mediating cardiomyocyte hypertrophy and lead us to propose that GATA-4 is responsible for the maintenance of cardiac gene expression in basal, unstimulated conditions and that under stimulated conditions, activation of RhoA-dependent

signaling pathways would lead to potentiation of GATA-4 transcriptional activity and upregulation of a subset of cardiac genes, including sarcomeric protein genes and other hypertrophic markers, such as ANF and BNP. In turn, the upregulation of these genes would affect the cardiomyocyte mechanical and biological properties, leading to cardiomyocyte hypertrophy. This hypothesis will need to be tested *in vivo*.

In conclusion, the study of cardiac transcription is critical for elucidating the molecular basis controlling cardiogenesis and cardiac homeostasis. This, in turn, will undoubtedly help us understand and perhaps manipulate the profound genetic and cellular changes that are associated with cardiac diseases.

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APPENDICES

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Cooperative Interaction between GATA-4 and GATA-6 Regulates Myocardial Gene Expression

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Two members of the GATA family of transcription factors, GATA-4 and GATA-6, are expressed in the developing and postnatal myocardium and are equally potent transactivators of several cardiac promoters. However, several in vitro and in vivo lines of evidence suggest distinct roles for the two factors in the heart. Since identification of the endogenous downstream targets of GATA factors would greatly help to elucidate their exact functions, we have developed an adenovirus-mediated antisense strategy to specifically inhibit GATA-4 and GATA-6 protein production in postnatal cardiomyocytes. Expression of several endogenous cardiac genes was significantly down-regulated in cells lacking GATA-4 or GATA-6, indicating that these factors are required for the maintenance of the cardiac genetic program. Interestingly, transcription of some genes like the α- and β-myosin heavy-chain (α- and β-MHC) genes was preferentially regulated by GATA-4 due, in part, to higher affinity of GATA-4 for their promoter GATA element. However, transcription of several other genes, including the atrial natriuretic factor and B-type natriuretic peptide (ANF and BNP) genes, was similarly down-regulated in cardiomyocytes lacking one or both GATA factors, suggesting that GATA-4 and GATA-6 could act through the same transcriptional pathway. Consistent with this, GATA-4 and GATA-6 were found to colocalize in postnatal cardiomyocytes and to interact functionally and physically to provide cooperative activation of the ANF and BNP promoters. The results identify for the first time bona fide in vivo targets for GATA-4 and GATA-6 in the myocardium. The data also show that GATA factors act in concert to regulate distinct subsets of genes, suggesting that combinatorial interactions among GATA factors may differentially control various cellular processes.

The vertebrate GATA transcription factors share a highly conserved domain composed of two zinc fingers (39). This domain is responsible for specific binding to a consensus WGATAR element. Based on sequence homology and tissue distribution, the vertebrate GATA family can be divided into two subgroups. The first subgroup is composed of GATA-1, -2, and -3. These three GATA factors are expressed in the hematopoietic system and are essential for normal hematopoiesis (36, 37, 39, 40, 44). The second subgroup is composed of GATA-4, -5, and -6, which are differentially expressed in the heart and gut (26). Within the heart, GATA-5 is restricted to the endocardium (22), whereas GATA-4 and -6 are expressed in the developing and postnatal myocardium (14, 18, 21, 33).

Several lines of evidence have implicated GATA-4 in diverse developmental processes including survival, differentiation, and/or migration of cardiomyocyte precursors. For example, in vitro experiments using embryonic stem cells showed that GATA-4 is essential for survival of cardioblasts and terminal cardiomyocyte differentiation (15, 16). In vivo, inactivation of the GATA-4 gene is embryo lethal at day 9.5 postcoitum due to failure of the GATA-4 null mice to develop a primitive heart tube (25, 31). Unfortunately, the requirement for GATA-4 at early stages of heart development has precluded analysis of its role in the postnatal myocardium either in vitro or in vivo. Nevertheless, two lines of evidence suggest that GATA-4 may play a critical role in postnatal cardiac transcription: GATA elements were found to be essential for activation of some cardiac promoters in adult myocardium (17, 19, 28, 30), and GATA-4 was shown to functionally and physically interact with NFAT-3, which would implicate it as a mediator of the calcineurin-dependent hypertrophic process in the myocardium (32).

Analyses of GATA-6 in the myocardium have been more limited, but the available data are consistent with a role for GATA-6 in myocardial development and gene expression. Thus, axis disruption experiments with *Xenopus* frogs showed that transcription of GATA-6, much like that of GATA-4 and -5, correlates with specification of cardiac progenitors and ectopic expression of either of those factors activates the α-myosin heavy-chain (α-MHC) and cardiac α-actin genes (21). Moreover, cotransfection experiments using heterologous cells showed that GATA-6 is as potent as GATA-4 in transactivating cardiac genes harboring GATA elements in their promoter, such as the cardiac troponin C (cTnC) and atrial natriuretic factor (ANF) genes (9, 33). Interestingly, Gove et al. have reported that in *Xenopus* embryos, GATA-6 overexpression blocks differentiation and stimulate proliferation of heart precursors (12). Recently, the inactivation of the GATA-6 gene in mice was reported to be embryo lethal at day 7.5 postcoitum, precluding analysis of its role in the heart (34). Finally, the inability of GATA-6 to compensate for the GATA-4 deficiency in GATA-4 null mice suggests that GATA-4 and GATA-6 play different roles in vivo.

The molecular basis for the differential roles of GATA-4 and -6 in the myocardium remains largely unknown but could occur via at least three nonexclusive mechanisms: differential expres-

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sion of GATA-4 and GATA-6 in subsets of cardiomyocytes, differential affinity of GATA factors for GATA elements and therefore different in vivo target genes, or differential interaction of GATA-4 and -6 with cofactors. Support for this latter possibility was recently provided by Durocher et al., who showed functional and physical interaction between GATA-4, but not GATA-6, and the cardiac homeodomain protein Nkx2-5 (9, 10). However, the relative affinities of GATA-4 and -6 for their DNA-binding sites have not been determined, and whether GATA-4 and -6 localize differentially in the heart and target distinct genes is not known.

To address these issues, we have developed antibodies specific for the different cardiac GATA factors and analyzed at the cellular level the localization of GATA-4 and -6 in the myocardium. We also developed an adenovirus-mediated antisense strategy to specifically inhibit GATA-4 and GATA-6 protein production in neonatal cardiomyocytes and assess its effect on cardiac gene expression. The results indicate that several endogenous cardiac genes, including those encoding ANF, B-type natriuretic peptide (BNP), α-MHC, β-MHC, cTnI, and platelet-derived growth factor receptor β (PDGFRβ), are down-regulated in cardiomyocytes lacking either GATA-4 or GATA-6, suggesting that these genes are bona fide targets for both GATA-4 and GATA-6. Interestingly, the α- and β-MHC genes are preferential targets for GATA-4, likely due to the higher affinity of GATA-4 for their promoter GATA element. Remarkably, GATA-4 and GATA-6 colocalize in postnatal cardiomyocytes and interact functionally and physically to provide cooperative activation of the ANF and BNP promoters. These results suggest that GATA factors are involved in the maintenance of the cardiac phenotype and that expression of cardiac genes is controlled by combinatorial interactions of the different GATA proteins.

MATERIALS AND METHODS

Plasmids and adenovirus vectors. The recombinant replication-deficient adenovirus type 5 (Ad5) expressing antisense regions directed specifically toward GATA-4 or -6 was generated by using the cloning system developed and generously provided by F. L. Graham (29). Briefly, a 358-bp *Eco*RI/*Hin*dIII fragment encoding the extreme N-terminal portion of rat GATA-4 and a 359-bp *Xba*I/*Stu*I fragment from the 5′ untranslated region (UTR) of rat GATA-6 were subcloned into *Hin*dIII/*Eco*RI and *Eco*RV/*Xba*I, respectively, between left-end adenovirus sequences in Ad5 shuttle vector pΔE1sp1B/CMV/BGH, a plasmid generously provided by B. A. French (1). This plasmid was constructed by inserting the 1,276-bp *Bgl*II/*Pvu*II fragment (containing the cytomegalovirus [CMV] immediate-early promoter, polylinker, and bovine growth hormone polyadenylation signal) from pcDNA3 (Invitrogen Corp., San Diego, Calif.) between the *Bgl*II/Klenow-blunted *Cla*I sites of the polylinker in Ad5 shuttle vector pΔE1sp1B (6). Each shuttle vector was cotransfected into 293 embryonic kidney cells with pJM17, which contains a circularized *dl*309 adenovirus genome, to generate replication-deficient viruses with substitution of the Ad5 E1 genes for the antisense GATA-4 and GATA-6 sequences (AS4 and AS6). The virus Ad5/CMV/NLS-*lacZ*, carrying an expression cassette in which the CMV immediate-early promoter transcribes sequences encoding the simian virus 40 (SV40) large T-antigen nuclear localization signal (NLS) fused to the *Escherichia coli lacZ* reporter gene, was used as control (Ctl; a generous gift from B. A. French) (11). Putative Ad5 clones were plaque purified, screened for antisense inserts, propagated, isolated, and titered according to the protocol of Graham and Prevec (13), to produce viral stocks with titers of $>2 \times 10^9$ PFU/ml.

Wild-type rat ANF and BNP reporter plasmids and the wild-type rat GATA-4 expression vector (pCG-GATA-4) were described previously (14). The various deletions or mutations of the ANF promoter and GATA-4 cDNA were performed by PCR or by the Altered Sites in vitro mutagenesis system (Promega Corp., Madison, Wis.) as described by the manufacturer. The polyhistidine-tagged GATA-4 constructs used for in vitro transcription and translation were generated by insertion of the *Xba*I-*Bam*HI fragment of the corresponding pCG-GATA-4 construct into the *Nhe*I-*Bam*HI or *Nhe*I-*Bgl*II sites of pRSETA (Invitrogen Corp.). The rat GATA-6 cDNA was cloned by PCR and subcloned into the pcDNA3 expression vector. All constructs were confirmed by sequencing.

Neonatal cardiomyocyte preparation, infection, and transfection. Primary cultures of cardiac myocytes were prepared from 4-day-old Sprague-Dawley rats as previously described (4), with minor modifications. Essentially, ventricles and atria of ~60 to 72 hearts were digested four to five times, for 15 min each time, in Joklik's modified Eagle's medium (Canadian Life Technologies Inc.) containing 18 mM HEPES (pH 7.4), 0.1% collagenase (~250 U/ml; Worthington Biochemical Corp.), and DNase I (5 μg/ml; ~2,000 U/mg; Boehringer Mannheim Canada). The enzymatic digestion was stopped with fetal bovine serum (FBS, qualified grade; Canadian Life Technologies Inc.), and the undigested tissue was removed by filtration through nylon mesh (pore size, 100 μm). Cardiomyocytes were purified by three preplatings of 20 min each to remove residual nonmyocytes by differential adhesiveness, then plated at a density of 0.5×10^6 cells/35-mm-diameter dish (Primeria; Falcon), and cultured for 16 to 24 h in Dulbecco's modified Eagle's medium (DMEM; Canadian Life Technologies Inc.) containing 10% FBS. The following day, the medium was exchanged for serum-free hormonally defined medium (SFHD) as previously described (4).

Transfections were carried out by using calcium phosphate precipitation 24 h after plating. For assay of basal ANF promoter activity, cardiomyocytes were transfected with 6 μg of a wild-type or mutant ANF-luciferase reporter. At 36 h posttransfection, cells were harvested and luciferase activity was assayed with a Berthold LB 953 luminometer.

Serial dilutions of recombinant Ad5 (Ctl, AS4, or AS6) were prepared in OptiMEMI (Canadian Life Technologies Inc.). Cardiomyocytes were exposed to 200 μl of OptiMEMI containing 0.5×10^6, 2×10^6, or 8×10^6 PFU of recombinant Ad5 for 30 min at 25°C, and 2 ml of SFHD was added to each petri dish. The medium was replaced 16 h later with fresh SFHD. The cells were kept for 3 to 5 days, with the medium replaced every 24 h with fresh SFHD. Just before replacement, aliquots of the medium were taken for determination of ANF concentration.

β-Galactosidase detection. One day after infection, cardiomyocytes were fixed in 0.5% glutaraldehyde–phosphate-buffered saline (PBS) for 10 min, washed twice for 30 min each time with PBS containing 0.02% Nonidet P-40, and stained with a mixture of 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Boehringer Mannheim Corp.) per ml, 0.01% deoxycholic acid, and 0.02% Nonidet P-40 in PBS for 1 to 2 h at 25°C in the dark. The stained cardiomyocytes were washed with PBS, and 2 ml of 70% glycerol was added to each petri dish. The stained cells were kept at 4°C until photographed.

RNA extraction and Northern blotting. Total RNA was isolated from cardiomyocytes by the guanidium thiocyanate-phenol-chloroform method as previously described (3). RNA was denatured with formaldehyde and formamide, size fractionated on a 1.2% agarose gel, transferred to a nylon membranes (MSI, Westborough, Mass.) by capillary blotting with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and cross-linked to the membrane with a UV Stratalinker 2400 (120 mJ; Stratagene). Blots were hybridized with random prime-labeled rat cDNA probes for GATA-4, GATA-6, ANF, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GATA-4 and -6 cDNA fragments are the same ones used to generate the AS4 and AS6 adenoviruses. The ANF cDNA was described previously (5), and the GAPDH cDNA was generously provided by P. Jolicoeur. Blots were exposed in a PhosphorImager cassette and analyzed with ImageQuant (Molecular Dynamics).

Cell cultures and transfections. HeLa or L cells were grown in DMEM supplemented with 10% FBS. Transfections were carried out by using calcium phosphate precipitation 24 h after plating. For overexpressions, 800,000 L cells were plated per 100-mm-diameter petri dish and transfected with 40 μg of pCG, pCG-GATA-4, pCG-GATA-4 mutant, or pCDNA3-GATA-6 expression vector. At 36 h posttransfection, cells were harvested and nuclear extracts were prepared as previously described (14). Nuclear extract protein concentrations were quantitated by the Bradford assay (Bio-Rad Laboratories, Hercules, Calif.).

For ANF promoter transactivation assays, 100,000 HeLa cells were plated per 35-mm-diameter petri dish and transfected with 3 μg of ANF-luciferase reporter plasmid and 1 μg of GATA expression vector. For synergy, 200 ng of GATA-6 and 200 ng of wild-type or mutant GATA-4 expression vector were used. The total amount of DNA was kept constant at 4 μg. At 36 h posttransfection, cells were harvested and luciferase activity was assayed.

Electrophoretic mobility shift assays (EMSAs). Binding reactions were performed in 20-μl reaction mixtures containing 3 μg of nuclear extracts from L cells overexpressing GATA-4 or GATA-6 in a buffer containing 12 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 60 mM KCl, 4 mM Tris-HCl (pH 7.9), 0.6 mM EDTA, 0.6 mM dithiothreitol, 0.5 mg of bovine serum albumin (BSA) per ml, 1 μg of poly(dI-dC), 12% glycerol, 20,000 cpm of radiolabeled double-stranded −120 ANF GATA probe, and increasing amounts of the appropriate unlabeled competitor for 20 min at room temperature. Reactions were then loaded on a 4% polyacrylamide gel and run at 200 V at room temperature in 0.25× Tris-borate-EDTA. The gel was then dried and exposed to a PhosphorImager (Molecular Dynamics) cassette for quantitative analysis. Relative bindings were quantitated and plotted as a function of the unlabeled competitor amount. Probes used were, from 5′ to 3′ (only the coding strand is shown), rat ANF −120 (proximal; GATCTCGCTGGACTGATAACTTTAAAAGG), rat ANF −120mut (TGAC AAGCTTCGCTGGACTCCTAACTTTAAAAG), rat ANF −280 (distal; GAT CTCCCAGGAAGATAACCAAGGACTCG), and rat α-MHC −265 bp (GAT CCTCCTCTATCTGCCCATCA), where the WGATAR consensus motifs are underlined and the mutations are in boldface.

For DNA-binding affinity measurement, EMSAs were performed as described above but with increasing amounts of radiolabeled ANF proximal or distal GATA probe with a constant amount (3 μg) of nuclear extracts from L cells

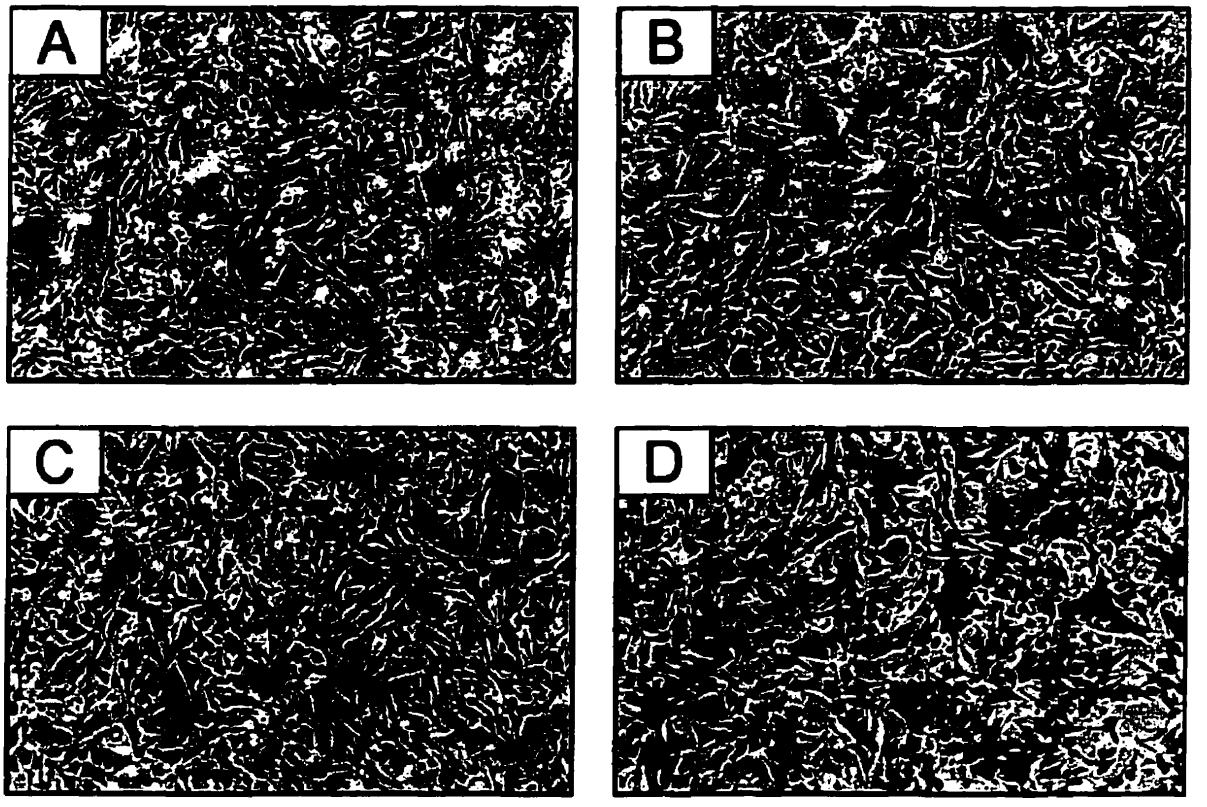


FIG. 1. Cardiomyocytes are efficiently infected by adenovirus. Ventricular cardiomyocytes isolated from 4-day neonatal rats were mock infected (A) or infected at MOIs of 1 (B), 4 (C), and 16 (D) with the Ctl adenovirus, which expresses an NLS-*lacZ* gene. Twenty hours later, cells were assayed for β-galactosidase activity.

overexpressing GATA-4 or GATA-6. Scatchard analysis was then performed on the binding data by plotting the bound/free DNA ratio as a function of the bound DNA. The resulting dissociation constant (K_d) was calculated by using Microsoft Excel.

Western blots. Three days after infection, cardiomyocytes were harvested and nuclear extracts were prepared; then 20 μg of cardiomyocyte nuclear extracts and 2 μg of GATA-4 or GATA-6-overexpressing L-cell nuclear extracts were boiled in Laemmli buffer and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a Hybond polyvinylidene difluoride membrane and immunoblotted by using the Renaissance chemiluminescence system (NEN Life Sciences, Boston, Mass.) as described by the manufacturer. Goat GATA-4 supershift antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) was used at a dilution of 1/1,000 and was revealed with an anti-goat horseradish peroxidase-conjugated antibody (Sigma, St. Louis, Mo.) at a dilution of 1/100,000. The GATA-6 antibody was made in rabbits by injection of a GATA-6-specific peptide linked to keyhole limpet hemocyanin as described elsewhere (2). The purified GATA-6 antibody was used at a dilution of 1/1,000 and was revealed with an anti-rabbit horseradish peroxidase-conjugated antibody (Sigma) at a dilution of 1/100,000.

RIA. Immunoreactive ANF (irANF) concentration was determined in the cardiomyocyte culture medium by radioimmunoassay (RIA) as previously described (3).

Pull-down assays. Polyhistidine-tagged GATA-4 and wild-type GATA-6 were in vitro cotranscribed and cotranslated in the presence of radiolabeled methionine according to the manufacturer's protocol (TNT reticulocyte lysate kit; Promega Corp.). The proteins were then allowed to interact at 4°C with agitation in 400 μl of binding buffer (150 mM NaCl, 50 mM Tris-Cl [pH 7.5], 0.3% Nonidet P-40, 10 mM $ZnCl_2$, 1 mM dithiothreitol, 0.25% BSA) as described previously (9). After 2 h, 50 μl of nickel resin (ProBond resin; Invitrogen Corp.) was added, and the reaction mixture was incubated further for 2 h at 4°C. The resin was then washed three times with binding buffer and twice with binding buffer minus BSA. Interacting proteins were resolved by SDS-PAGE (15% gel). The gel was dried and exposed to a PhosphorImager cassette.

Cross-linking. Cardiomyocyte whole-cell extracts were prepared in radioimmunoprecipitation assay (RIPA) buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, cocktail of protease inhibitors) by repeated aspiration through a syringe needle, followed by centrifugation to pellet cellular debris. The supernatant was then incubated on ice for increasing amounts of time in the presence of 0.02% glutaraldehyde, and the reaction was blocked by the addition of 1 M glycine (pH 7.6). To reduce nonspecific binding, the cellular extracts were preincubated at 4°C for 30 min with 1 μg of normal goat serum and 20 μl of protein A/G PLUS-Agarose (Santa Cruz Biotechnology). GATA-4/GATA-6 complexes were immunoprecipitated with 1 μl of anti-GATA-4 antibody (Santa Cruz Biotechnology) and 20 μl of protein A/G PLUS-Agarose at 4°C overnight with agitation. Subsequently, the immunoprecipitates were washed four times in RIPA buffer, resolved by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. The presence of the GATA-4/GATA-6 complexes was revealed by Western blotting using an anti-GATA-6 antibody.

Immunofluorescence. Paraformaldehyde-fixed heart sections from neonatal mice were processed for immunofluorescence as described elsewhere (41). The GATA-4 antibody was used at a dilution of 1/500 and was revealed with a biotinylated anti-goat antibody (1/200; Vector Laboratories Inc., Burlingame, Calif.) followed by avidin-rhodamine (1/200; Vector Laboratories). The purified GATA-6 antibody was used at a dilution of 1/50 and was revealed with a fluorescein isothiocyanate-conjugated anti-rabbit antibody (1/200; Sigma).

RESULTS

In vivo target genes for GATA-4 and GATA-6 in postnatal atrial and ventricular cardiomyocytes. To analyze the consequence of inhibiting GATA-4 or GATA-6 expression for endogenous myocardial gene expression, we used adenovirus-mediated delivery of antisense gene regions to cardiomyocytes in primary culture. For this, we initially determined the dose of adenovirus that would efficiently infect ventricular cardiomyocytes isolated from 4-day neonatal rats, using a replication-deficient adenovirus expressing a *lacZ* gene fused to an SV40 NLS (Ctl adenovirus). At a multiplicity of infection (MOI) of 1, all the cardiomyocytes showed β-galactosidase activity (blue staining) in the nucleus, with staining intensity increasing in a dose-dependent manner up to an MOI of 16 (Fig. 1B to D). No staining was observed in mock-infected cardiomyocytes (Fig. 1A). Similar results were obtained with atrial neonatal cardiomyocytes (data not shown). Higher dose of the Ctl adenovirus led to cytotoxicity (data not shown). Thus, MOIs ranging from

1 to 16, which achieve efficient infection without any apparent cytotoxicity (Fig. 1), were used in subsequent experiments.

To specifically inhibit GATA-4 or GATA-6 protein production, replication-deficient adenoviruses expressing an antisense cDNA directed specifically toward GATA-4 or GATA-6 were generated. A 358-bp fragment from the extreme N-terminal portion of GATA-4 was used for the AS4 production (Fig. 2A). For AS6, a 359-bp fragment from the 5′ UTR of GATA-6 was used. These regions were chosen because of their low overall homology with other GATA factors, and as shown in Fig. 2B, the DNA fragments used to generate AS4 and AS6 show no cross-hybridization with each other's mRNA.

Ventricular neonatal cardiomyocytes were infected at MOIs of 1, 4, and 16 with Ctl, AS4, and AS6, and RNA was isolated 3 days postinfection. Northern blot analysis showed that both antisense transgenes were efficiently expressed in a dose-dependent manner, to a level exceeding 100-fold that of endogenous GATA-4 or -6 (Fig. 2B). AS4 and AS6 were also specific and efficient at reducing GATA-4 and GATA-6 protein levels in a dose-dependent manner, as evidenced by Western blot analysis (Fig. 2C). As shown in Fig. 2D, AS4 reduced GATA-4 levels by 80% whereas AS6 reduced GATA-6 levels by 60% at an MOI of 4. The effect of AS4 and AS6 extended also to GATA-binding activity as assessed by EMSA; a 50% decrease in GATA binding over the ANF −120 GATA probe was observed with AS4 or AS6, indicating that indeed both GATA-4 and GATA-6 contribute to the GATA-binding activity in postnatal cardiomyocytes (data not shown). Moreover, AS4 and AS6 had no effect on GATA-5 mRNA levels, suggesting that GATA-5 does not compensate for the lack of GATA-4 or GATA-6 in postnatal cardiomyocytes (data not shown).

To assess the effects of reduced GATA-4 or GATA-6 activity on endogenous gene expression, we initially analyzed changes in ANF mRNA and protein levels. The ANF promoter contains two conserved GATA elements (see Fig. 4A) and was previously shown to be transactivated by GATA-4 and GATA-6 in heterologous cells (9, 14). In ventricular neonatal cardiomyocytes with reduced GATA-4 or GATA-6 activity, irANF secretion was decreased in a time-dependent manner (Fig. 3A). The effect was first observed at 3 days postinfection (50% decrease), and irANF secretion was decreased by 60 to 80% at 5 days postinfection. The decrease of secreted irANF was dose dependent (Fig. 3B). Interestingly, the reduction of both GATA-4 and GATA-6 activities (AS4 plus AS6) had the same effect on irANF secretion as reduction of GATA-4 (AS4) or GATA-6 (AS6) activity alone (Fig. 3B), suggesting that either a maximal threshold is reached or GATA-4 and GATA-6 are in the same transcriptional pathway. The effect of attenuation of GATA-4 or GATA-6 on ANF expression extended to the transcript level (Fig. 3C). Similar results were obtained with atrial cardiomyocytes (data not shown) and indicate that the ANF gene is an in vivo transcriptional target for GATA-4 and GATA-6 in postnatal cardiomyocytes.

We also verified that other cardiac genes were affected by the decrease in GATA-4 or GATA-6. BNP, α-MHC, β-MHC, cTnI, and PDGFRβ mRNA levels were down-regulated by AS4 and AS6 (Fig. 3D). Interestingly, α-MHC and β-MHC were preferentially down-regulated by AS4, suggesting that these genes are preferential targets for GATA-4. The lack of GATA factors did not affect all cardiac genes; for example, cardiac α-actin, myosin light-chain 1, and GAPDH mRNA levels were not altered by AS4 or AS6 (Fig. 3C and D and data not shown). Similar results were obtained by semiquantitative reverse transcription-PCR analysis (data not shown). Thus, GATA-4 and GATA-6 are involved in regulating specific subsets of cardiac genes in postnatal cardiomyocytes.

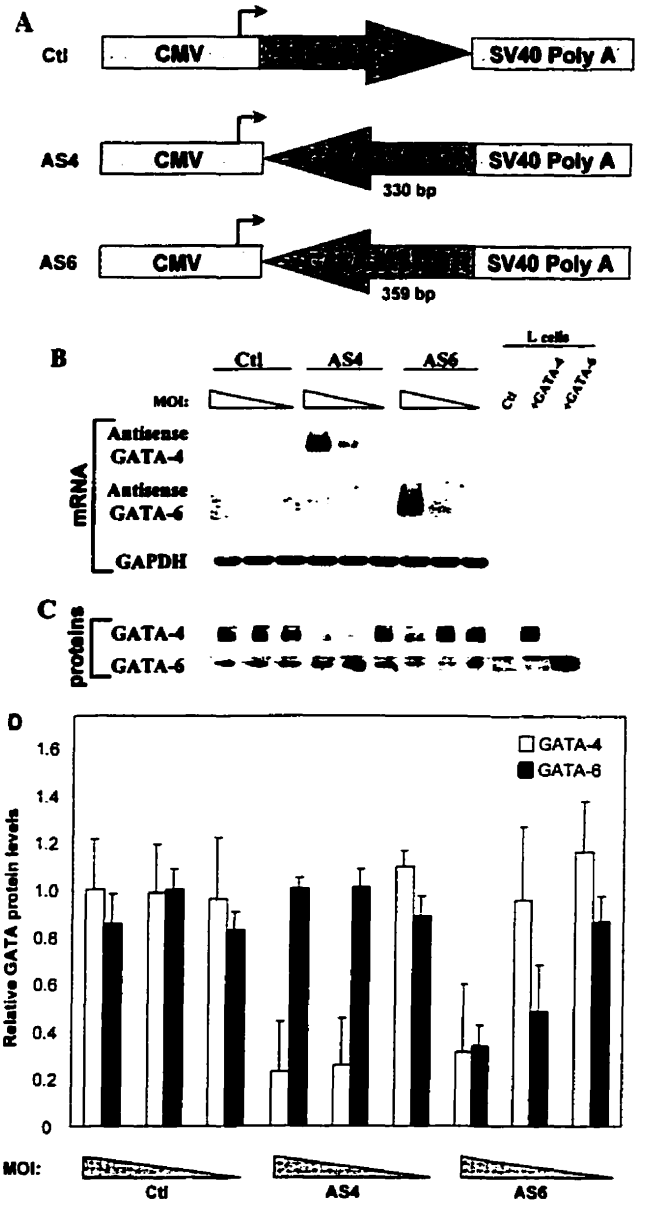


FIG. 2. Characterization of AS4 and AS6. (A) Schematic representation of the constructs used to generate the recombinant adenoviruses. NLS-*lacZ*, a 358-bp fragment from the extreme N-terminal portion of GATA-4, or a 359-bp fragment from the 5′ UTR of GATA-6 was cloned downstream of the CMV promoter and upstream of an SV40 poly(A) sequence in order to generate the recombinant adenoviruses. (B) Transgene expression is dose dependent. Ventricular cardiomyocytes were infected at MOIs of 16, 4, and 1 (corresponding to the progressively narrowing triangle) with Ctl, AS4, and AS6. RNA was isolated 3 days postinfection. Northern blot analysis showed that both antisense transgenes were efficiently expressed in a dose-dependent manner. (C) AS4 and AS6 are specific and efficient at decreasing GATA-4 and GATA-6 protein levels. Ventricular cardiomyocytes were infected as described above, and nuclear extracts were isolated 3 days postinfection and analyzed by Western blotting. L cells overexpressing GATA-4 or GATA-6 were used as controls for the specificity of the antibodies. (D) Quantification of the effects of AS4 and AS6 on GATA-4 and GATA-6 protein levels. The data represent the means of two independent Western blots performed as described for panel C and quantified by densitometry. At an MOI of 4, GATA-4 levels were decreased specifically by AS4 (80% reduction), while GATA-6 levels were reduced specifically by AS6 (60% reduction).

ANF is a direct transcriptional target for both GATA-4 and GATA-6. To determine if ANF is a direct transcriptional target for both GATA-4 and GATA-6, a detailed analysis of the ANF promoter was performed. Comparison of the ANF promoter

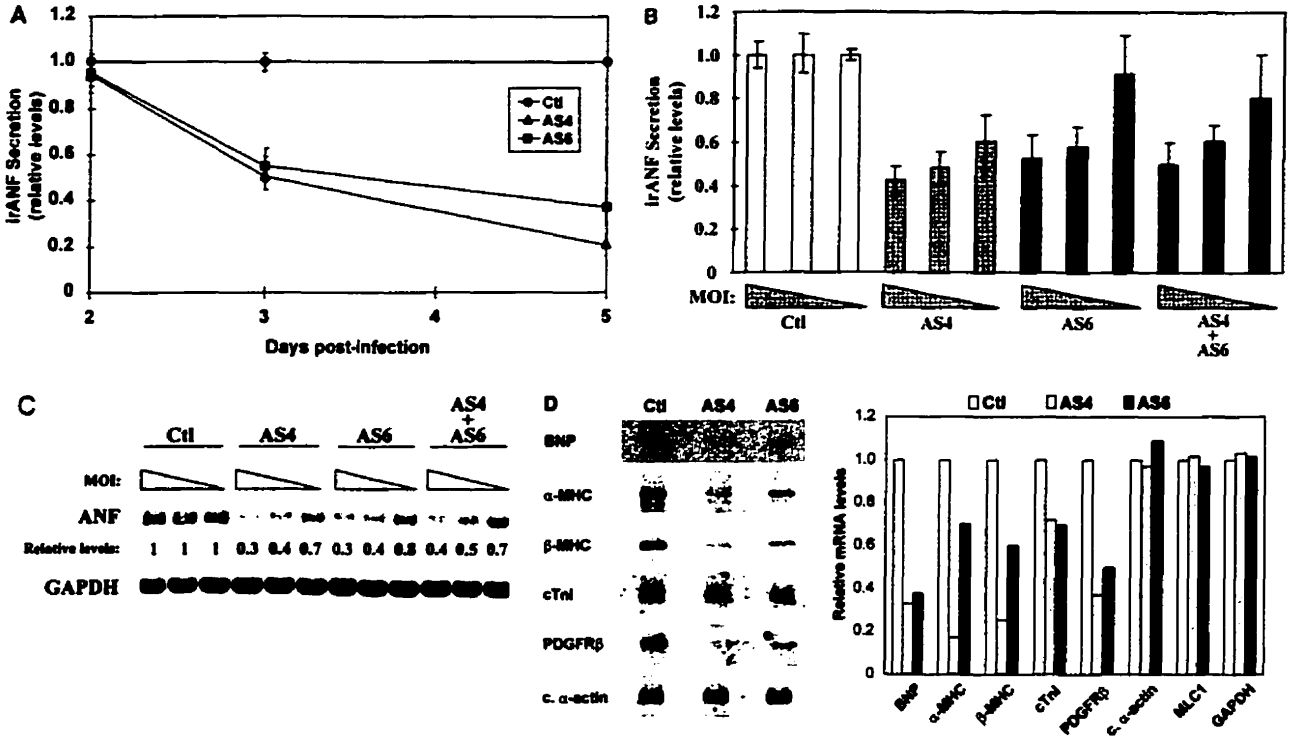


FIG. 3. In vivo transcriptional targets for GATA-4 and GATA-6 in postnatal cardiomyocytes. (A) irANF secretion is decreased in a time-dependent manner by AS4 and AS6. Ventricular cardiomyocytes were infected at an MOI of 4 with Ctl, AS4, and AS6. Secreted irANF was assayed in the cardiomyocyte culture medium after a 24-h accumulation period at 2, 3, and 5 days postinfection. Since the effect of the antisense DNA on irANF secretion is clearly visible at 3 days postinfection, subsequent analyses were performed at this time point. (B) irANF secretion is decreased in a dose-dependent manner by AS4, AS6, and AS4 plus AS6. Ventricular cardiomyocytes were infected at MOIs of 16, 4, and 1 (corresponding to the progressively narrowing triangle) with Ctl, AS4, AS6, and AS4 plus AS6. (C) ANF mRNA levels are decreased in a dose-dependent manner by AS4, AS6, and AS4 plus AS6. Ventricular cardiomyocytes were infected as for panel B, and total RNA was analyzed by Northern blotting. The ANF mRNA levels are expressed relative to that of Ctl-infected cardiomyocytes. The GAPDH mRNA levels were unaffected. (D) Many cardiac genes are decreased by AS4 and AS6. Ventricular cardiomyocytes were infected at an MOI of 4 with Ctl, AS4, and AS6, and Northern blot analysis was performed on poly(A)$^+$ RNA (left). Relative mRNA levels were quantified with a PhosphorImager (right). Note how α-MHC and β-MHC mRNAs were preferentially down-regulated by AS4, whereas cardiac (c.) α-actin, myosin light-chain 1 (MLC1), and GAPDH mRNA levels were not affected by AS4 or AS6.

sequences available in the database (from human, rat, mouse, sheep, and bovine genomes) shows that two consensus WGATAR elements, a proximal one at −120 bp and a distal one at −280 bp, are entirely conserved across species (Fig. 4A), suggesting that these GATA elements may play important evolutionarily conserved functions in ANF gene regulation. Indeed, deletion or point mutation of the proximal or the distal GATA element decreased by about 50% ANF promoter activity in postnatal cardiomyocytes (Fig. 4B). A more drastic effect was observed when both elements were mutated, leaving

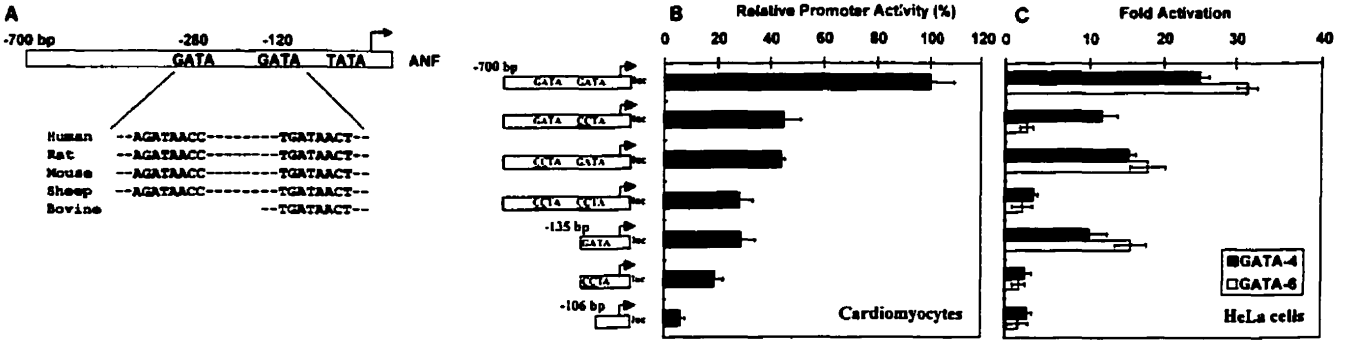
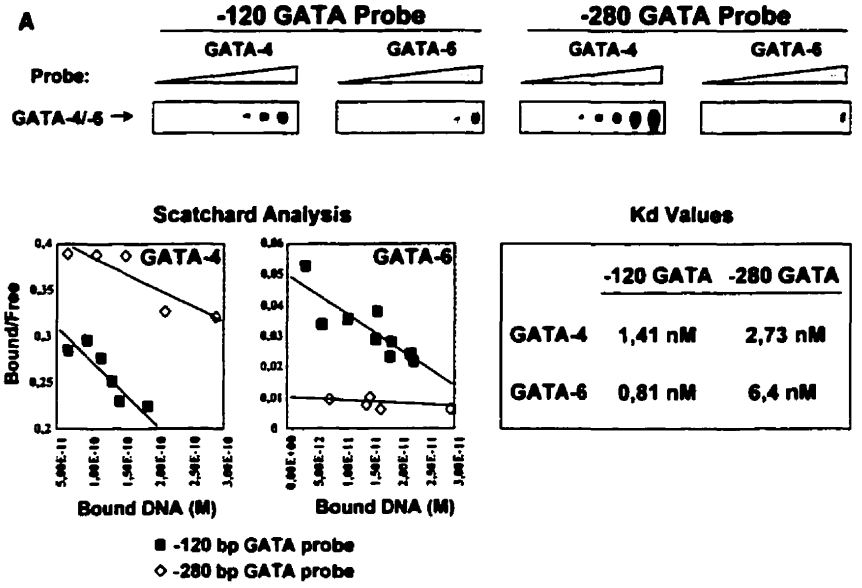


FIG. 4. ANF is a direct transcriptional target for both GATA-4 and GATA-6. (A) Alignment of sequences of human, rat, mouse, sheep, and bovine (for which only a partial sequence is available) ANF promoters. Note that two consensus WGATAR elements, a proximal one at −120 bp and a distal one at −280 bp, are entirely conserved across species. (B) The proximal and distal GATA elements are major contributors of ANF promoter activity in neonatal cardiomyocytes. Wild-type and mutated ANF promoters fused to the luciferase (luc) reporter gene were transiently transfected into ventricular cardiomyocytes isolated from 4-day neonatal rats. Luciferase activity was assayed 36 h posttransfection. The results are expressed relative to the −700 bp ANF promoter activity. (C) GATA-4 and GATA-6 transactivate the ANF promoter. HeLa cells were cotransfected with a GATA-4 or GATA-6 expression vector and wild-type or mutated ANF promoters fused to the luciferase reporter gene. Luciferase activity was assayed 36 h posttransfection. The results are expressed as fold activation by GATA-4 or GATA-6. In all cases, the data represent the mean ± standard deviation of two or three independent experiments carried out in duplicate.



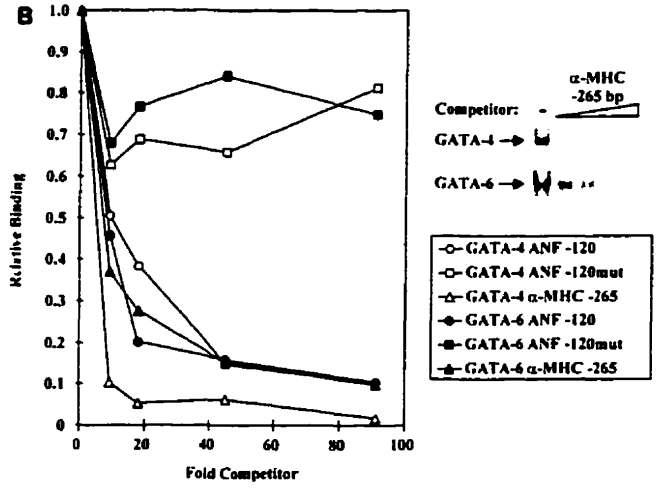


FIG. 5. GATA-4 and GATA-6 bind GATA elements with different affinities. (A) EMSAs were performed with increasing amounts of radiolabeled probe (−120-bp GATA or −280-bp GATA) and a constant amount of nuclear extracts from L cells overexpressing GATA-4 or GATA-6. GATA binding is shown in the upper panel. Scatchard analysis were performed on the binding data, and the relative affinities (K_d values) are shown. (B) GATA-4 has higher affinity than GATA-6 for the −265-bp α-MHC GATA element. EMSAs were performed with nuclear extracts from L cells overexpressing GATA-4 or GATA-6 incubated with a radiolabeled −120-bp ANF GATA element and increasing amounts of an unlabeled competitor (−120-bp ANF GATA, −120-bp ANF GATAmut, or −265-bp α-MHC GATA; top right panel). Relative bindings were quantitated and plotted as a function of the amount of unlabeled competitor (left).

only 30% of wild-type promoter activity. Similar results were observed for all constructs in 1-day ventricular or atrial cardiomyocytes. Thus, both proximal and distal GATA elements are major contributors of ANF promoter activity in postnatal cardiomyocytes.

Since both GATA-4 and GATA-6 are present in neonatal cardiomyocytes, we tested their relative efficiencies at transactivating the ANF promoter. The results indicate that both GATA factors are potent activators of the ANF promoter, exhibiting about 25- to 30-fold activation (Fig. 4C). However, while mutation of the proximal or distal GATA element reduced GATA-4 transactivation by approximately 50%, mutation of the proximal GATA element fully abrogated GATA-6 transactivation.

To test whether this was due to differential affinity of GATA-4 and -6 for certain GATA elements, we analyzed the binding affinities of GATA-4 and GATA-6 for both ANF GATA elements. EMSAs were performed with increasing amounts of radiolabeled probes and a constant amount of nuclear extracts from L cells overexpressing GATA-4 or GATA-6 (Fig. 5A). Scatchard analysis revealed that GATA-4 has similar relative affinities for the proximal and distal GATA elements (relative K_ds of 1.41 and 2.73 nM, respectively). However, the relative affinity of GATA-6 for the distal element was 8-fold lower than that for the proximal GATA element (relative K_ds of 6.4 and 0.81 nM, respectively). Similar K_ds were obtained with bacterially expressed proteins (data not shown). These results show for the first time that GATA-4 and GATA-6 possess differential affinities for naturally occurring sites.

Since differential activation of the ANF promoter GATA elements by GATA-4 and -6 correlated well with the relative affinities for these sites, we tested whether the preferential regulation of α-MHC by GATA-4 was due to differential affinities of GATA factors for the α-MHC GATA element (30). As shown in Fig. 5B, the α-MHC GATA element was more efficient at competing GATA-4 than GATA-6 binding. Thus, the higher affinity of GATA-4 for this element may explain the preferential regulation of the α-MHC gene by GATA-4.

GATA-4 and GATA-6 functionally and physically interact to cooperatively activate cardiac promoters. The fact that the reduction of both GATA-4 and GATA-6 protein levels had the same effect on ANF gene expression as the reduction of either factor alone suggests that GATA-4 and GATA-6 are members of a single functional complex and that ablation of GATA-4 or GATA-6 in that complex is sufficient to disrupt its transcriptional activity.

To test whether GATA-4 and GATA-6 act cooperatively, the −700-bp ANF promoter fused to luciferase was cotransfected with various doses of GATA-4 and GATA-6 expression

vectors. At a low dose of expression vector where neither GATA-4 nor GATA-6 could activate transcription by itself, synergistic activation of the ANF promoter was achieved when both GATA factors were added (Fig. 6A). Interestingly, cooperative activation by GATA-4 and -6 occurred through a single GATA-binding site, as evidenced by the activation of the −135-bp ANF promoter construct. In fact, the proximal GATA element was necessary and sufficient for synergy, and the distal GATA element (−700-bp ANF ΔGATA −120 bp) could not mediate cooperative activation. Synergistic activation by GATA-4 and GATA-6 was also observed on the BNP promoter but not on a shorter ANF promoter lacking GATA elements (−106-bp ANF).

The domain(s) of GATA-4 required for synergy with GATA-6 was mapped by cotransfection in HeLa cells of GATA-6 and various GATA-4 mutant expression vectors (Fig. 6C; summarized in Fig. 7). All mutants were tested for expression and nuclear localization. The mutants used in transfection assays were expressed at similar levels, as evidenced by EMSAs and Western blot analysis (Fig. 6B and data not shown). Progressive deletion of the N-terminal (127 to 443 and 201 to 443) activation domain of GATA-4 did not drastically affect synergy with GATA-6. However, deletion of the C-terminal activation domain of GATA-4 (1 to 332) or GATA-4 mutants harboring no transcriptional activation domain (201 to 332 and 242 to 332) did not exhibit synergy with GATA-6. DNA binding by GATA-4 was not required since G4m, a GATA-4 mutant in the second zinc finger bearing no DNA-binding activity (but that still localizes to the nucleus) was able to provide synergistic activation with GATA-6. Thus, while the N-terminal activation domain of GATA-4 and GATA-4 DNA-binding ability are dispensable, the C-terminal activation domain of GATA-4 is required for synergy with GATA-6.

To test whether this functional cooperation between GATA-4 and -6 involves direct interaction, polyhistidine-tagged GATA-4 and wild-type GATA-6 were in vitro cotranscribed and cotranslated in presence of radiolabeled methionine. As shown in Fig. 6D and summarized in Fig. 7, GATA-4 interacted specifically with GATA-6, and this interaction required the zinc fingers and the basic region of GATA-4. The ability of GATA-4 and GATA-6 to contact each other was further confirmed in vivo by chemical cross-linking of cardiomyocyte extracts followed by immunoprecipitation with a GATA-4 antibody and Western blotting with a GATA-6 antibody. As shown in Fig. 6E, this resulted in immunoprecipitation of a heterodimer composed of endogenous GATA-4 and GATA-6 proteins.

GATA-4 expression and GATA-6 expression colocalize in postnatal cardiomyocytes. To ascertain that the immunoprecipitated GATA-4 and -6 complex reflects the ability of these proteins to associate with each other intracellularly, we verified the coexpression of GATA-4 and GATA-6 in cardiomyocytes. Immunofluorescence studies using specific anti-GATA-4 and anti-GATA-6 antibodies were performed on sections from neonatal mouse hearts and on primary rat cardiomyocyte cultures (Fig. 8 and data not shown). These studies revealed that GATA-4 and GATA-6 colocalize in most postnatal ventricular cardiomyocytes.

DISCUSSION

Members of the GATA family of transcription factors play critical roles in diverse cellular processes. Although some members are coexpressed in specific cell types, each family member appears to fulfill essential, nonredundant functions during development. However, the mechanisms by which GATA factors control gene expression and cell fate as well as the molecular basis for their specificity remain poorly understood.

The data presented in this paper provide evidence for the existence of specific in vivo downstream targets for two members of the GATA family, GATA-4 and -6, which are coexpressed in myocardial cells. The results also show that GATA factors act in concert to regulate transcription of subsets of cellular genes, suggesting that combinatorial interactions among GATA factors may differentially control various cellular processes.

Identification of in vivo targets for GATA factors in cardiomyocytes. Regulatory elements containing GATA-binding sites have been identified in many cardiac promoters, including BNP, α-MHC, cTnI, and cTnC, and GATA-4 was shown to transactivate several of these promoters (8, 14, 20, 28, 30, 35, 38, 42). However, several cardiac markers examined which were previously proposed to be GATA-4 targets were still expressed at high levels in GATA-4 null hearts, raising the possibility that these genes are not bona fide GATA-4 targets or that other cardiac factors, including GATA-6, whose expression is up-regulated in GATA-4 null mice, provide compensatory pathways (25, 31). Thus, determining the in vivo targets for GATA-4 and GATA-6 is crucial for understanding their role in the heart. The data obtained in this study indicate that while some cardiac genes are regulated preferentially by GATA-4, others are targets for both GATA-4 and GATA-6. This is consistent with a specialized role for each factor in heart development and the requirement for both in normal heart formation. At present, GATA-6 has been linked to cardioblast proliferation whereas GATA-4 has been associated with terminal differentiation (12, 15). In this respect, it is noteworthy that markers of later stages of cardiomyocyte differentiation—α- and β-MHC genes—appear to be preferential GATA-4 targets whereas genes expressed in precardiomyocytes prior to the beating stage, such the natriuretic peptide (ANF and BNP) and PDGFR genes (16, 24), are targeted by both GATA-4 and GATA-6. Since the adenovirus-mediated antisense strategy revealed that GATA-4 and GATA-6 are required for maintenance of the differentiated phenotype in postnatal cardiomyocytes, it could now be used to determine the role of these factors in embryonic myocytes. Finally, in light of recent reports suggesting a role for GATA-4 in mediating hypertrophic signals (17, 19), it will be interesting to use the adenovirus tools developed in this study to directly test the implication of GATA-4 or GATA-6 in cardiomyocyte hypertrophy.

Transcriptional mechanisms of GATA factors. The data presented here show that a subset of cellular genes may be bona fide targets for more than one GATA factor, whereas others are under the control of a specific GATA factor. It may be significant that this is the first time that specific in vivo targets for cardiac GATA factors have been reported.

Differential affinity could be one of the mechanisms by which GATA factors target distinct downstream genes. In the case of the hematopoietic GATA-1, -2, and -3 proteins, in vitro binding site selection experiments have shown differences in DNA-binding specificity, although they have not yet been correlated with natural GATA elements present on hematopoietic promoters (23).

In this study, we show that GATA-4 and GATA-6 bind to the two ANF GATA elements with different relative affinities that correlate well with their ability to transactivate the ANF promoter. The results also show that the higher affinity of GATA-4 for the α-MHC GATA element correlates with the finding that the endogenous α-MHC gene is a preferential GATA-4 target. Interestingly, the ANF and α-MHC sequences

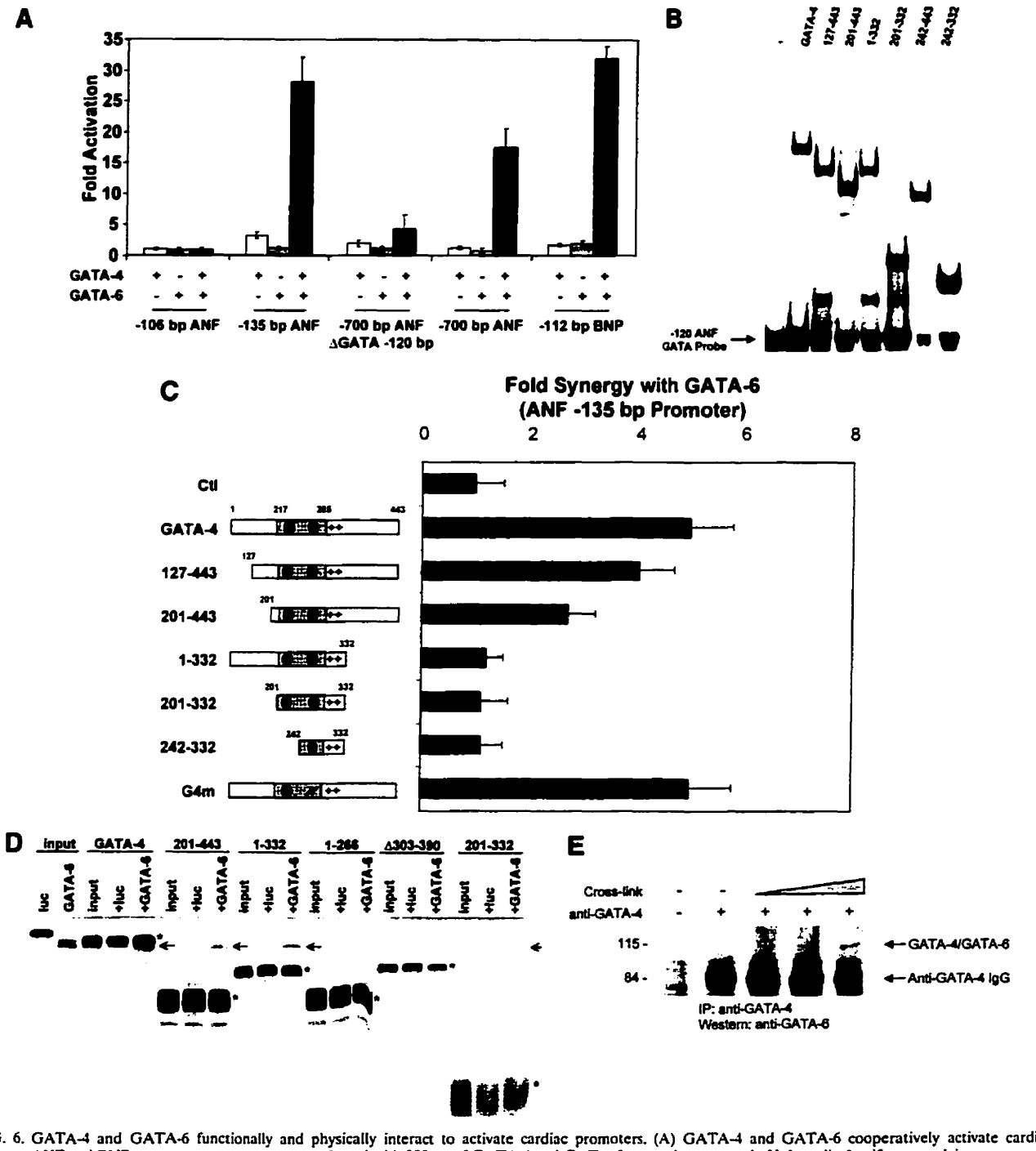


FIG. 6. GATA-4 and GATA-6 functionally and physically interact to activate cardiac promoters. (A) GATA-4 and GATA-6 cooperatively activate cardiac promoters. ANF and BNP reporter vectors were cotransfected with 200 ng of GATA-4 and GATA-6 expression vectors in HeLa cells. Luciferase activity was assayed 36 h posttransfection. The results are expressed as fold activation by GATA-4 and/or GATA-6. (B) DNA binding by the GATA-4 mutants. EMSAs were performed on the −120-bp ANF GATA element, using various GATA-4 mutants overexpressed in L cells. The results are summarized in Fig. 7. (C) Mapping of the domain(s) required for synergy between GATA-4 and GATA-6. HeLa cells were cotransfected with GATA-6 and various GATA-4 mutant expression vectors. Luciferase activity was assayed 36 h posttransfection. The results are expressed as fold synergy of the −135-bp ANF promoter, which is defined by the activation of the −135-bp ANF promoter by both GATA-4 and GATA-6 divided by the sum of the activation by GATA-4 and GATA-6 alone. (D) The zinc fingers and the basic region of GATA-4 are sufficient for physical interaction with GATA-6. Polyhistidine-tagged GATA-4 and wild-type GATA-6 were in vitro cotranscribed and cotranslated in the presence of radiolabeled methionine. The reaction mixture was then incubated with a nickel resin in order to pull down His-tagged GATA-4 from the mixture. Interacting proteins were resolved by SDS-PAGE. Luciferase (luc) was used as a negative control for interaction. The various His-tagged GATA-4 proteins are indicated with asterisks, and the interacting GATA-6 proteins are marked by arrows. Note that due to the His tag, GATA-4 has a slightly lower electrophoretic mobility than GATA-6. (E) GATA-4 and GATA-6 interact in vivo in postnatal cardiomyocytes. Cardiomyocyte whole-cell extracts were cross-linked by using glutaraldehyde for 15, 30, and 150 min, followed by immunoprecipitation (IP) with an anti-GATA-4 antibody. The immunoprecipitates were resolved by SDS-PAGE, and the GATA-4/GATA-6 complexes were revealed by Western blotting using an anti-GATA-6 antibody. Positions of molecular weight standards (in kilodaltons) are indicated on the left. The strong signal is due to the GATA-4 antibody immunoglobulin G (IgG) heavy chains. Note that the GATA-4/GATA-6 complex migrates at about 110 kDa, which corresponds to the sum of the molecular masses of GATA-4 and GATA-6.

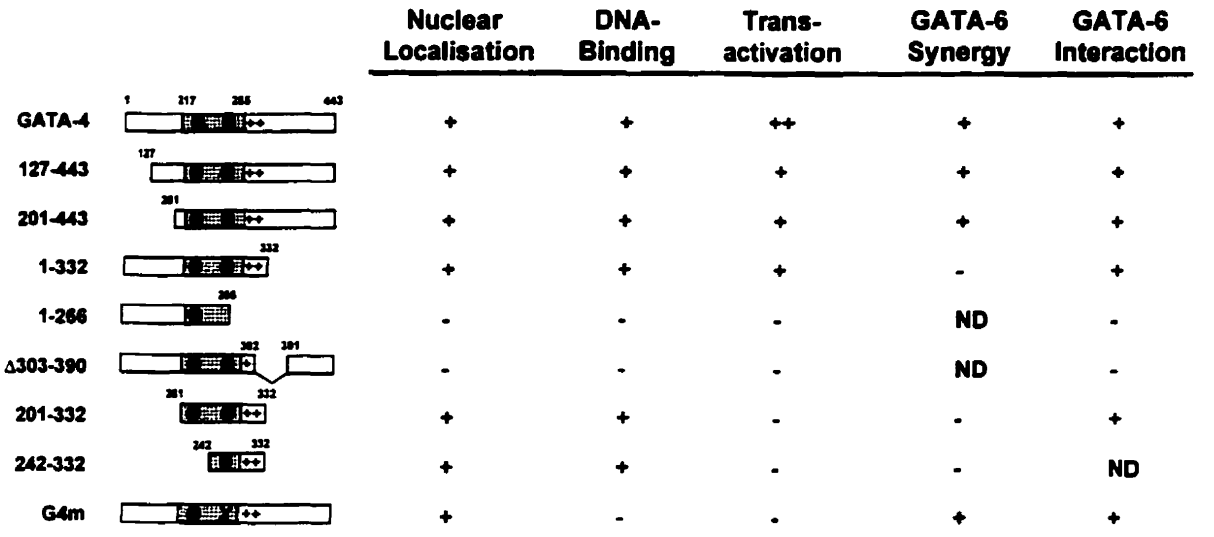


FIG. 7. Summary of GATA-4 functional domains. The GATA-4 constructs used in this study are shown, along with some of their functional properties. ND, not determined.

that are preferential GATA-4 binding sites have an A residue at the W position of the consensus WGATAR. While ascertainment of the generality of this observation awaits additional studies, it is noteworthy that at least one other natural AGATAA site present on the BNP promoter (at −30 bp) also appears to be a preferential GATA-4-binding site (6a). The results raise the possibility of finding specific targets for GATA-4 or -6 based on differential affinities for their DNA sequences.

An important outcome of this study is the demonstration that some cardiac genes such as the ANF and BNP genes are bona fide targets for both GATA-4 and GATA-6 and that the two GATA factors form transcriptionnally active complexes over a single GATA element. Several lines of evidence supporting the existence of functional interaction between GATA-4 and GATA-6 are presented. First, the reduction of both GATA-4 and GATA-6 protein levels had the same effect on ANF expression as that of either factor alone, suggesting that GATA-4 and GATA-6 are members of a single functional complex and that the ablation of GATA-4 or GATA-6 in that complex is sufficient to disrupt its transcriptional activity. Second, when GATA-4 and GATA-6 are cotransfected in heterologous cells, they cooperatively activate ANF and BNP reporter genes. The presence of a functional complex between GATA-4 and GATA-6 is further supported by the finding that GATA-4 and GATA-6 physically interact in vitro and in vivo in postnatal cardiomyocytes. Finally, the colocalization of GATA-4 and GATA-6 in postnatal cardiomyocytes lends further credibility to the likelihood of in vivo relevance of a GATA-4 and GATA-6 interaction.

Homotypic (for GATA-1) and heterotypic interactions between hematopoietic GATA factors (GATA-1 and GATA-2 or

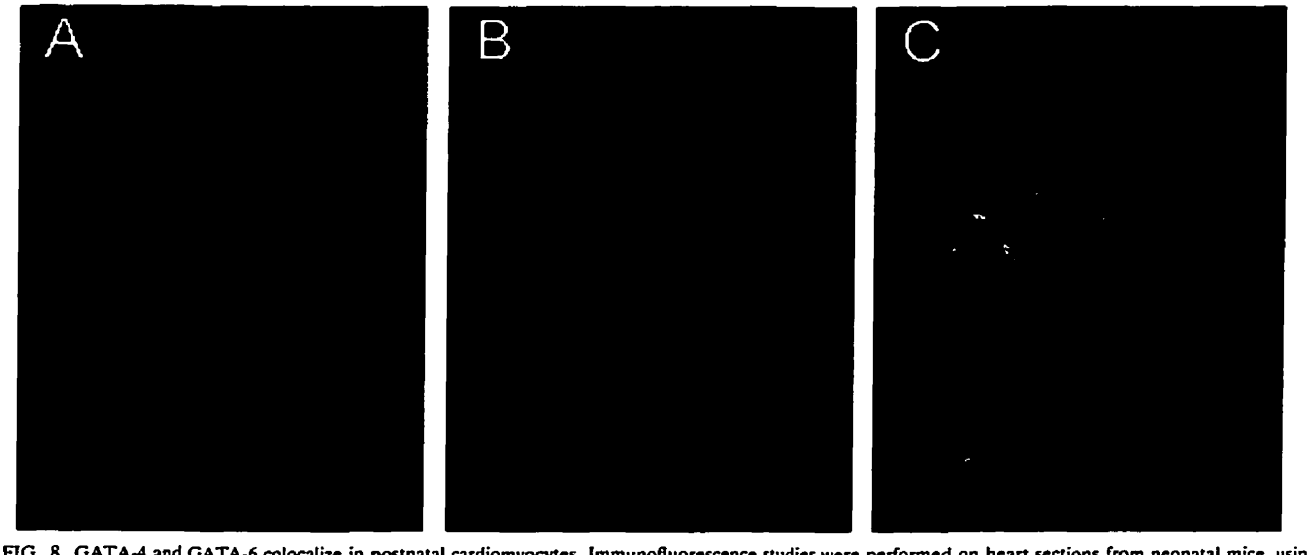


FIG. 8. GATA-4 and GATA-6 colocalize in postnatal cardiomyocytes. Immunofluorescence studies were performed on heart sections from neonatal mice, using a specific anti-GATA-4 antibody (A) and a specific anti-GATA-6 antibody (B). (C) Superposition of panels A and B indicates that GATA-4 and GATA-6 colocalize in most postnatal cardiomyocytes.

GATA-3) via the DNA-binding domain of GATA-1 have also been reported (7, 27, 43). In the case of GATA-4 and GATA-6, physical interaction also occurs via the DNA-binding zinc finger domain; however, functional cooperativity requires the C-terminal activation domain of GATA-4, suggesting that the transcriptionally active complex includes additional cofactors that are involved in specific protein-protein interactions with one but not the other GATA member. In the case of ANF, such a cofactor may be Nkx2-5, which was shown to specifically interact with GATA-4 but not GATA-6 (9, 10). Thus, heterotypic interactions may be an intrinsic property of GATA factors, and the combinatorial interaction of different GATA factors present in various cell types with each other and with other cellular cofactors may contribute to their cell-specific mode of action. The finding that GATA-4 and GATA-6 cooperatively target the ANF and BNP genes in neonatal cardiomyocytes provides biological relevance for these heterotypic interactions.

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GATA-dependent recruitment of MEF2 proteins to target promoters

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The myocyte enhancer factor-2 MEF2) proteins are MADS-box transcription factors that are essential for differentiation of all muscle lineages but their mechanisms of action remain largely undefined. In mammals, the earliest site of MEF2 expression is the heart where the MEF2C isoform is detectable as early as embryonic day 7.5. Inactivation of the *MEF2C* gene causes cardiac developmental arrest and severe down-regulation of a number of cardiac markers including atrial natriuretic factor ANF). However, most of these promoters contain no or low affinity MEF2 binding sites and they are not significantly activated by any MEF2 proteins in heterologous cells suggesting a dependence on a cardiac-enriched cofactor for MEF2 action. We provide evidence that MEF2 proteins are recruited to target promoters by the cell-specific GATA transcription factors, and that MEF2 potentiates the transcriptional activity of this family of tissue-restricted zinc finger proteins. Functional MEF2/GATA-4 synergy involves physical interaction between the MEF2 DNA-binding domain and the carboxy zinc finger of GATA-4 and requires the activation domains of both proteins. However, neither MEF2 binding sites nor MEF2 DNA binding capacity are required for transcriptional synergy. The results unravel a novel pathway for transcriptional regulation by MEF2 and provide a molecular paradigm for elucidating the mechanisms of action of MEF2 in muscle and non-muscle cells.

Keywords: GATA factors/heart development/MEF2 proteins/muscle transcription

Introduction

Members of the myocyte enhancer factor-2 MEF2) family of MADS MCM1, Agamous, Deficiens, Serum response factor)-box transcription factors are evolutionarily conserved proteins that are expressed at high levels in all muscle cells. MEF2 proteins are also found in non-muscle cells including brain and lymphoid tissue reviewed in Black and Olson, 1998). In mammals, the MEF2 family is composed of four members, MEF2A, MEF2B, MEF2C and MEF2D, which form homo- and heterodimers that bind the consensus DNA sequence T/C)TA A/T)$_4$TA G/A) present in many muscle and non-muscle promoters. MEF2 proteins contain a conserved N-terminal 56 amino acid MADS domain and an adjacent 29 amino acid MEF2 domain, which together mediate DNA binding and dimerization.

Genetic studies have provided evidence for an essential role of MEF2 proteins in muscle-specific gene expression and differentiation of all three muscle lineages. In *Drosophila*, mutation of the D-*mef2* gene results in embryos lacking differentiated skeletal, cardiac and visceral muscle cells Bour *et al.*, 1995; Lilly *et al.*, 1995; Ranganayakulu *et al.*, 1995). In mice, inactivation of the MEF2C gene, which is the first MEF2 isoform expressed during embryonic development, leads to cardiac morphogenetic defects, vascular abnormalities and lethality by embryonic day 9.5 Lin *et al.*, 1997, 1998; Bi *et al.*, 1999). The mechanisms by which MEF2 proteins regulate myogenesis of both striated and smooth muscle cells and the identity of their downstream targets in these various tissues are only starting to be elucidated. At present, the mechanisms of action of MEF2 have been analyzed mostly in skeletal muscle where MEF2 appears to act as cofactors for the myogenic basic helix–loop–helix bHLH) proteins, MyoD, Myf5, myogenin and MRF4 Kaushal *et al.*, 1994; Molkentin *et al.*, 1995; Black *et al.*, 1998). Thus, MEF2 proteins strongly potentiate the transcriptional activity of the bHLH myogenic factors and cooperate with them for inducing and maintaining the skeletal muscle phenotype. This cooperativity is mediated by direct interaction between the DNA-binding domains of MEF2 and myogenic proteins, and necessitates a DNA-binding site for only one of the two factors. Therefore, in skeletal myocytes, MEF2 may modulate transcription by two distinct pathways: one involving DNA binding to MEF2 sites and another one involving recruitment of MEF2 to E-boxes in target promoters via the myogenic bHLH factors. Whether similar mechanisms underlie the action of MEF2 in cardiac and visceral muscle cells where the MyoD family of transcription factors is not expressed remains unknown.

MEF2-binding sites have been reported in several cardiac promoters and their mutation was shown to decrease promoter activity in cardiomyocytes; they include the MEF2 sites in the ventricular myosin light chain MLC2V), cardiac troponin T, cardiac troponin I, α-myosin heavy chain αMHC) and Desmin Iannello *et al.*, 1991; Zhu *et al.*, 1991; Yu *et al.*, 1992; Molkentin and Markham, 1993; Kuisk *et al.*, 1996; Di Lisi *et al.*, 1998). Analysis of cardiac gene expression in mice with targeted mutation of the MEF2C gene confirmed that some of these

genes, like αMHC, required MEF2C for optimum transcription Lin *et al.*, 1997). In addition to αMHC, two other cardiac-specific genes not previously associated with MEF2 proteins, atrial natriuretic factor ANF) and α-cardiac actin α-CA), were completely absent in the hearts of MEF2C-deficient embryos. How MEF2C regulates transcription of these target genes remains unclear; the two αMHC MEF2 sites are low-affinity MEF2-binding sites Yu *et al.*, 1992; Molkentin and Markham, 1993), and MEF2 proteins are unable to activate αMHC-driven reporters in cotransfection assays although they can potentiate transactivation of the αMHC promoter by the thyroid hormone receptor Lee *et al.*, 1997). Moreover, ectopic expression of MEF2 proteins in explanted *Xenopus* ectoderm failed to activate endogenous αMHC or α-CA genes Chambers *et al.*, 1994; Fu and Izumo, 1995). However, forced expression of MEF2 proteins in whole *Xenopus* embryos results in precocious expression of endogenous αMHC and enlarged hearts Fu and Izumo, 1995). Together, these studies suggest that MEF2 regulates transcription of αMHC and possibly other cardiac genes in conjunction with a cell-specific cofactor present in embryonic mesoderm or endoderm) but not in ectoderm.

The ANF promoter, a known downstream target for the cardiac-specific transcription factors GATA-4 and Nkx2-5, does not contain MEF2 consensus binding sites Durocher *et al.*, 1996, 1997; Charron *et al.*, 1999); this suggests an indirect action of MEF2 on ANF transcription possibly through modulation of GATA-4, Nkx2-5 or other ANF regulators. However, neither GATA-4 nor Nkx2-5 levels are altered in MEF2C$^{-/-}$ embryos Lin *et al.*, 1997).

In this study, we provide evidence that MEF2 proteins are recruited by the cardiac-specific transcription factor GATA-4 to synergistically activate ANF and several other MEF2C target promoters including αMHC and α-CA. The MEF2–GATA-4 synergy is mediated by physical interaction between the respective DNA-binding domains and requires the transactivation domains of both factors. GATA-binding sites are necessary and sufficient for cooperativity with MEF2. Other GATA factors, including GATA-6, which is expressed in cardiac and smooth muscle cells, and GATA-2 and -3, which are present in hemopoietic and neuronal cells, are also able to cooperate with MEF2 proteins. Together, the data suggest that in addition to cooperating with the myogenic bHLH proteins in skeletal muscle differentiation, MEF2 proteins act as cofactors for the tissue-restricted zinc finger GATA proteins in cardiac myogenesis and raise the possibility that GATA factors may be essential components of MEF2 action in several other cell types.

Results

MEF2 proteins activate the ANF promoter via two distinct mechanisms

The absence of ANF transcripts in the heart of mice homozygous for a null mutation of the MEF2C gene Lin *et al.*, 1997) indicates that ANF is a downstream target for MEF2 proteins. We tested whether the effect of MEF2C was due to direct action on the ANF promoter be it via DNA binding or through recruitment by protein–protein interactions. The first 700 bp of the rat ANF promoter are

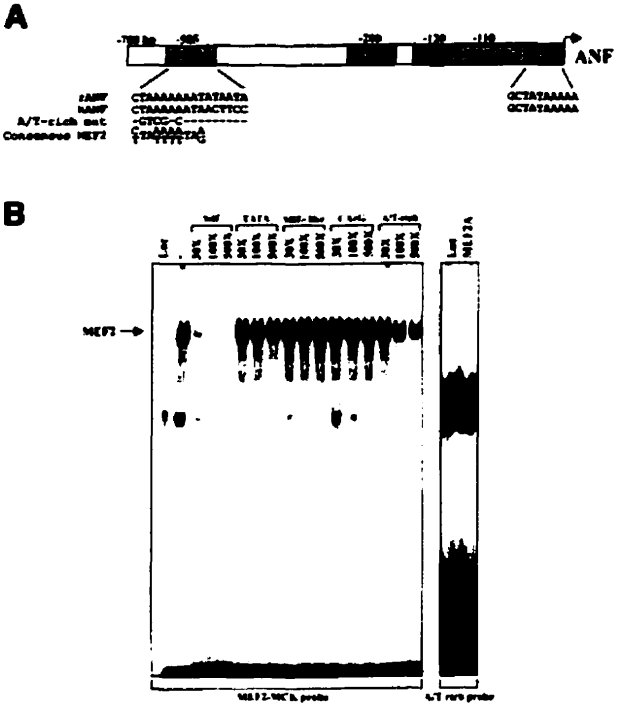

Fig. 1. The ANF promoter harbors a low-affinity MEF2-binding site. **A)** Schematic representation of the ANF promoter. Regulatory elements are boxed and their location relative to the transcription start site is indicated. All these elements are evolutionarily conserved on the ANF promoter from many species. SRE-like is a low-affinity serum response element; the GATAd and GATAp are the distal and proximal GATA-binding sites, respectively. The consensus MEF2-binding site is also shown. The A/T-rich mut sequence indicates the mutations introduced to abolish the A/T-rich element. rANF and hANF are the rat and human ANF promoter, respectively. **B)** The A/T-rich element is a low-affinity MEF2-binding site. EMSAs were performed on the MEF2 element of the MCK promoter MEF2-MCK, left panel) or the A/T-rich element of ANF A/T-rich, right panel) using *in vitro* translated MEF2A. In the left panel, the MEF2A binding was competed with different unlabeled ANF probes described in Materials and methods. Only the A/T-rich element of the ANF promoter was able to compete the MEF2A binding. Similar results were obtained with *in vitro* translated MEF2C and MEF2D.

sufficient to recapitulate cardiac specificity and spatio-temporal regulation of the endogenous gene in cultured cardiomyocytes Argentin *et al.*, 1994) and in transgenic mice Durocher *et al.*, 1998).

Sequence analysis of the entire 700 bp rat ANF promoter revealed no consensus MEF2 sites; the closest sequence homologies to MEF2-binding sites mapped to an A/T-rich element sharing similarities with a MEF2 consensus Figure 1A). To verify whether this A/T-rich element could be recognized by cardiac-derived or recombinant MEF2 proteins, it was used in gel shift assay to compete MEF2 binding on the well characterized muscle creatine kinase MCK) MEF2 site. As seen in Figure 1B, several A/T-rich ANF elements including the TATA-box, the SRE-like and the CArG-box could not compete the MEF2 binding on the MCK probe even when used at a 500-fold molar excess. On the other hand, the distal A/T-rich element was able to displace the MEF2 binding although at a much lower efficiency than the MCK site. Identical results were obtained using MEF2C and

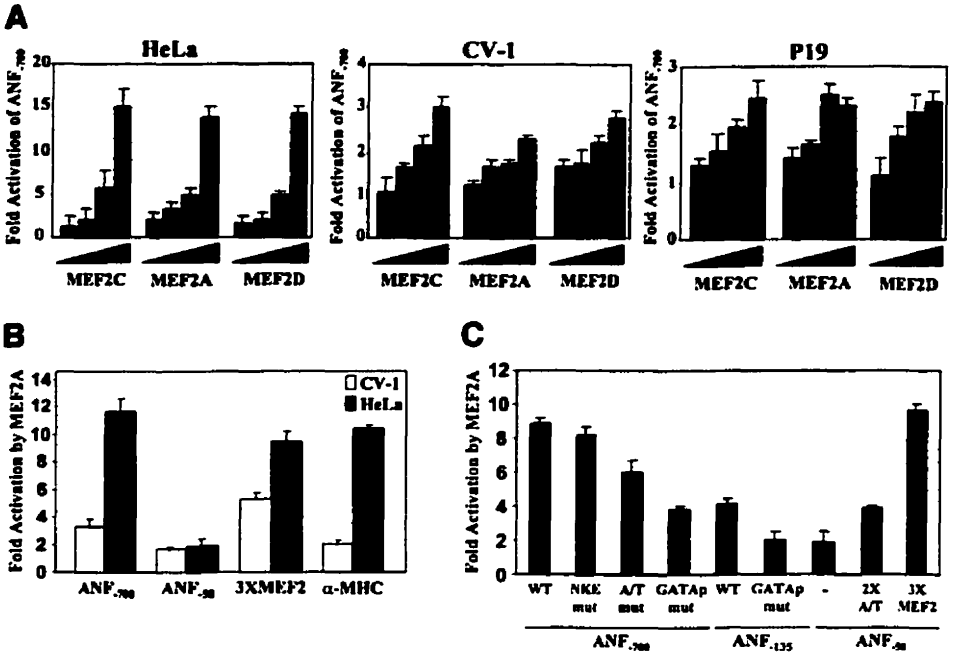

Fig. 2. The low-affinity A/T-rich and the proximal GATA elements contribute to MEF2-dependent ANF promoter activation. **A)** Dose-dependent ANF_{-700} promoter activation by MEF2A, MEF2C and MEF2D in HeLa, CV-1 and P19 cell lines. Transient transfections were performed using 50 ng, 100 ng, 500 ng and 1 μg of MEF2 expression vector. Note the fold-activation difference between HeLa and CV-1 or P19 cells. **B)** Preferential activation of the ANF_{-700} and αMHC promoters, but not an artificial MEF2 reporter, in HeLa cells. Transfections were performed using 1 μg of MEF2A expression vector. Similar results were obtained using MEF2C and MEF2D. **C)** The low-affinity A/T-rich and the proximal GATA elements contribute to MEF2-dependent ANF promoter activation. Transfections were performed in HeLa cells using 1 μg of MEF2A expression vector. Similar results were obtained using MEF2C and MEF2D. 3XMEF2 and 2XA/T are the MEF2-MCK and the ANF A/T-rich elements trimerized and dimerized, respectively, in front of the ANF_{-50} minimal promoter.

MEF2D or cardiomyocyte nuclear extracts data not shown). Consistent with its ability to recognize MEF2 proteins, the ANF A/T-rich probe was able to bind all three recombinant MEF2 proteins tested MEF2A, MEF2C and MEF2D) albeit with low affinity Figure 1B and data not shown). Finally, when cloned upstream of a minimal promoter, the ANF A/T-rich element could be transactivated 4-fold by cotransfection with MEF2 expression vectors in heterologous cells Figure 2C). These results suggest that the ANF promoter contains a very low-affinity MEF2-binding site that could mediate MEF2 action.

Indeed, MEF2C and other MEF2 proteins activate the ANF promoter in a dose-dependent manner in several non-cardiac cells Figure 2A). Interestingly, the magnitude of activation was much greater 4- to 6-fold) in HeLa cells than in most cell lines tested including CV1, P19 and C2C12), with maximal ANF promoter induction of 15-fold. This difference in MEF2 responsiveness was also observed with the cardiac αMHC promoter, which was induced by 10-fold in HeLa cells and was barely responsive in CV1 or P19 cells Figure 2B and data not shown); in contrast, a synthetic promoter harboring a multimerized MCK MEF2 site upstream of a minimal ANF promoter was more similarly activated by MEF2 in HeLa 9-fold) and CV1 6-fold) cells Figure 2B). The transfected MEF2 vectors produced similarly high levels of MEF2 proteins in all cell lines as assessed by gel shift assays data not shown). Thus, the differences observed in the level of MEF2-dependent ANF and αMHC promoter activation may reflect cooperative interaction between

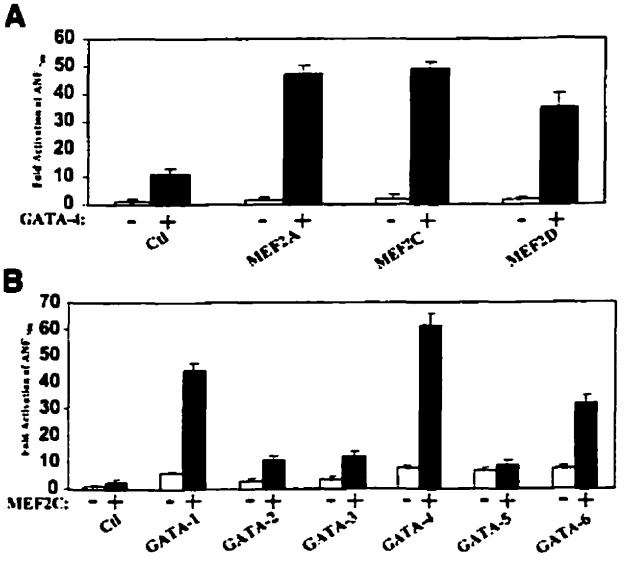

Fig. 3. The MEF2 and GATA transcription factors cooperatively activate the ANF_{-700} promoter. **A)** MEF2A, MEF2C and MEF2D functionally interact with GATA-4. Cotransfections were performed in HeLa cells using the ANF-luc_{-700} construct and 1 μg of MEF2A, MEF2C or MEF2D expression vector in the absence –) or presence +) of 1.5 μg of GATA-4 expression vector. **B)** MEF2 proteins functionally interact with a subset of GATA proteins. Cotransfections were performed as in A) using 1.5 μg of various GATA expression vectors in the absence –) or presence +) of 1 μg of MEF2C expression vector. Note that cooperative interaction between MEF2A and the different GATA factors was identical to the one shown here for MEF2C and GATA-1 to -6. Similar results were also obtained in the CV1 cell line.

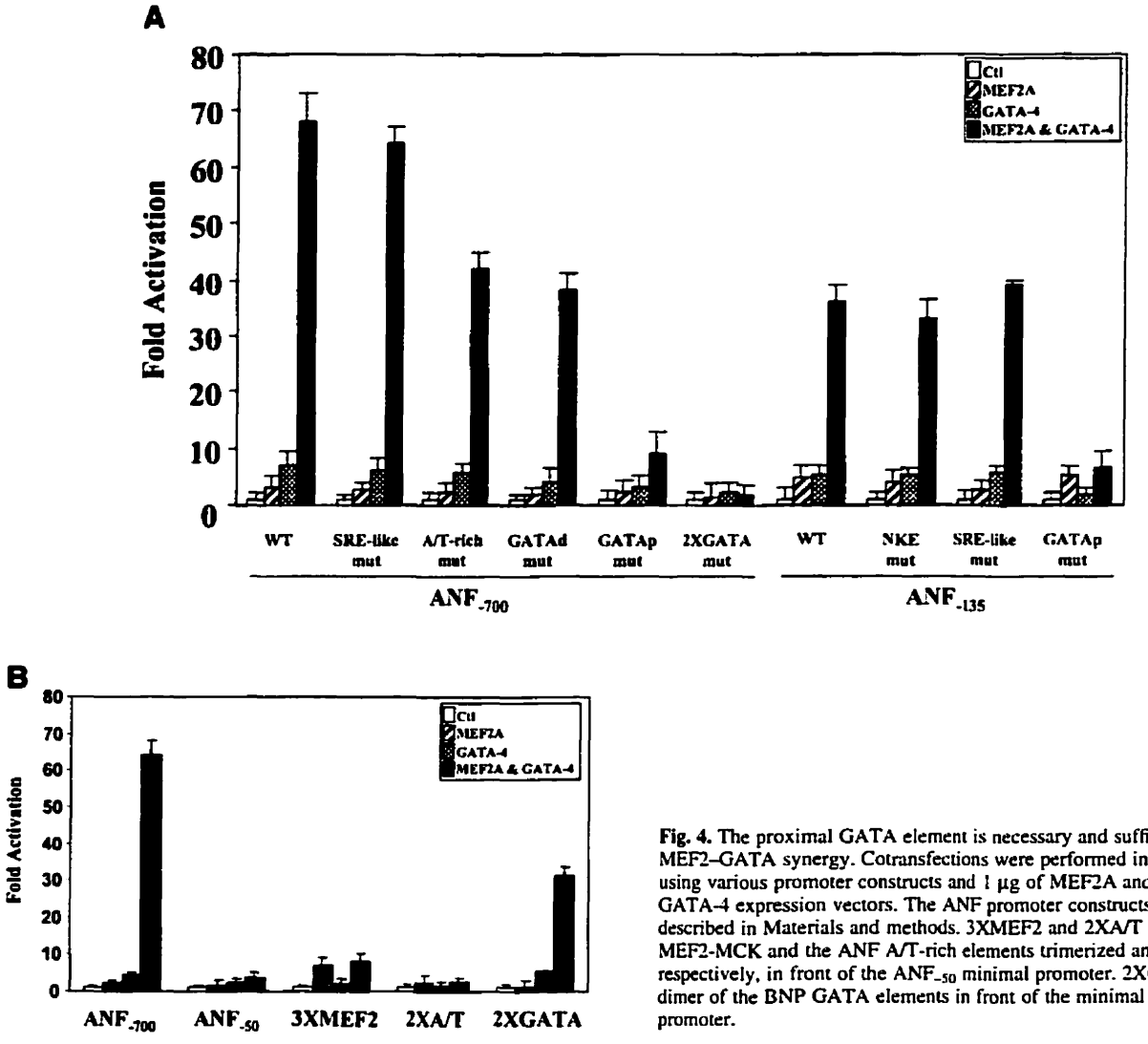

Fig. 4. The proximal GATA element is necessary and sufficient for MEF2–GATA synergy. Cotransfections were performed in HeLa cells using various promoter constructs and 1 μg of MEF2A and/or 1.5 μg of GATA-4 expression vectors. The ANF promoter constructs used are described in Materials and methods. 3XMEF2 and 2XA/T are the MEF2-MCK and the ANF A/T-rich elements trimerized and dimerized, respectively, in front of the ANF_{-50} minimal promoter. 2XGATA is a dimer of the BNP GATA elements in front of the minimal BNP promoter.

transfected MEF2 proteins and other cellular factors bound to the promoters.

Mutational analysis was used to test which DNA elements on the ANF promoter are required for activation by MEF2 Figure 2C). Consistent with its characterization as a weak-affinity MEF2 site, the A/T-rich element was necessary for maximal MEF2 activation but its deletion or mutation reduced promoter activation by only 30%. Surprisingly, mutation of the proximal GATA element that can bind endogenous GATA-2 protein present in HeLa cells Grépin *et al.*, 1994) had a more drastic effect on MEF2 responsiveness, suggesting that GATA factors may cooperate with MEF2. In fact, the proximal ANF promoter ANF_{-135}), which lacks any MEF2-binding site, was induced 4- to 5-fold by MEF2 proteins, and mutation of the GATA site therein abrogated MEF2 responsiveness Figure 2C). This element is a high-affinity binding site for GATA factors Charron *et al.*, 1999) but does not bind MEF2 proteins Figure 1B). Together, these results suggest that MEF2 could act as a cofactor of promoter-bound GATA proteins to activate the ANF and possibly other cardiac promoters.

Synergistic activation of the ANF promoter by MEF2 and GATA factors

Two members of the GATA family of zinc finger transcription factors, GATA-4 and GATA-6, are expressed in cardiac muscle cells and bind to and activate the ANF promoter Charron *et al.*, 1999). To test whether either factor could recruit MEF2 proteins to target promoters, the effect of co-expressing them with MEF2 in heterologous cells was assayed on ANF promoter activity. Cotransfection of GATA-4 with MEF2A, MEF2C or MEF2D leads to a synergistic 40- to 50-fold activation of the ANF promoter Figure 3A). MEF2 proteins were also able to cooperate to varying degrees with other GATA factors including the hemopoietic GATA-1, -2 and -3, and the other cardiac GATA factor, GATA-6, but not GATA-5 Figure 3B). The inability of MEF2C and MEF2A to synergize with GATA-5 and the more modest synergy achieved with GATA-2 and -3 are not due to different levels of GATA proteins produced as all expression vectors have been shown to produce similar protein levels Viger *et al.*, 1998; Nemer *et al.*, 1999).

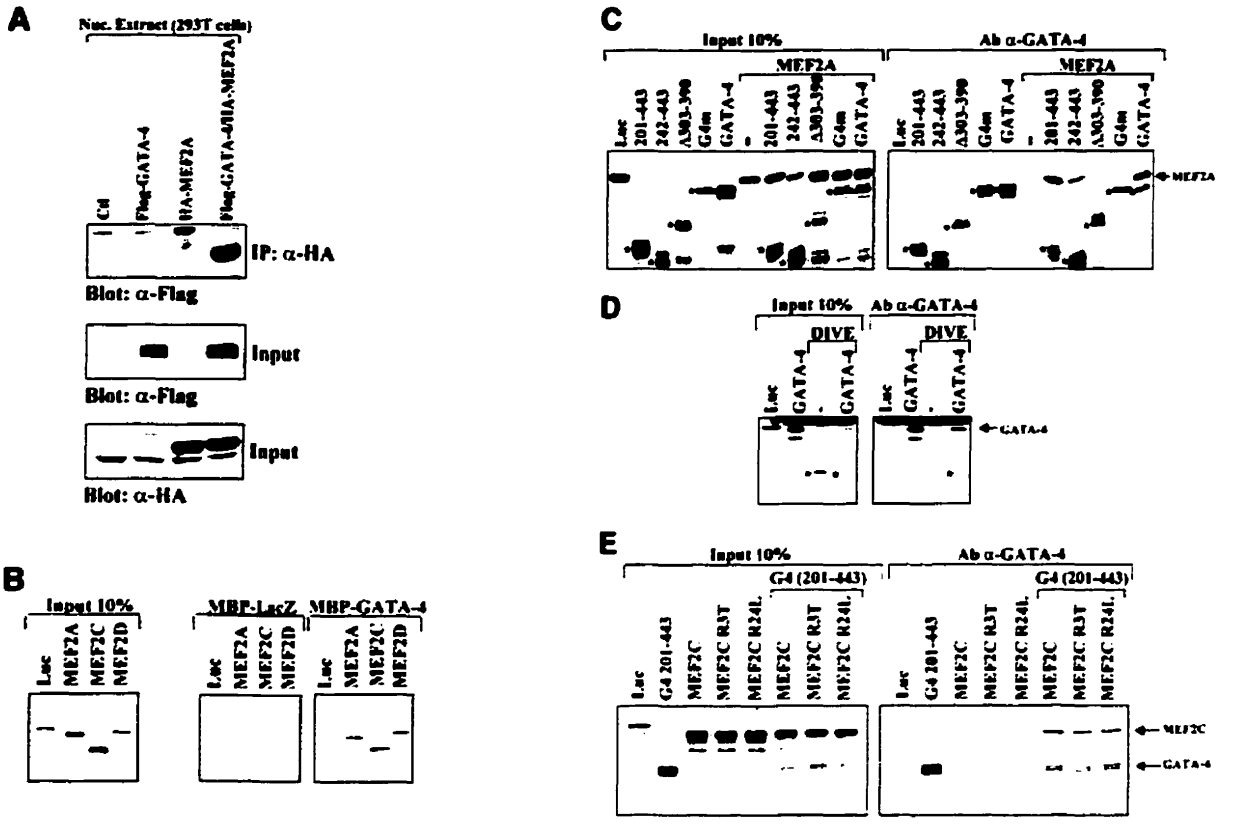

Fig. 5. MEF2 proteins physically interact with GATA-4. **A)** MEF2A interacts *in vivo* with GATA-4. Nuclear extracts from 293T cells transfected with empty vectors Ctl), Flag-GATA-4 and/or HA-MEF2A were immunoprecipitated using an anti-HA antibody, separated on 10% SDS–PAGE, transferred to PVDF membranes, and subjected to immunoblotting using an anti-Flag antibody top panel). The lower two panels are Western blots carried out on the same nuclear extracts using either HA to reveal tagged MEF2A proteins) or Flag to reveal tagged GATA-4 proteins) antibodies. **B)** MEF2A proteins interact *in vitro* with GATA-4. Pull-down assays were performed using immobilized, bacterially produced MBP fusions MBP-GATA-4 and MBP-LacZ as control) and *in vitro* translated ^{35}S-labeled MEF2A, MEF2C, MEF2D or luciferase luc) protein. The protein complexes were resolved on 10% SDS–PAGE. **C)** The physical interaction between GATA-4 and MEF2 requires the C-terminal zinc finger DNA-binding domain of GATA-4. Full-length GATA-4 and various GATA-4 mutants depicted in Figure 6A) were *in vitro* cotranslated with MEF2A and co-immunoprecipitated using an antibody directed against the extreme C-terminus of GATA-4. The protein complexes were resolved on 15% SDS–PAGE. The asterisks highlight GATA protein bands. **D)** The DNA-binding domain of MEF2 is sufficient for interaction with GATA-4. MEF2A DIVE aa 1–86) retains the MADS and MEF2 domains. Co-immunoprecipitations were performed as described in C). The asterisks highlight the MEF2A DIVE band. The protein complexes were resolved on 20% SDS–PAGE. **E)** MEF2 DNA-binding-defective mutants interact with GATA-4. MEF2C R3T and MEF2C R24L do not bind DNA but are still able to dimerize. A deleted GATA-4 construct [G4 201–443)] was used to differentiate between GATA-4 and MEF2C, which have similar electrophoretic mobility. Co-immunoprecipitations were performed as described in C). The protein complexes were resolved on 10% SDS–PAGE.

In order to map the promoter element s) required for the synergy between MEF2 and GATA-4, various ANF promoter mutations were tested. Mutation of the low-affinity MEF2-binding element A/T-rich) or the distal GATA element reduced maximal MEF2–GATA-4 synergy by 35–40% Figure 4A). Mutation of the proximal GATA element in the context of the –700 or the –135 bp promoter completely abolished synergy, indicating that this element is essential for MEF2–GATA-4 cooperation. Interestingly, the –135 bp ANF promoter was sufficient to produce 50% of the maximal synergy obtained with the longer promoter ANF$_{-700}$) and displayed the same response to the various MEF2–GATA combinations as the ANF$_{-700}$ promoter Figure 3), suggesting that binding of GATA factors to the proximal GATA element may be sufficient to recruit MEF2 proteins to the promoter.

Indeed, an artificial reporter driven by multimerized GATA-binding sites could be synergistically activated by GATA and MEF2 proteins; however, neither the high-affinity MCK MEF2-binding site nor the lower affinity ANF MEF2 element was sufficient to support MEF2–GATA cooperativity Figure 4B).

MEF2 factors physically interact in vitro and in vivo with GATA-4

The observation that MEF2A–GATA-4 synergy required only the GATA-binding site implied that GATA-4 recruits MEF2 proteins to the ANF promoter through protein–protein interaction. Indeed, MEF2A and GATA-4 could be co-immunoprecipitated *in vivo* Figure 5A), suggesting physical interaction between the two proteins.

To determine whether this interaction was direct, we performed *in vitro* pull-down assays using immobilized MBP-GATA-4 and *in vitro* translated ^{35}S-labeled MEF2 proteins. MBP-GATA-4 was able to retain specifically MEF2A, MEF2C and MEF2D but not the control luciferase Figure 5B), confirming that GATA-4 and MEF2 directly interact.

In order to map the interaction domain between MEF2 and GATA proteins, different mutants of GATA-4 were *in vitro* cotranslated with MEF2A and co-immunoprecipitated using an antibody directed against the extreme C-terminus of GATA-4 present in all mutants tested). MEF2A was able to interact with the full-length GATA-4 and the N-terminal activation domain-deleted mutant 201–443) Figure 5C). Deletion of the N-terminal zinc finger of GATA-4 242–443) reduced but did not abrogate interaction with MEF2A. However, MEF2A was unable to interact with the G4m [which harbors a point mutation in the C-terminal zinc finger, abolishing DNA binding Charron *et al.*, 1999)] or the Δ303–390 mutant, indicating that the C-terminal zinc finger structure and the basic region are essential for physical interaction with MEF2. The same approach was also used to map the GATA-4 interaction domain on MEF2 and revealed that the DNA-binding domain consisting of the MADS and MEF2 domains MEF2A DIVE, aa 1–86) is sufficient for interaction with GATA-4 Figure 5D). Interestingly, within the MADS domain, interaction with GATA factors and binding to DNA could be segregated as two DNA-binding-defective mutants MEF2C R3T and MEF2C R24L) retained the ability to bind GATA-4 Figure 5E). These results suggest that GATA-4 and MEF2 physically interact through their DNA-binding domains.

MEF2–GATA synergy does not require MEF2 DNA binding

To determine whether the activation domains of either or both GATA and MEF2 proteins are required, various GATA-4 mutants were tested for their capacity to activate the ANF promoter synergistically with MEF2A. The GATA-4 mutants that delete the N-terminal region 127–443 and 201–443) and the first zinc finger 242–443) were able to synergize with MEF2A Figure 6A). However, the GATA-4 mutants that delete the C-terminal transactivation domain 1–332, 201–332 and 242–332) were all unable to support MEF2 synergy. Consistent with a requirement for GATA-4 DNA binding Figure 4A) and the GATA-4 DNA-binding domain for physical interaction with MEF2 Figure 5C), a point mutation in the second zinc finger that destroys DNA binding G4m) abrogated MEF2 synergy Figure 6A).

Functional synergy also required the activation domain of MEF2, as deletion of the C-terminal activation domain MEF2A DIVE) completely abolished the synergy with GATA-4 Figure 6B). However, consistent with the requirement for GATA- but not MEF2-binding sites, MEF2C mutants that are DNA-binding defective retained the ability to synergize with GATA-4 Figure 6B). These results indicate that GATA-4 is able to recruit DNA-binding-defective MEF2 proteins to transcriptionally active complexes.

MEF2–GATA synergy: a mechanism for MEF2 action in the heart

We next tested whether transcription of other cardiac genes is cooperatively activated by MEF2 and GATA-4. As seen in Figure 7A, in addition to ANF, the αMHC, α-CA and B-type natriuretic peptide BNP) promoters are also synergistically activated by MEF2 and GATA-4. Both

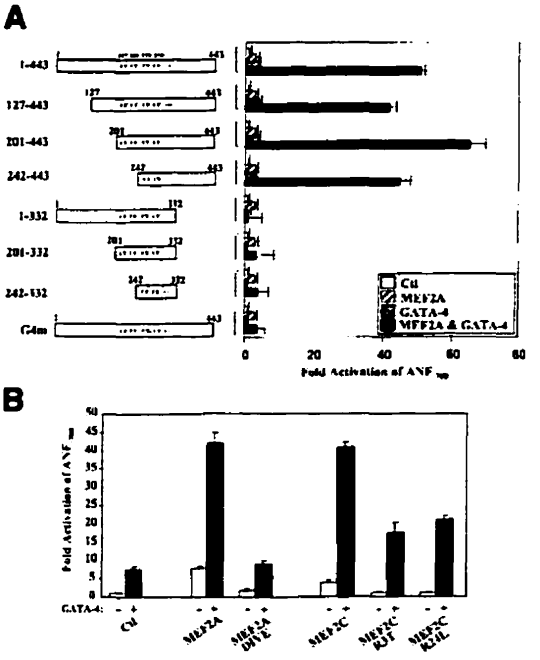

Fig. 6. Mapping of the GATA-4 and MEF2 domains required for synergy. **A)** The C-terminal activation domain of GATA-4 is required for MEF2 synergy. Cotransfections were performed in HeLa cells on the ANF_{-700} promoter construct using 1 μg of MEF2 and 1.5 μg of GATA-4 expression vectors. **B)** The C-terminal activation domain of MEF2, but not its DNA-binding capacity, is required for synergy with GATA-4, as shown by the ability of MEF2C R3T and MEF2C R24L to synergize with GATA-4. Note that the DNA-binding domain MEF2A DIVE) is not sufficient to support functional synergy although it interacts physically with GATA-4 as shown in the previous figure.

αMHC and α-CA are downregulated in MEF2C null mice and neither contain a high-affinity MEF2-binding site although both are GATA targets Sepulveda *et al.*, 1998; Charron *et al.*, 1999). However, as shown by the βMHC promoter, not all GATA target promoters are synergistically activated by MEF2, suggesting that functional GATA–MEF2 synergy is promoter context dependent and may be targeted to a specific subset of cardiac genes.

Finally, to ascertain whether, in cardiac cells, MEF2 proteins are GATA cofactors, the effect of a dominant-negative MEF2 protein on the activity of the proximal ANF promoter was determined. This promoter contains a GATA- but no MEF2-binding site. Cotransfection of a MEF2 mutant that retains the ability to associate physically with GATA-4 but lacks the activation domain reduces by 50% the activity of the ANF promoter in primary cardiomyocyte cultures; in contrast, cotransfection with wild-type MEF2A induces promoter activity by 80% Figure 7B). Both activation by wild-type MEF2 and inhibition by its dominant-negative form are blunted by point mutation of the GATA-binding site Figure 7B). These data are consistent with a role for MEF2 proteins as co-activators of GATA factors in cardiac muscle cells and point to a novel GATA-dependent pathway for transcriptional activation by MEF2.

Discussion

The MEF2 transcription factors are key regulators of cardiac myogenesis and morphogenesis, but the molecular

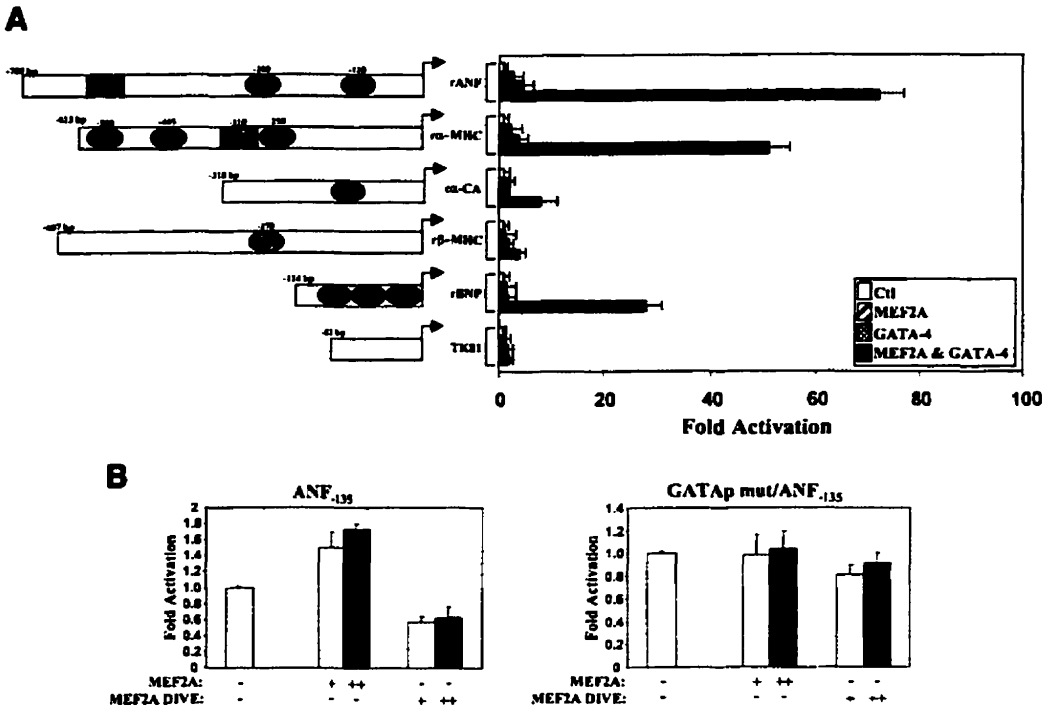

Fig. 7. The MEF2–GATA-4 synergy: a mechanism for MEF2 action in the heart. **A)** The MEF2–GATA-4 synergy is not limited to the ANF promoter. HeLa cells were cotransfected with 1 μg of MEF2A and 1.5 μg of GATA-4 expression vectors together with various cardiac promoters. Except for the cardiac α-actin promoter that was from chicken, all other promoters used are from rat and are described in Materials and methods. TK81 is the thymidine kinase –81 bp promoter. Elements shaded in black and gray are high- and low-affinity sites, respectively, as determined by DNA-binding assays. **B)** A dominant-negative MEF2 protein decreases ANF promoter activity in cardiomyocytes. Primary culture of cardiomyocytes was transfected with the wild-type ANF_{-135} left panel) or GATA-mutated ANF_{-135} promoter GATAp mut/ANF_{-135}, right panel) and no –), 50 ng +) or 1000 ng ++) of MEF2A or a dominant-negative form of MEF2A MEF2A DIVE). The results shown represent the mean ± SD of two independent experiments each carried out in duplicate.

basis for their actions is poorly understood. The data presented here provide evidence that, in cardiac myocytes, MEF2 proteins are recruited by the cardiac-specific GATA transcription factors to target promoters and functionally synergize with this family of tissue-restricted zinc finger proteins. This observation is reminiscent of the cooperative interaction between MEF2 proteins and the myogenic bHLH factors in skeletal muscles, and suggests that MEF2 proteins are able to interact with and potentiate the action of other classes of cell-specific transcription factors. Given the co-expression of MEF2 and GATA factors in several cell types including smooth muscle, neuronal and T cells, the GATA-dependent MEF2 pathway described in this work may provide a molecular paradigm for understanding the mechanisms of action of MEF2 in many target cells.

GATA proteins are evolutionarily conserved cell-restricted transcription factors that play crucial roles in differentiation. In vertebrates, six GATA factors have been identified and they are all expressed in a lineage-restricted and developmentally controlled manner. GATA-1, -2 and -3 are predominantly expressed in hemopoietic cells while GATA-4, -5 and -6 are largely restricted to the heart and gut. Genetic and biochemical studies have revealed crucial roles for specific family members in hemopoietic, cardiac, neuronal and endodermal cells Pevny *et al.*, 1991; Tsai *et al.*, 1994; Grépin *et al.*, 1995; Pandolfi *et al.*, 1995; Molkentin *et al.*, 1997; Morrisey *et al.*, 1998). In addition to their essential roles in development, GATA factors are also required for the proper function of adult organs. GATA-binding sites are present on many hemopoietic and cardiac promoters, which are potently activated by GATA factors reviewed in Charron and Nemer, 1999). Moreover, GATA proteins act cooperatively with other classes of transcription factors including several zinc finger proteins, such as SP1 and FOG-1 friend of GATA-1) and homeodomain-containing factors like the cardiac-specific Nkx2-5 Durocher *et al.*, 1997; Tsang *et al.*, 1997). The differential interactions of GATA proteins with other transcription factors are likely to be important for functional specificity of GATA proteins during embryonic development and in differentiated and adult cells.

In this study, we report for the first time that, in addition to interacting with homeodomain- and zinc finger-containing proteins, GATA factors are also able to interact with members of the MADS-box family of transcription factors. This interaction involves physical contact between the C-terminal zinc finger DNA-binding domain and the adjacent basic region of GATA-4 and the MADS domain of MEF2. This, in turn, leads to synergistic activation of the ANF and other MEF2 target promoters independently of the DNA-binding activity of MEF2. Synergy is observed with two of the three cardiac GATA factors: GATA-4 and -6 but not with GATA-5; MEF2 synergy was also detected with GATA-1, -2 and -3 although at varying levels. Thus, all GATA factors are not equally competent to synergize with MEF2. The observation that GATA-4 and -6 but not -5 synergize with MEF2 is interesting given

that GATA-4 and -5 but not -6 synergize with the cardiac homeodomain protein Nkx2-5 Durocher *et al.*, 1997); this suggests that while MEF2 and Nkx2-5 interact with the same domain of GATA-4, they apparently do not recognize the same molecular determinants.

Although GATA factors physically contact MEF2 proteins through the DNA-binding domain, DNA binding and GATA interaction are dissociable and neither physical nor functional interaction with GATA-4 on natural promoters requires MEF2 DNA binding. The ability of MEF2 to cooperate with MyoD in skeletal myogenesis and to synergize with MyoD in activating an artificial promoter driven by multimerized MyoD-binding sites was also shown to be independent of MEF2 DNA-binding capacity Molkentin *et al.*, 1995). DNA binding has been shown to be dispensable for some of the actions of two other sequence-specific DNA-binding proteins, the glucocorticoid receptor Reichardt *et al.*, 1998) and the cell-specific homeodomain protein Pit1 Dasen *et al.*, 1999). However, in both cases, DNA-binding-independent activities involved transcriptional repression. In the present study, we show that transcriptional activation of several natural promoters by MEF2 proteins is independent of MEF2-binding sites and MEF2 DNA-binding ability. Thus, both suppressive and activating functions of transcription factors may involve DNA-binding-independent pathways.

In addition to the MADS domain, the activation domain of MEF2 is required for functional synergy with GATA-4, suggesting that MEF2 proteins potentiate transcriptional activity of GATA factors through recruitment and/or stabilization of co-activators in the GATA transcription complex. Such co-activators may include the CBP/p300 family, as both MEF2 and GATA proteins have been shown to interact independently with these co-activators Sartorelli *et al.*, 1997; Blobel *et al.*, 1998; Kakita *et al.*, 1999). Alternatively, MEF2–GATA interaction may displace or overcome a corepressor of either or both factors. For example, MEF2 interaction with GATA-4 or -6 in the heart may displace FOG-2, a GATA-associated cofactor that represses GATA-4 activity Lu *et al.*, 1999; Svensson *et al.*, 1999). Conversely, recruitment of MEF2 by GATA factors may displace the MEF2-associated corepressors MITR or the HDAC4 deacetylase Miska *et al.*, 1999; Sparrow *et al.*, 1999).

In addition to cardiomyocytes, MEF2 proteins are co-expressed with members of the GATA family in several other cell types. Most notable is the presence of MEF2 proteins with GATA-6 in smooth muscle cells Narita *et al.*, 1996; Suzuki *et al.*, 1996), and with GATA-3 in T lymphocytes Zheng and Flavell, 1997), somites George *et al.*, 1994) and brain Pandolfi *et al.*, 1995). Given the demonstrated role of GATA factors in cell differentiation, the GATA–MEF2 synergy provides a general paradigm for understanding the role of MEF2 proteins as determining factors in diverse cell lineages.

Finally, it is tempting to speculate on the role of the MEF2–GATA synergy as a nuclear target of several signaling cascades including calcineurin and p38 MAP kinase. Both pathways, which are highly relevant to many human disorders such as ischemia, heart failure and inflammatory diseases, have been shown to activate MEF2 in cardiac Kolodziejczy *et al.*, 1999), skeletal muscle Zetser *et al.*, 1999), neuronal Mao *et al.*, 1999) and T cells Han *et al.*, 1997; Blaeser *et al.*, 2000). GATA factors have also been suggested as downstream targets of calcineurin in cardiac and skeletal muscle hypertrophy in connection with the calcineurin-activated NFAT factor Molkentin *et al.*, 1998; Musaro *et al.*, 1999; Semsarian *et al.*, 1999). It would be interesting to test whether calcineurin or p38-dependent post-translational modifications of MEF2 or GATA proteins modulate the MEF2–GATA physical interaction and/or the resulting functional synergy.

Materials and methods

Cell cultures and transfections

HeLa cells were grown in Dulbecco's modified Eagle's medium DMEM) supplemented with 10% fetal bovine serum FBS). Transfections were carried out using calcium phosphate 24 h after plating. At 36 h post-transfection, cells were harvested and luciferase activity was assayed with a Berthold LB953 luminometer. The amount of reporter was kept at 1.5 μg per 35 mm dish and the total amount of DNA was kept constant usually 7 μg). The amount of expression vector used is indicated in the figure legends. Primary cardiomyocyte cultures were prepared from 4-day-old Sprague–Dawley rats as previously described Charron *et al.*, 1999). The results shown are the mean ± SD of at least two independent experiments carried out in duplicate.

Plasmids

ANF-luciferase promoter constructs were cloned in the PXP-2 vector as described previously Argentin *et al.*, 1994; Durocher *et al.*, 1996). The BNP-luc constructs were described in Grépin *et al.* 1994), the βMHC-luc and cardiac α-actin-luc reporters were described in Abdellatif *et al.* 1994). The αMHC-luc vector was kindly provided by P.M.Buttrick Buttrick *et al.*, 1993). The construction of the various pCG-GATA-4 vectors was based on the original rat GATA-4 cDNA as previously described Grépin *et al.*, 1994). The various deletions or point mutations of the ANF promoter and the pRSET-GATA-4 derivatives were generated as described previously Durocher *et al.*, 1997; Charron *et al.*, 1999). ANF constructs with mutations in the GATA elements or in the NKE were previously described Durocher *et al.*, 1996; Charron *et al.*, 1999). The A/T-rich mutation is shown in Figure 1; the SRE-like mutation replaces the TTT of the ANF-SRE by GGG thus destroying SRF binding. Heterologous promoters were generated by multimerizing the relevant oligonucleotides flanked by *Bam*HI and *Bgl*II sites upstream of the minimal –50 bp) ANF-luciferase reporter. pcDNA-MEF2A DIVE was constructed by insertion of the *Xba*I–*Bam*HI fragment of the corresponding pCGN-MEF2A DIVE construct into the *Xba*I–*Bam*HI sites of the pcDNA-3 vector. MEF2 plasmids were kindly provided by E.N.Olson Molkentin *et al.*, 1996a) and K.Walsh Andres *et al.*, 1995). The MBP-GATA-4 plasmid was prepared by subcloning a *Xba*I–*Bam*HI rat GATA-4 cDNA fragment containing the entire open reading frame and 1.2 kb of 3′ untranslated sequences Grépin *et al.*, 1994) into the MBP-expressing pMalc-2 vector New England Biolabs, Beverly, MA) cut with *Nhe*I–*Bam*HI.

Recombinant protein production

Recombinant MBP-GATA-4 was obtained according to our previously described protocol Durocher *et al.*, 1997). Essentially, individual colonies were picked and grown in 500 ml of LB up to an OD of 0.6 at 600 nm. Induction of the recombinant protein was carried out by adding isopropyl-β-D-thiogalactopyranoside IPTG) at a final concentration of 0.5 mM for 2 h at 37°C. The cultures were centrifuged and the bacteria were resuspended and lysed by sonication. Purification on amylose columns New England Biolabs, Beverly, MA) was performed according to the manufacturer's instructions.

In vitro transcribed/translated ^{35}S-labeled MEF2 and GATA proteins were produced in rabbit reticulocyte lysates using the TNT-coupled *in vitro* transcription/translation system Promega Corp., Madison, WI) from pcDNA-MEF2 derivatives using either T7 or Sp6 RNA polymerase.

In vitro protein–protein interactions

In vitro binding studies were performed using purified MBP-GATA-4 immobilized on an amylose–Sepharose resin New England Biolabs) and *in vitro* transcribed/translated MEF2 proteins. Typically, 2–6 μl of

^{35}S-labeled MEF2 proteins were incubated in the presence of 300 ng of immobilized GATA-4 fusion protein in 500 µl of binding buffer [150 mM NaCl, 50 mM Tris–Cl pH 7.5, 0.3% Nonidet P-40, 1 mM dithiothreitol DTT), 0.5 mM phenylmethylsulfonyl fluoride PMSF) and 0.25% bovine serum albumin BSA)] for 2 h at 4°C with agitation and then centrifuged for 2 min at 15 000 r.p.m. at room temperature. The resin was washed three times by vortexing in 500 µl of binding buffer at room temperature and three more times by vortexing in 500 µl of binding buffer without BSA. The protein complexes were released from the resin after boiling in Laemmli buffer and resolved by SDS–PAGE. Labeled proteins were visualized and quantified by autoradiography on phospho storage plates PhosphorImager, Molecular Dynamics).

To determine the domains of GATA-4 and MEF2 required for physical interaction, full-length GATA-4 or mutated GATA-4 plasmids were used for *in vitro* cotranscription/cotranslation with wild-type or mutant MEF2A and MEF2C. The cotranslated proteins were incubated in 500 µl of binding buffer with 1 µl of GATA-4 antibody Santa-Cruz Biotechnology) for 2 h at 4°C with agitation and for an additional 2 h with 20 µl of protein A/G Plus–agarose added Santa Cruz Biotechnology). Bound immunocomplexes were washed and visualized as mentioned above.

Electrophoretic mobility shift assays EMSAs)

Three microliters of the *in vitro* translated MEF2A, MEF2C and MEF2D proteins were used for the binding reactions performed essentially as previously described for GATA binding Charron *et al.*, 1999) except that 100 ng of dI-dC were included in the binding reaction. Reactions were loaded on a 4% polyacrylamide gel and run at 200 V at room temperature in 0.25× Tris-borate-EDTA. The MEF2-MCK probe is as described in Molkentin *et al.* 1996b). The rat ANF probes used were as follows: TATA-box, $^{-46}$TCAGGGAGCTGGGGGCTATAAAAACGGGAGACGCC^{-11}; SRE-like, $^{-124}$GATCCACTGATAACTTTAAAAGGGCATCTTCA^{-99}; CArG, $^{-417}$GATCCTCCCGCCCTTATTTGGAGCCCCTGA^{-390}; A/T-rich, $^{-597}$GATCCATACTCTAAAAAAATATAATAGCTCTTTCA^{-567}.

Immunoprecipitations and immunoblots

Co-immunoprecipitations of Flag-GATA-4 and HA-MEF2A were carried out using nuclear extracts of 293T cells overexpressing the relevant proteins. Nuclear extracts were prepared as follows: five million 293T cells transfected with 15 µg of expression vectors were harvested 48 h post-transfection in ice-cold phosphate-buffered saline PBS) containing 1 mM sodium orthovanadate and 1 mM EDTA. The cells were resuspended in hypotonic buffer 20 mM HEPES pH 7.9, 20 mM sodium fluoride, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, 0.25 mM sodium molybdate, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml pepstatin, 2 mM DTT, 0.5 mM PMSF and 100 nM okadaic acid) and swollen on ice for 15 min. Twenty-five microliters of 10% NP-40 were added and the microtubes were vortexed vigorously. The nuclei were then pelleted by centrifugation at 7000 r.p.m. at 4°C. The nuclear pellet was resuspended in 50–100 µl of high salt buffer hypotonic buffer containing 20% glycerol and 0.4% NaCl) and shaken vigorously at 4°C for 1 h. The nuclear extracts were cleared by centrifugation at 15 000 r.p.m. for 15 min at 4°C and the protein concentration was determined by the Bradford assay. Co-immunoprecipitation reactions were carried out on 50 µg of nuclear extracts using 1 µl of 12CA5 antibody in 500 µl of binding buffer without BSA, and bound immunocomplexes were washed and subjected to SDS–PAGE, as described previously Durocher *et al.*, 1997). Proteins were transferred on Hybond-PVDF membrane and subjected to immunoblotting. Anti-Flag M5 Sigma) and 12CA5 anti-HA) monoclonal antibodies were used at a dilution of 1/8000, revealed with an anti-mouse-HRP Sigma) at a dilution of 1/50 000 and visualized using ECL Plus Amersham Pharmacia Biotechnology).

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The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors

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The tissue-restricted GATA-4 transcription factor and Nkx2-5 homeodomain protein are two early markers of precardiac cells. Both are essential for heart formation, but neither can initiate cardiogenesis. Overexpression of GATA-4 or Nkx2-5 enhances cardiac development in committed precursors, suggesting each interacts with a cardiac cofactor. We tested whether GATA-4 and Nkx2-5 are cofactors for each other by using transcription and binding assays with the cardiac atrial natriuretic factor (ANF) promoter—the only known target for Nkx2-5. Co-expression of GATA-4 and Nkx2-5 resulted in synergistic activation of the ANF promoter in heterologous cells. The synergy involves physical Nkx2-5–GATA-4 interaction, seen *in vitro* and *in vivo*, which maps to the C-terminal zinc finger of GATA-4 and a C-terminus extension; similarly, a C-terminally extended homeodomain of Nkx2-5 is required for GATA-4 binding. Structure/function studies suggest that binding of GATA-4 to the C-terminus autorepressive domain of Nkx2-5 may induce a conformational change that unmasks Nkx2-5 activation domains. GATA-6 cannot substitute for GATA-4 for interaction with Nkx2-5. This interaction may impart functional specificity to GATA factors and provide cooperative crosstalk between two pathways critical for early cardiogenesis. Given the co-expression of GATA proteins and NK2 class members in other tissues, the GATA/Nkx partnership may represent a paradigm for transcription factor interaction during organogenesis.

Keywords: ANF/cardiogenesis/GATA factors/homeodomain/transcription

Introduction

The GATA family of transcription factors are key developmental regulators that have been conserved throughout evolution (Fu and Marzluf, 1990; Spieth *et al.*, 1991; Winick *et al.*, 1993; Stanbrough *et al.*, 1995; Coffman *et al.*, 1996; Platt *et al.*, 1996). Various family members have been shown to alter transcription of target genes via binding to the consensus WGATAR sequence through a DNA-binding domain consisting of two adjacent zinc-fingers of the C2/C2 family. Sequence-specific DNA-binding requires the C-terminal zinc-finger and the N-terminal finger may stabilize the DNA–protein complex via electrostatic interactions with the phosphate backbone (Whyatt *et al.*, 1993). This DNA-binding domain is the highest conserved region among the various GATA proteins. In vertebrates, six members have been identified so far and they can be divided into two subgroups based on sequence homology and tissue distribution. The first subgroup, which includes GATA-1, -2 and -3, is largely restricted to the hematopoietic system where all three GATA factors have been shown to play essential, non-redundant functions (Tsai *et al.*, 1994; Pandolfi *et al.*, 1995; Fujiwara *et al.*, 1996; Ting *et al.*, 1996). Remarkably, arrested proerythroblasts lacking GATA-1 express several GATA-1 target genes although they are unable to achieve terminal erythroid differentiation (Weiss *et al.*, 1994), raising the possibility that GATA-2—which is co-expressed with GATA-1 in proerythroblasts—may partially substitute for GATA-1. Consistent with this, GATA factors appear to be functionally interchangeable in some (Blobel *et al.*, 1995; Visvader *et al.*, 1995) but not all (Briegel *et al.*, 1993; Weiss *et al.*, 1994) *in vitro* assays. Taken together with the *in vivo* data, these results suggest that functional specificity of GATA proteins likely involves interactions with other cell-restricted cofactors. Consistent with this hypothesis, GATA-1 was found to interact with the erythroid-specific LIM protein RBTN2 and to be present in complexes containing RBTN2 and the hematopoietic basic helix-loop-helix protein SCL/TAL1 (Osada *et al.*, 1995). GATA-1 was also shown to cooperate with the ubiquitous SP1 protein and with two other erythroid factors, the basic leucine zipper NFE-2 (Walters and Martin, 1992; Gong and Dean, 1993) and the zinc finger EKLF (Merika and Orkin, 1995; Gregory *et al.*, 1996) for transcriptional activation of erythroid promoters/enhancers. At least, in the case of SP1 and EKLF, the interaction was also observed with GATA-2 and involved direct contact through the DNA-binding domains (Merika and Orkin, 1995). Thus, the identity of the proteins that serve as cofactor(s) to impart functional specificity of GATA proteins in the hematopoietic system remains essentially unknown.

The other subclass of vertebrate GATA factors includes GATA-4, -5 and -6 whose expression is restricted to the heart and gut (Arceci *et al.*, 1993; Kelley *et al.*, 1993; Grépin *et al.*, 1994; Laverriere *et al.*, 1994; Jiang and Evans, 1996). All three genes are transcribed at very early stages of *Xenopus*, avian and mouse cardiac development (Kelley *et al.*, 1993; Heikinheimo *et al.*, 1994; Laverriere *et al.*, 1994; Jiang and Evans, 1996; Morrisey *et al.*, 1996).

Within the heart, transcripts for GATA-4, -5 and -6 are found in distinct cell types with GATA-5 mRNA predominantly found in endocardial cells while GATA-4 and -6 are present in the myocardium (Kelley *et al.*, 1993; Grépin *et al.*, 1994; Morrisey *et al.*, 1996). The spatial and temporal expression of GATA-4 together with various functional studies are consistent with an important role of this GATA family member in cardiogenesis. Thus, GATA-4 was found to be a potent transactivator of several cardiac-specific promoters (Grépin *et al.*, 1994; Ip *et al.*, 1994; Molkentin *et al.*, 1994); inhibition of GATA-4 expression in an *in vitro* model of cardiogenesis altered survival of precardiac cells and inhibited terminal cardiomyocyte differentiation (Grépin *et al.*, 1995, 1997). Moreover, targeted inactivation of the GATA-4 gene blocks formation of the primitive heart tube, indicating a crucial role for GATA-4 in heart development (Kuo *et al.*, 1997; Molkentin *et al.*, 1997). However, ectopic expression of GATA-4 is not sufficient to initiate cardiac differentiation or to activate the cardiac genetic program, although it markedly potentiates cardiogenesis (Jiang and Evans, 1996; Grépin *et al.*, 1997) suggesting cooperative interaction between GATA-4 and other cardiac transcription factors.

Genetic studies in *Drosophila melanogaster* have identified the gene *Tinman* as a key regulator of heart differentiation. In *tin* embryos, flies lack the dorsal vessel, the fly structure homologous to the heart, as a result of defects in late mesoderm specification (Bodmer *et al.*, 1990; Azpiazu and Frasch, 1993; Bodmer, 1993). Presumptive homologues of *Tinman* have been cloned in vertebrates (Nkx2-3, Nkx2-5 and Nkx2-7) and are expressed in the myocardium (reviewed in Harvey, 1996; see also Lints *et al.*, 1993; Tonissen *et al.*, 1994; Evans *et al.*, 1995; Schultheiss *et al.*, 1995; Buchberger *et al.*, 1996; Chen and Fishman, 1996; Lee *et al.*, 1996). Targeted disruption of the Nkx2-5 gene in mice leads to embryonic death due to cardiac morphogenetic defects (Lyons *et al.*, 1995). However, gain-of-function studies in zebrafish *Danio rerio* and *Xenopus laevis* indicate that ectopic expression of Nkx2-5 results in enhanced myocyte recruitment but is not sufficient to initiate cardiac gene expression or differentiation (Chen and Fishman, 1996; Cleaver *et al.*, 1996), suggesting that Nkx2-5 acts in concert with other transcription factors to specify the cardiac phenotype.

Since the cardiac-specific atrial natriuretic factor (ANF) promoter is a transcriptional target for both GATA-4 and Nkx2-5 (Grépin *et al.*, 1994; Durocher *et al.*, 1996), we used it to investigate functional cooperation between Nkx2-5 and GATA-4 in the heart. We present data showing that Nkx2-5 and GATA-4 specifically cooperate in activating ANF and other cardiac promoters, and physically interact both *in vitro* and *in vivo*. This molecular interaction provides the interesting possibility that instead of being part of the same epistatic group, the two pathways collaborate in the early events of cardiogenesis.

Results

GATA-4 and Nkx2-5 synergistically activate cardiac transcription

Recent studies from our laboratory have demonstrated that two cardiac-specific transcriptional pathways converge on the ANF promoter (Grépin *et al.*, 1994; Durocher *et al.*, 1996), ANF being the major secretory product of embryonic and postnatal cardiomyocytes. The region of the ANF promoter which is essential for high basal cardiac activity (Argentin *et al.*, 1994) harbors a GATA element located at −120 bp in the rat promoter which binds with high affinity all the members of the cardiac GATA subfamily (F.Charron *et al.*, manuscript in preparation), and the NKE which binds Nkx2-5 and is required for ANF promoter and enhancer function (Durocher *et al.*, 1996). As seen in Figure 1A, the nucleotide composition of these elements as well as their phasing are conserved across species, suggesting an evolutionary pressure to maintain important regulatory pathways. This led us to investigate whether GATA proteins and Nkx2-5 could functionally interact at the level of the ANF promoter. We tested this hypothesis by co-transfecting GATA-4 and Nkx2-5 expression vectors in non-cardiac cells (HeLa cells) at limiting DNA concentrations (Grépin *et al.*, 1994; Durocher *et al.*, 1996) in order to avoid squelching. Under these conditions, GATA-4 and Nkx2-5 were able to activate synergistically the ANF promoter but not control promoters lacking GATA and NKE sites such as TK109 (Figure 1B). This cooperative response was not caused by transactivation of the CMV promoter which drives Nkx2-5 and GATA-4 expression since the co-expression of both vectors does not alter the level of either Nkx2-5 or GATA-4 protein (see Figure 6B).

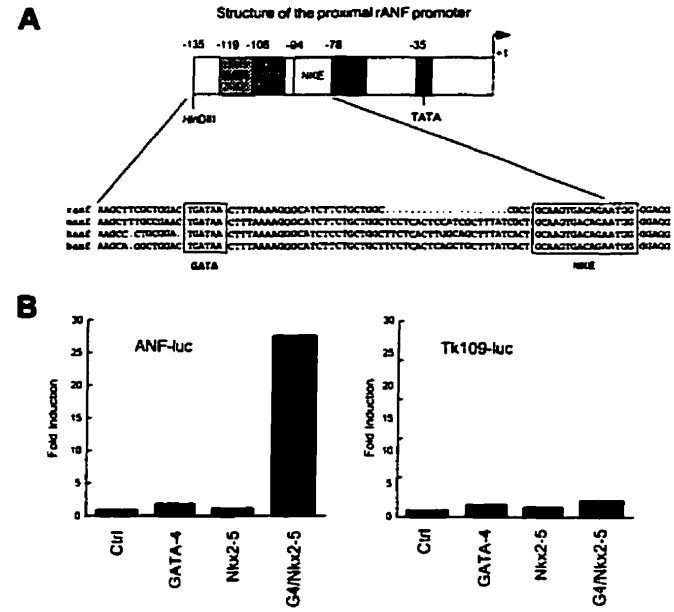

Fig. 1. Nkx2-5 and GATA-4 can cooperate transcriptionally. (**A**) Structural organization of the proximal ANF promoter. Regulatory elements of the ANF promoter are boxed, and their location relative to the transcription start are indicated. The PERE corresponds to the phenylephrine response element (Ardati and Nemer, 1993), the NKE to the NK2 response element (Durocher *et al.*, 1996). The Nkx2-5 binding site and the GATA elements of the ANF promoter are conserved among species (rat, human, bovine and mouse promoters) and their spacing is conserved (20 bp, two turns of the DNA double helix). (**B**) GATA-4 and Nkx2-5 synergistically activate the ANF promoter. HeLa cells were transiently co-transfected as described in Materials and methods using CMV-driven expression vectors for GATA-4 and Nkx2-5 or the backbone vector as control (pCGN) in conjunction with either ANF-luciferase or Tk109-luciferase reporters. The results, expressed as fold induction of reporter constructs, are from one representative experiment (out of at least four) and represent the mean of a duplicate.

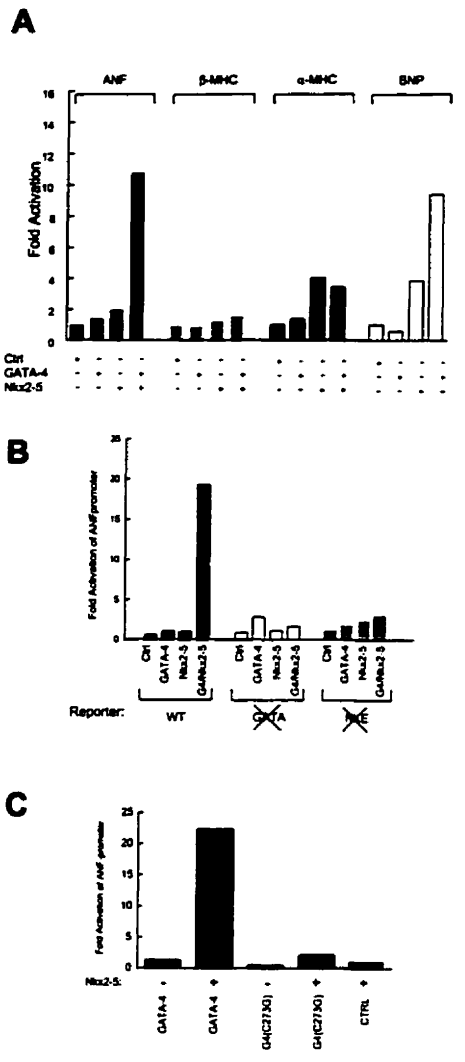

Fig. 2. A subset of cardiac promoters are synergistically activated by the Nkx2-5–GATA-4 combination. (**A**) HeLa cells were transiently co-transfected as described in Figure 1 using various cardiac promoters linked to the luciferase reporter. ANF represents the rat ANF –135 construct; β-MHC, the rat –667 bp promoter; α-MHC, the rat –613 bp promoter whereas BNP represents the rat –2 kbp promoter. (**B**) The synergy between GATA-4 and Nkx2-5 requires both binding sites in the context of the ANF promoter. Transient co-transfections in HeLa cells were carried out as described in the previous figures and the promoter described represents either the –135 bp promoter (WT), the Δ–106/–135 bp promoter which removes the GATA element and the Δ–57/–106 bp promoter which removes the NKE site. (**C**) GATA-4 binding to DNA is required for synergy. A point mutant of GATA-4 (C273G) which does not bind DNA *in vitro* and which cannot activate GATA-dependent promoters was used in a co-transfection assay with or without Nkx2-5.

The relevance of this synergy to cardiac transcription was further assessed by co-transfecting Nkx2-5 and GATA-4 with other cardiac promoters including ANF, β-MHC, α-MHC and the B-type natriuretic peptide (BNP) reporters. Under the conditions used in Figure 1, a subset of promoters that contain both NKE and GATA elements could be synergistically activated by the combination of Nkx2-5 and GATA-4 (Figure 2A). Thus, BNP promoter which is a GATA-4 target (Argentin *et al.*, 1994) responds synergistically to Nkx2-5 and GATA-4. Interestingly, sequences with high homology to the NKE are present around –385 and –437 bp and are conserved across species; promoter fragments lacking these putative NKEs are no longer responsive to Nkx2-5/GATA synergy (data not shown). On the other hand, neither additive nor synergistic effects were observed on the α-MHC and the β-MHC promoters in response to Nkx2-5 and GATA-4 at all different DNA concentrations tested (Figure 2A and data not shown). These data suggest that only a subgroup of cardiac genes are targeted by both transcription factors and that both NKE and GATA sites are required for synergy. This hypothesis was further tested using ANF promoter mutants deleted of either the GATA or the NKE elements. As shown in Figure 2B, there appears to be an absolute requirement for both elements to achieve synergy. The same results were obtained using BNP promoters containing only GATA sites or heterologous promoters with multimerized GATA elements (data not shown). This suggests that, in natural promoters, both proteins have to be recruited at the promoter or require a conformational change induced upon DNA-binding. Indeed, a GATA-4 mutant that no longer binds DNA because one of the zinc-coordinating cysteines in the carboxy-terminal zinc finger was mutated, no longer supports Nkx2-5 synergy (Figure 2C).

Since multiple GATA and homeobox proteins are expressed in the heart, we investigated the specificity of the synergy. In co-transfection assays using the proximal −135 bp ANF promoter as reporter, we found that Nkx2-5 was able cooperatively to activate transcription of the ANF reporter only with GATA-4 and GATA-5 (Figure 3A). No synergy was observed with either GATA-1 or GATA-6. Since GATA-6 is as potent as GATA-4 in transactivating the ANF promoter (our unpublished data), the results suggest that transcriptional cooperativity between Nkx2-5 and GATA proteins requires specific molecular/structural determinants on the GATA-4 and -5 proteins. The same approach was used to identify homeoproteins that could cooperate with GATA-4, including other NK2 proteins (TTF-1/Nkx2-1; Guazzi *et al.*, 1990; Lints *et al.*, 1993), Hox proteins (HoxB3), Pou proteins (Oct1; Sturm *et al.*, 1988) or *bicoid*-related homeoboxes (Ptx1; Lamonerie *et al.*, 1996). Transcriptional cooperativity was observed with the members of the *Antp* superfamily Nkx2-5, TTF-1 and HoxB3, but not with Oct1 and Ptx1 (Figure 3B). These results suggest that the *Antp*-type homeodomain plays an important role in the specificity of the synergy.

We then used deletion mutants of both Nkx2-5 and GATA-4 to map the domains involved in synergy over the ANF promoter. All mutant proteins were tested for expression level and nuclear localization (data not shown). The analyses revealed that, in addition to the DNA-binding domain (Figure 2C), two GATA-4 domains are required for the synergy, one located within the N-terminal 119 amino acids of the protein, and the second in the C-terminal 121 amino acids (Figure 4). Both domains contain GATA-4 activation domains although the presence of either domain is sufficient for transactivation of GATA-dependent promoters in heterologous cells (Figure 4B). The observation that both domains are required for cooperative interaction with Nkx2-5, suggests that synergistic interaction between these two domains may be required for Nkx2-5-induced transcriptional activation of the ANF promoter or that each domain fulfills a distinct function. Mutational analysis

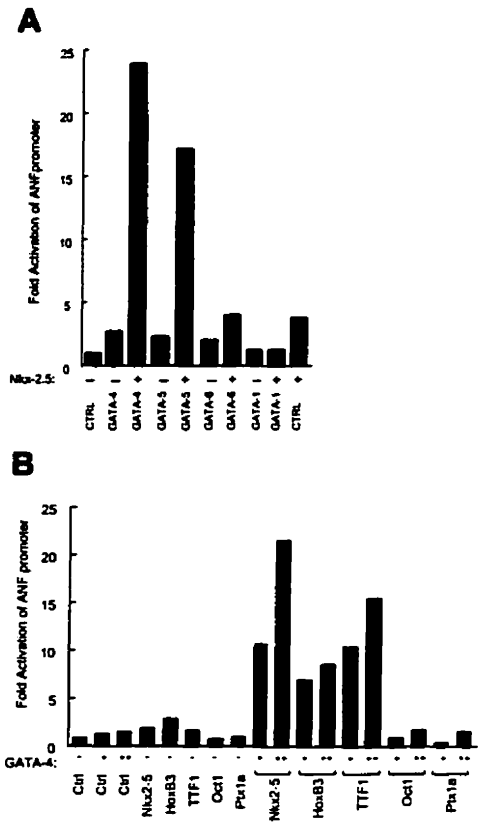

Fig. 3. The synergy is specific for a subset of cardiac GATA proteins and *Antp*-type homeoproteins. (**A**) Co-transfection assays in HeLa cells using various GATA expression vectors were done in presence (+) or absence (–) of the Nkx2-5 expression vector. Ctrl represents the backbone vector for most of the GATA constructs (pCGN). (**B**) Co-transfection assays in HeLa cells were performed in the presence of various homeodomain protein expression vectors in absence (–) or in presence (+) of GATA-4 (+, 0.1 μg; ++, 0.25 μg).

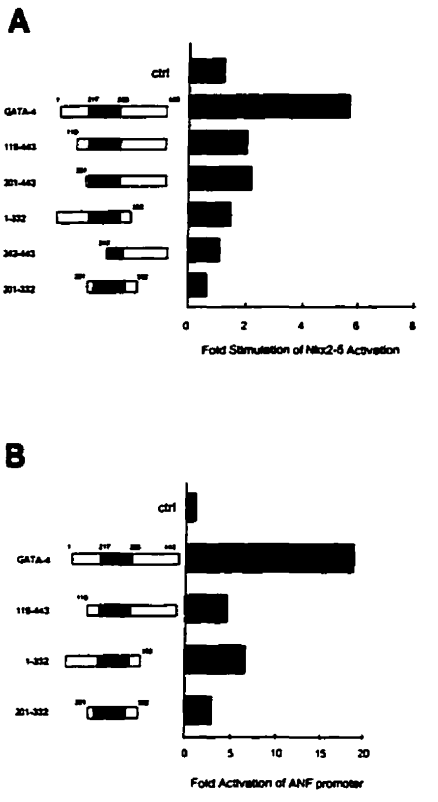

Fig. 4. The synergy requires both activation domains of GATA-4. (**A**) GATA-4 vectors (50 ng/35 mm dish) expressing truncated GATA-4 proteins able to translocate to the nucleus were used in co-transfection assays with or without the Nkx2-5 expression vector. ctrl represents the backbone vector. The results are expressed as fold stimulation of Nkx2-5 activation (equivalent to fold synergy where the value of 1 represents no synergy, i.e. the ratio between the activity of the reporter in the presence of the GATA deletion mutant plus Nkx2-5 over the activity of the reporter only in the presence of the GATA deletion mutant). (**B**) GATA-4 activation domains are located both at the C- and N-termini. GATA-4 vectors were transfected in HeLa cells at the dose of 0.2 μg/dish with the ANF –135 bp luciferase reporter.

of Nkx2-5 showed that, while the homeodomain is critical for Nkx–GATA synergy, domains outside the homeobox, particularly sequences C-terminus of the homeodomain, are also important (Figure 5A). Thus, neither the homeodomain (122–203), nor in fusion with the N-terminal regions of Nkx2-5 (1–203) is able to stimulate GATA-4 activity. Deletion of the entire C-terminal region totally impairs the ability of Nkx2-5 to stimulate GATA-4 transcription while partial deletions of the C-terminus (1–246 and Δ204–246) reduce consistently the extent of synergy observed without completely abolishing it. This result suggests that these two regions of the C-terminus are only partially redundant or that the functional interaction between Nkx2-5 and GATA-4 requires an 'extended' homeodomain in the C-terminus. The C-terminus is not known as a transcriptional activation domain, in fact; it appears to be an autorepression domain since its deletion leads to superactivation (Figure 5B). Thus, the requirement for the C-terminus suggests that GATA-4 physically interacts with Nkx2-5 to cause a conformational change and derepress (or unmask) Nkx2-5 activation domains.

GATA-4 and Nkx2-5 physically interact in vitro and in vivo

We first assessed possible physical interaction between Nkx2-5 and GATA-4 using pull-down assays with immobilized MBP–Nkx2-5 and *in vitro*-translated, ^{35}S-labeled GATA-4 (Figure 6A). MBP–Nkx2-5 was able to retain GATA-4 specifically while a MBP–LacZ control fusion could not retain GATA-4 on the column and the immobilized Nkx2-5 could not interact with labeled control luciferase (Figure 6A). The interaction between GATA-4 and Nkx2-5 was also observed in the presence of ethidium bromide, suggesting that this interaction occurs without DNA bridging (data not shown). In order to ascertain the *in vivo* relevance of this interaction, co-immunoprecipitations were performed on nuclear extracts from 293 cells transfected with expression vectors for wild-type GATA-4 or HA-tagged Nkx2-5 alone or in combination. Nuclear extracts from these transfected cells were then incubated with the monoclonal antibody 12CA5 which recognizes the HA epitope. Immunocomplexes were separated on SDS–PAGE, subjected to Western blotting and visualized with the anti-GATA-4 antibody. As seen in Figure 6B, GATA-4 protein was precipitated by the 12CA5 mAb solely when both proteins were expressed, implying either a direct or indirect contact with Nkx2-5.

Next, deletion mutants of GATA-4 were generated in order to map the region(s) of GATA-4 protein involved in physical interaction with Nkx2-5. Figure 7A displays

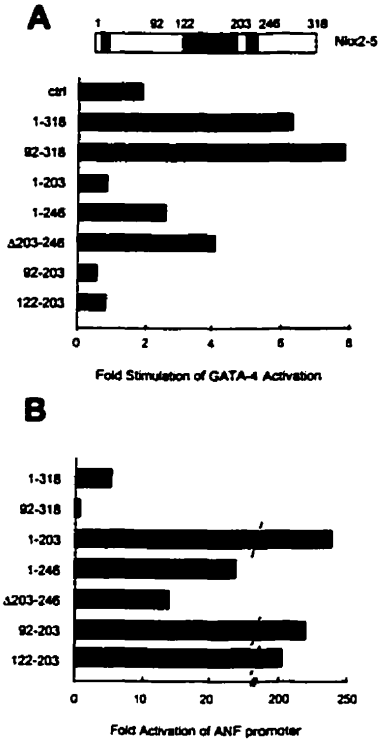

Fig. 5. The synergy requires the C-terminus of Nkx2-5.
(**A**) CMV-driven vectors, expressing various deletions of Nkx2-5, were used in co-transfection assays as described in Figure 4A, where Nkx2-5 concentration was kept at 0.5 μg/35 mm dish. ctrl represents the backbone vector without insert. The data are expressed as fold stimulation of GATA-4 activation which is calculated by the ratio of the reporter activation when GATA-4 and Nkx2-5 expression vectors are present over the reporter activation when GATA-4 alone is present. (**B**) The C-terminus domain of Nkx2-5 is an auto-inhibitory domain that masks an activation domain located N-terminal of the homeodomain. Co-transfections in HeLa cells were carried out with an optimal dose of pCGN–Nkx2-5 constructs (2 μg/dish) on the ANF −135 bp promoter. The results depict the mean of six independent experiments.

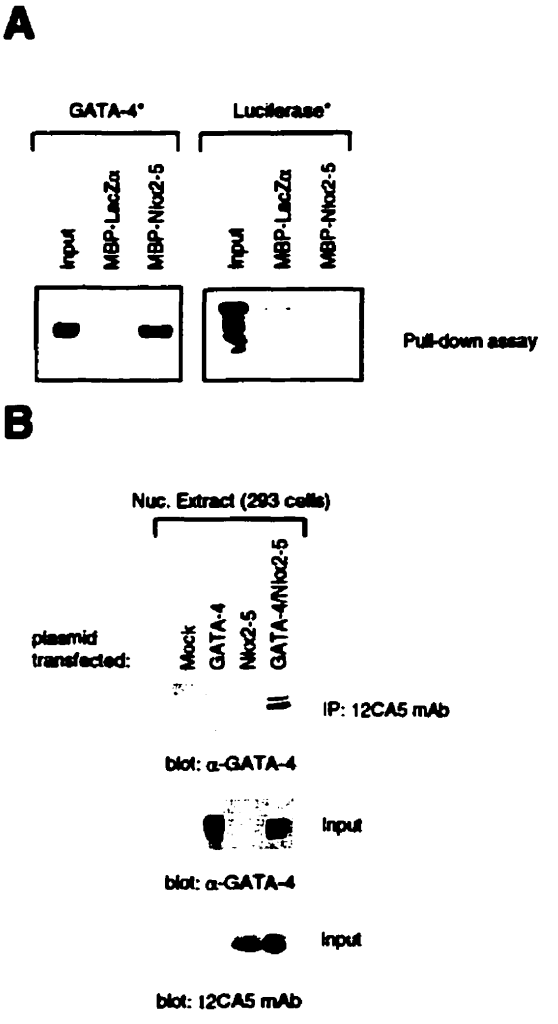

Fig. 6. GATA-4 and Nkx2-5 physically interact *in vitro* and *in vivo*.
(**A**) GATA-4 and Nkx2-5 interact *in vitro*. Pull-down protein–protein binding assays were performed using immobilized, bacterially produced MBP fusions (MBP–Nkx2-5 and MBP–LacZ as control) and either ^{35}S-labeled GATA-4 or luciferase protein. After incubation, the protein complexes were spun, extensively washed and separated on a 10% SDS–PAGE. Labeled proteins were visualized and quantified by autoradiography on phosphor plates. (**B**) GATA-4 and Nkx2-5 interact *in vivo*. Nuclear extracts from the simian kidney cell line 293 transfected with either the backbone vectors (mock), GATA-4 expression vector alone (GATA-4), HA-tagged Nkx2-5 (Nkx2-5), or a combination of GATA-4 and HA–Nkx2-5 (GATA-4/Nkx2-5) were used for immunoprecipitation. 60 μg of nuclear extract were incubated with the mAb 12CA5 and precipitated with protein-G–agarose. The top panel represents the immunocomplex separated by electrophoresis and blotted with an anti-GATA-4 polyclonal antibody. The bottom two panels represent Western blots on the transfected nuclear extracts (20 μg) using either the anti-GATA-4 Ab (middle panel) or the anti-HA (12CA5) mAb. The white ghost bands are produced by the immuglobulin heavy chains that co-migrate with GATA-4 on SDS–PAGE.

the results of the binding studies and the left panel of Figure 8A shows the structure of the deletion mutants and summarizes their behavior in pull-down and transfection assays. The Nkx2-5 interaction domain seems to map to the second zinc-finger and a C-terminal basic region that is not part of any known activation domain of GATA-4. This localization is consistent with the observation that the physical interaction requires zinc ions, since pull-down assays in the presence of chelating agents (EDTA and phenanthroline) abolish the interaction (data not shown). Unfortunately the requirement of the Nkx2-5 binding domain for the synergy could not be assessed since it is part of a basic region essential for the nuclear targeting of GATA-4 (F.Charron *et al.*, unpublished results). Interestingly, neither GATA-1 nor GATA-6, which do not transcriptionally synergize with Nkx2-5, could be retained on the MBP–Nkx2-5 column, suggesting that physical interaction is required for functional cooperativity.

The same approach was also used to map the GATA-4 interaction domain on Nkx2-5. A series of Nkx2-5 deletion mutants were bacterially produced in fusion with MBP, quantified and assayed for their ability to interact with ^{35}S-labeled GATA-4. The results of these binding assays revealed that both the homeodomain and its C-terminal region are required for physical interaction (Figures 7B and 8A). The homeodomain by itself (122–203) or extended to contain the N-terminal domain (1–203) were insufficient for physical interaction. However, when the homeodomain was fused to parts of the C-terminal (1–246 and Δ204–246) the fusion proteins regained the ability to bind GATA-4, suggesting that the C-terminal extension provided an essential docking site for the GATA protein or was required for the proper folding of the homeodomain.

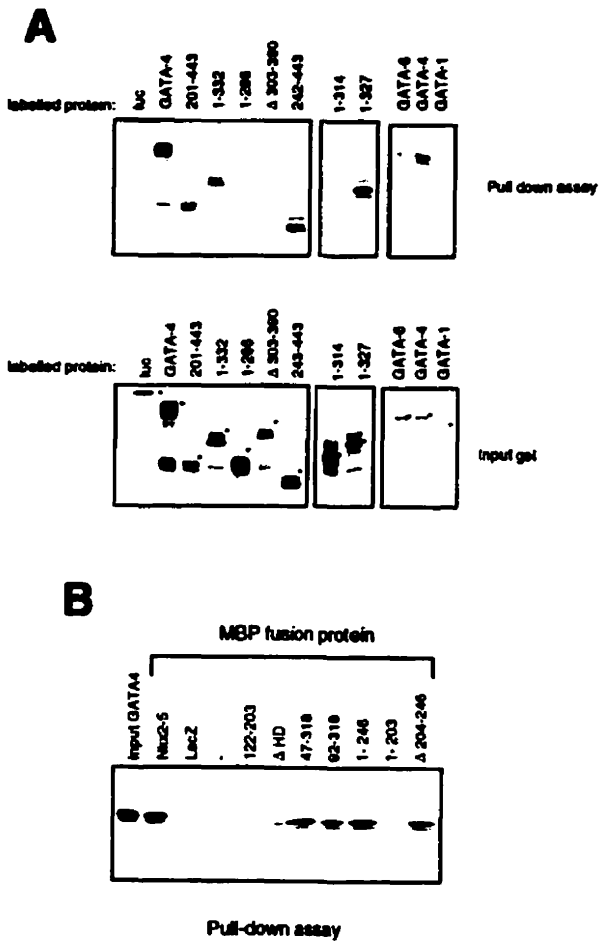

Fig. 7. The physical interaction maps near the C-terminal zinc-finger of GATA-4 and to the C-terminally extended homeodomain of Nkx2-5. (**A**) Luciferase (luc) or deletion mutants of GATA-4 were translated and labeled with [^{35}S]methionine to be subsequently used in pull-down assays with full-length MBP–Nkx2-5 as described in Figure 6. (**B**) A series of immobilized deletion mutants of Nkx2-5, in fusion with the maltose binding protein (MBP), were produced in bacteria, quantified on gel, and used in pull-down assays with *in vitro* translated GATA-4. ΔHD represents Nkx2-5 Δ122–203. Protein complexes were separated by electrophoresis and GATA-4 protein was visualized by autoradiography on phosphor plates.

It is noteworthy that these results are in complete agreement with the transfection data and indicate that the determinants of Nkx2-5 and GATA-4 interaction reside mostly in the homeodomain and a C-terminal extension. Collectively, the results also suggest that functional synergy between Nkx2-5 and GATA-4 requires physical interactions of the two proteins.

Discussion

Transcription factors GATA-4 and Nkx2-5 are two of the earliest markers of precardiac cells and, as evidenced by gene inactivation studies (Lyons *et al.*, 1995; Kuo *et al.*, 1997; Molkentin *et al.*, 1997), both play critical roles in cardiogenesis. The data presented here show that GATA-4 and Nkx2-5 interact physically and synergistically to activate cardiac transcription, suggesting functional convergence of two critical cardiac pathways.

Modulation of Nkx2-5 activity by GATA-4

Members of the GATA family of transcription factors (GATA-1, -2 and -3) have been shown to interact with other classes of nuclear proteins containing Lim domain (Osada *et al.*, 1995), zinc finger (Merika and Orkin, 1995; Gregory *et al.*, 1996), and basic leucine zipper (Walters and Martin, 1992; Gong and Dean, 1993; Kawana *et al.*, 1995) motifs. The present work demonstrates that GATA factors are also able to interact with homeodomain-containing proteins of the NK2 and *Antp* type. This GATA–Nkx interaction is so far the first example of zinc finger–homeodomain interaction in vertebrates. The only other known zinc finger–homeodomain cooperation is in *Drosophila*, where it was recently shown that the orphan nuclear receptor αFtz-F1 is a cofactor for the homeodomain protein Ftz (Guichet *et al.*, 1997; Yu *et al.*, 1997); in this case, the physical association between αFtz-F1 and Ftz is thought to enhance the binding of the Ftz to its lower-affinity target sequences (Guichet *et al.*, 1997; Yu *et al.*, 1997), much in the same way that Extradenticle and Pbx modulate the DNA binding activity of Hox proteins (Phelan *et al.*, 1995; Lu and Kamps, 1996; Peltenburg and Murre, 1997). The interaction of GATA-4 with Nkx2-5 does not appear to result in cooperative DNA binding since neither protein appears to alter the affinity or sequence specificity of the other; moreover, the presence of both GATA and NKE sites does not enhance either GATA-4 or Nkx2-5 binding to their sites, as evidenced by gel shift assays using nuclear extracts containing both proteins or each one separate (data not shown). Instead, the data suggest that GATA-4 interaction with Nkx2-5 serves to unmask the activation domains of Nkx2-5 as illustrated in Figure 8B; this would be reminiscent of the Extradenticle-induced conformational change, that switches Hox proteins from repressors to activators (Chan *et al.*, 1996; Peltenburg and Murre, 1997).

The region of GATA-4 that contacts Nkx2-5 spans the second zinc finger and a ~40 amino acid C-terminal extension (Figure 8A, left panel). This represents a highly conserved segment among the cardiac GATA-4, -5 and -6 proteins with an overall 85–95% homology; notable differences between GATA-4 and -6 (but not GATA-4 and -5) that may account for the differential interaction with Nkx2-5 are found in the hinge region (aa 243–270) preceding the second zinc finger and three non-conservative changes that affect phosphorylatable residues (H244S, N250S, S262P). The differential interaction of GATA proteins with Nkx2-5 reveals for the first time differences between GATA proteins in an *in vitro* assay.

Is Nkx2-5 a specificity cofactor for GATA-4?

Two GATA proteins, GATA-4 and -6, are present in the myocardium and both are potent activators of cardiac transcription. However, inactivation of the GATA-4 gene arrests cardiac development at a very early stage, despite marked up-regulation of GATA-6 arguing for specificity of GATA-4 and -6 function (Kuo *et al.*, 1997; Molkentin *et al.*, 1997). The up-regulation of GATA-6 might, at least partially, account for ANF expression in presumed cardiogenic cells of the GATA-4$^{-/-}$ embryos (Molkentin *et al.*, 1997), much like up-regulation of GATA-2 in GATA-1$^{-/-}$ pre-erythroblasts might explain globin gene expression in the absence of GATA-1. However, it should

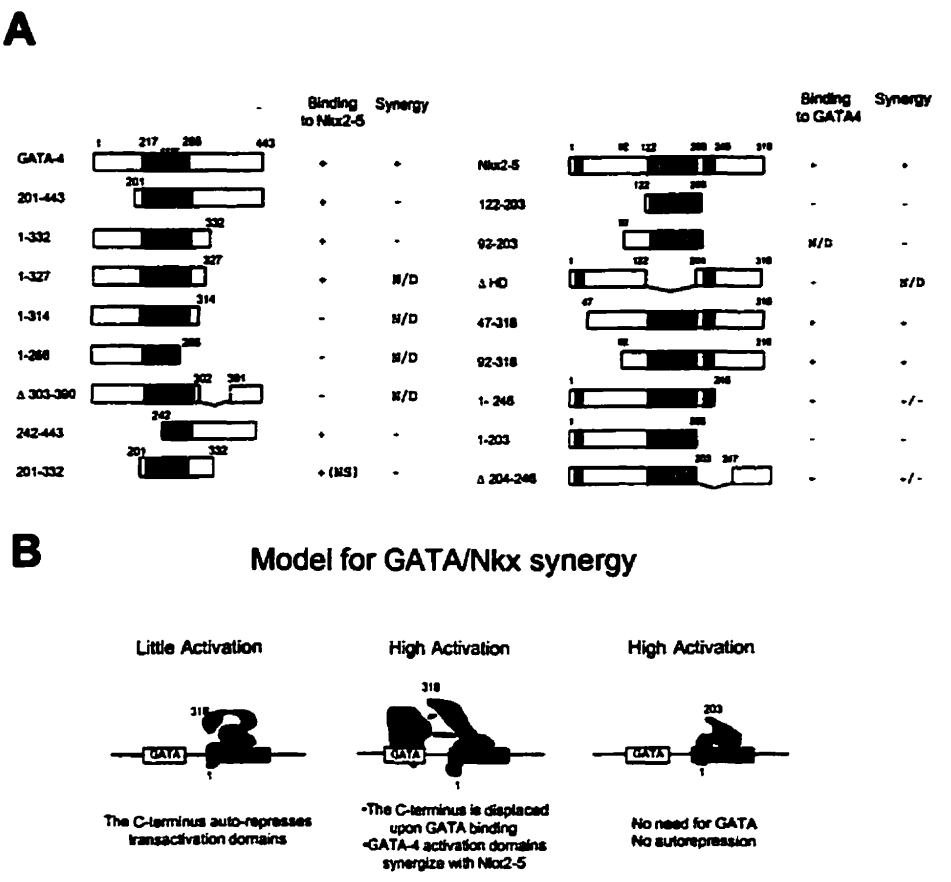

Fig. 8. (A) Schematic summary of the Nkx2-5–GATA-4 interaction. The left panel represents the activity of GATA-4 deletions both in transfection experiments and in pull-down assays. N/D represents constructs that were not tested. Constructs deleting the 266–332 region cannot be used in co-transfections since they do not translocate into the nucleus. The right panel summarizes the activities of Nkx2-5 deletion mutants in co-transfections with GATA-4 or in pull-down assays. The asterisks (*) on the GATA-4 molecule represents the amino acids that are conserved between GATA-4 and -5 but not in GATA-6, they are: H244S, N250D, L261V, S262P where the second amino acid represents the residue present on GATA-6 at the equivalent position. (B) Model of Nkx/GATA synergy. Synergistic interactions between Nkx2-5 and GATA-4 require the binding of GATA-4 and Nkx2-5 to their cognate binding sites. GATA-4 displaces the C-terminal auto-inhibitory region of Nkx2-5 and liberates the Nkx2-5 activation domain. The GATA-4 activation domains can then synergize with Nkx2-5 activation domains.

be pointed out that ANF transcription is controlled by multiple pathways in complex spatiotemporal manner (Argentin *et al.*, 1994; Durocher *et al.*, 1996); thus, the presence of ANF transcripts in GATA-4$^{-/-}$ cells may reflect activation or maintenance of more complex compensatory pathways; moreover, GATA-5—which can also cooperate with Nkx2-5 in the heart—does not seem to be restricted to the endocardial cells before the primitive heart tube stage (Morrisey *et al.*, 1997). The exact reason for which GATA-4 is obviously essential for primitive heart development must await further biochemical and genetic studies. Nevertheless, the available evidence clearly indicates that GATA-6 is unable to substitute fully for GATA-4 with respect to cardiogenesis. Similarly, despite their seemingly interchangeable role in some *in vitro* assays (Blobel *et al.*, 1995; Visvader *et al.*, 1995), the hematopoietic members of the GATA family are clearly non-redundant (Tsai *et al.*, 1994; Pandolfi *et al.*, 1995; Fujiwara *et al.*, 1996; Ting *et al.*, 1996). Unfortunately, the molecular basis for GATA factor specificity has yet to be unraveled. The data presented suggest that interaction of GATA proteins with other tissue-restricted transcription factors might be the underlying mechanism for functional specificity of the GATA family members. Thus, Nkx2-5 may be the specificity cofactor for GATA-4 while other homeodomain proteins of the NK2 or *antennapedia* class may fulfill a similar function for GATA-6 in the myocardium.

The presence of a cofactor for GATA proteins is likely the case for the hematopoietic system. Indeed, in a recent publication, Weiss *et al.* demonstrated that the presence of the GATA-1 zinc fingers was essential for erythroid differentiation. Interestingly, the homologous region of GATA-3 (which is not co-expressed with GATA-1) but not the entire GATA-2 (which is up-regulated in GATA-1$^{-/-}$ pre-erythroblasts) could functionally substitute for GATA-1 zinc fingers, suggesting that interaction of zinc fingers with an as yet unidentified nuclear factor may be an important determinant for definitive erythropoiesis (Weiss *et al.*, 1997). Since GATA proteins and other members of the NK2 family are also co-expressed in other tissues such as spleen (GATA-5 and Nkx2-5) and gut (GATA-5, -6 and Xbap) (Lints *et al.*, 1993; Morrisey *et al.*, 1996, 1997; Newman *et al.*, 1997), it is tempting to speculate whether the GATA–Nkx partnership may represent a paradigm for transcription factor interaction during cell fate determination.

It is noteworthy that, at least in cardiac muscle, such paradigm appears to have been evolutionarily conserved. Indeed, in *Drosophila*, the cardiac promoter of the transcription factor D-mef which is a target for Tinman,

Table I. Evolutionary conservation of the GATA–NK2 interaction on muscle promoters

Gene	Species	Promoter sequence	GATA	NK2	Reference
ANF	vertebrates	**TGATAA**CTT (N_{20}) CGCC**GCAAGTG**	GATA-4	Nkx2-5	this study
Myo-2	*C.elegans*	**TAAAGTGG**TTGTGT**GGATAA**	elt-2 (?)	Ceh-22	Okkema and Fire (1994)
D-Mef	*Drosophila*	**GGATAA**GGGGC**TCAAGTGG** **CACTTGA**GACCGGGGCTCGC**TATCG**	pannier (?)	Tinman	Gajewski *et al.* (1997)

Conserved GATA or NKE motifs are depicted in bold letters.

contains juxtaposed GATA and NKE sites (Table I); while the NKEs are necessary, they are not sufficient for cardiac expression, thus raising the possibility of an interaction with other factors (Gajewski *et al.*, 1997). Moreover, in *Caenorhabditis elegans*, two members of the GATA family have been described (elt-1 and -2; Spieth *et al.*, 1991; Hawkins and McGhee, 1995) whose expression is found in gut and perhaps pharyngeal muscles, and GATA elements are necessary for tissue-specific transcription in those tissues (Okkema and Fire, 1994; Egan *et al.*, 1995). Moreover, at least one member of the NK2 family, CEH-22, is also expressed in *C.elegans* pharyngeal muscle and has been implicated in activation of the muscle-specific myosin heavy chain (Myo-2) enhancer (Okkema and Fire, 1994). Interestingly, the Myo-2 enhancer requires the closely linked GATA and NKE sites (Table I) for muscle expression (Okkema and Fire, 1994). Thus, at least in muscle cells the GATA and Nkx interactions appear to have been evolutionarily conserved.

Materials and methods

Cell cultures and transfections

HeLa and 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Transfections were carried out using calcium phosphate precipitation 24 h after plating. At 36 h post-transfection, cells were harvested and luciferase activity was assayed with an LKB luminometer and the data were recorded automatically. In all experiments, RSV-hGH was used as internal control and the amount of reporter was kept at 3 μg per dish; the total amount of DNA was kept constant (usually 8 μg). Unless otherwise stated, the results reported were obtained from at least four independent experiments with at least two different DNA preparations for each plasmid. Primary cardiocyte cultures were prepared from 1- or 4-day-old Sprague-Dawley rats and kept in serum-free medium as described previously (Argentin *et al.*, 1994).

Plasmids

ANF-luciferase promoter constructs were cloned in the PXP-2 vector as described previously (Argentin *et al.*, 1994; Durocher *et al.*, 1996). The construction of the various pCG–GATA-4 vectors was based on the original rat GATA-4 cDNA described by Grépin *et al.* (1994). The position of the mutation/deletion is indicated on the figures. All constructs were sequenced and functionally tested for nuclear translocation and DNA-binding activity following transfection in L cells as previously described (Grépin *et al.*, 1994). pRSET–GATA-4 derivatives for *in vitro* translation were constructed by insertion of the *XbaI–BamHI* fragment of the corresponding pCG–GATA-4 construct into the *NheI–BamHI* sites or *NheI–BglII* sites of pRSETA (Invitrogen Corp.). MBP–Nkx2-5 (Δ203–246) was obtained by the insertion of an oligonucleotide corresponding to aa 198–203 in the *PflMI–SacII* sites of Nkx2-5. The *SphI–SacII* fragment corresponding to the deletion was then transferred in MBP–Nkx2-5. The other MBP–Nkx2-5 deletions were described by Chen and Schwartz (1995).

Recombinant protein production

After transformation of BL21(DE3) *Escherichia coli* strain with the MBP fusion vectors derived from pMalc-2 (New England Biolabs), individual colonies were picked and grown in 50 ml 2XYT up to an OD of 0.6 at 600 nm. Induction of the recombinant proteins and their purification were carried out as previously described (Durocher *et al.*, 1996). *In vitro* translation of GATA-4 and Nkx2-5 derivatives were performed with rabbit reticulocyte lysates using the TNT-coupled *in vitro* transcription/translation system (Promega Corp., Madison, WI)

Protein–protein binding assays

In vitro binding studies were performed with MBP–Nkx2-5 derivatives purified from bacteria and coupled to amylose–Sepharose beads (New England Biolabs). GATA-4 derivatives were labeled with [^{35}S]methionine during *in vitro* translation and typically 2–8 μl of labeled GATA proteins were incubated in the presence of 300 ng of immobilized Nkx2-5 fusion proteins in 400 μl of 1× binding buffer (150 mM NaCl, 50 mM Tris–Cl, pH 7.5, 0.3% Nonidet P-40, 10 mM $ZnCl_2$, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.25% BSA) for 2 h at 4°C with agitation and then centrifuged for 2 min at 13 000 r.p.m. at room temperature. Beads were washed three times by vortexing in 500 ml of binding buffer at room temperature, the protein complexes were released after boiling in Laemmli buffer and resolved by SDS–PAGE. Labeled proteins were visualized and quantified by autoradiography on phosphor storage plates (PhosphorImager, Molecular Dynamics).

Immunoprecipitations and immunoblots

Immunoprecipitations on nuclear extracts of transfected 293 cells were done using 60 μg of nuclear extract. Extracts were pre-cleared with 20 μl of normal rabbit serum and 15 μl of agarose–protein G beads (Sigma Chemicals) for 2 h at 4°C. Binding reactions were carried out with 40 μl of 12CA5 antibody in 500 μl of 1× binding buffer without BSA as described in the protein–protein binding assays paragraph for 2 h at 4°C, with agitation without protein G beads and for an additional 2 h with 15 μl of protein G beads. Bound immunocomplexes were washed four times in 1× binding buffer and were resuspended in 20 μl of 1× Laemmli buffer, boiled and subjected to SDS–PAGE electrophoresis. Proteins were transferred on Hybond-PVDF membrane and subjected to immunoblotting. GATA-4 antibody (Santa-Cruz Biotechnolgy) was used at a dilution of 1/1000 and was revealed with biotinylated anti-goat antibody (dilution 1/12 000) and avidin-biotinylated horseradish peroxidase (HRP) complex (Vectastain). The 12CA5 (anti-Ha) antibody was used at a dilution of 1/500 and was a generous gift of Benoit Grondin and Muriel Aubry (Grondin *et al.*, 1996). The secondary antibody was anti-mouse-HRP and the antigens were visualized with chemiluminescence (Kodak).

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GATA transcription factors and cardiac development

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Three members of the GATA family of transcription factors, GATA-4, -5, and -6, are expressed in the developing heart. One family member, GATA-5, is restricted to the endocardium while the other two, GATA-4 and -6, are present in the myocardium where they apparently fulfil distinct functions. The mechanisms underlying GATA factor specificity are not fully understood but may involve interaction with other tissue-restricted or ubiquitous co-factors. Thus, combinatorial interaction among GATA factors or between GATA factors and other co-factors may differentially control various stages of cardiogenesis.

Key words: embryonic development / GATA factors / heart / transcription / ventricular hypertrophy

Introduction

The GATA family of transcription factors

MEMBERS OF THE GATA family of transcription factors are zinc finger proteins that have been shown to play critical non-redundant roles in cell growth and differentiation. The founding member of this family, GATA-1, is largely restricted to the hematopoietic lineage, where it was initially shown to be a key activator of globin genes by binding to conserved GATA sites in the promoters and locus control region (reviewed in ref 1). Two other GATA factors, GATA-2 and -3, were subsequently identified and shown to be also restricted to hematopoietic cells.[2] GATA-2 expression is initially required for the proliferation of the hematopoietic precursors and its overexpression in culture leads to proliferation at the expense of differentiation.[3] GATA-1 expression characterizes more differentiated states since transcripts are found in mature erythroid cells and megakaryocytes. In hematopoietic cells, GATA-3 expression is restricted to T lymphocytes and is required for T cell differentiation.[4] These GATA factors are also found later in development in non-hematopoietic cells, namely gonads for GATA-1 and brain and kidney for GATA-3. Targeted disruption of GATA-1, -2, and -3 has confirmed the critical function of each of these factors in hematopoiesis.[4–7] Thus, although GATA factors may be co-expressed in specific cells, they apparently play distinct, non-redundant roles during development.

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Roles of GATA factors in embryonic heart development

Spatial and temporal expression of GATA-4, -5, and -6

Analysis of the cardiac regulatory elements of the B-type natriuretic peptide promoter (BNP) led to the characterization of tissue-specific regulatory elements containing GATA motifs and cloning of an additional member of the GATA family, GATA-4, whose expression is mainly restricted to the heart and gonads.[8,9] GATA-4 can be detected in the precardiac mesoderm as early as 7.0–7.5 dpc at the late primitive streak stage, where it precedes the expression of the earliest cardiac differentiation markers, such as contractile protein genes and natriuretic peptide genes. In the mesoderm, GATA-4 expression is confined to the cardiogenic crescents, on each side of the embryo; GATA-4 transcripts are also detected in the visceral endoderm. This expression pattern is very similar to that of Nkx2-5 and coincides with the heart-forming region in mouse, chicken, and *Xenopus*.[10–12] At later stages, GATA-4 transcripts are detected throughout the myocardium and endocardium where they are present at high level in the postnatal heart.[8,12] Thus,

GATA-4 is an early marker of the cardiac cells where it may regulate various stages of cardiogenesis.

In *Xenopus* and chicken, two additional GATA factors, GATA-5 and -6, were isolated by low stringency hybridization to the GATA-1 DNA binding domain and they were shown to be expressed in a broad ventral area that includes the heart progenitors and later predominantly in the heart and gut.[10,11] Rat and mouse homologues of these factors were also characterized.[13–15] GATA-4, -5, and -6 are homologous in their amino acid sequence, particularly in the DNA binding domain (90% homology), and form a distinct subclass of GATA factors. The proteins are more divergent outside of the zinc finger region, with 45% homology in the N-terminal domain and 35% homology in the C-terminal domain. Consistent with a conserved specialized function for GATA factors, the amino acid sequence of each protein is highly conserved among species, as shown in Figure 1. Within the heart, the three GATA factors are differentially regulated throughout development with GATA-4 being the predominant transcript in cardiomyocytes at all stages. GATA-6 is also expressed in the precardiac mesoderm at the late primitive streak stage and is later found in myocardial cells and also in the vascular smooth muscles.[11,15,16] GATA-5 transcripts are largely restricted to endocardial cells.[17] These data are summarized in Table 1.

Functional analysis of GATA-4, -5, and -6

In vitro, transfection studies in non-cardiac cells established that GATA-4 is a potent transactivator of numerous cardiac promoters including atrial natriuretic factor (ANF),[18] BNP,[8] cardiac troponin C (cTnC),[19] cardiac troponin I (cTnI),[20,21] m2 muscarinic acetylcholine receptor,[22] and slow myosin heavy chain 3 (slow MyHC 3).[23] Moreover, structure-function analysis revealed the presence of two transcriptional activation domains located in the N- and C-terminal regions of GATA-4.[18,24]

The role of GATA proteins in cardiogenesis has been assessed through gene inactivation and loss- or gain-of-function studies. The first evidence for a role of GATA-4 in heart differentiation came from our *in vitro* studies where GATA-4 expression was knocked-down by an antisense strategy in the pluripotent P19 embryonal carcinoma cells which provide a cellular model of inducible cardiac differentiation. In GATA-4$^-$ lines, terminal cardiac differentiation could not be achieved and massive apoptosis of precardiac cells was observed, suggesting that GATA-4 is a mediator of survival, proliferation, and/or differentiation signals.[25,26] Subsequent inactivation of the GATA-4 gene in transgenic mice confirmed that GATA-4 is essential for normal heart development.[27,28] Mice lacking GATA-4 fail to develop a linear heart tube and die *in utero* by day 9. The *cardia bifida* phenotype may be due to absence of ventral closure of the embryo resulting in the absence of morphogenetic movements required for the fusion of the bilateral primordia. Nevertheless, differentiated cardiomyocytes are observed in the GATA-4$^{-/-}$ mice, suggesting that the function of GATA-4 in the heart might be compensated by other proteins and/or signals in the developing mouse. One candidate for this compensation is GATA-6 whose levels are up-regulated in GATA-4$^{-/-}$ mice.[27,28] The exact mechanism underlying the role of GATA-4 during early cardiac development remains presently unclear. Studies with chimeric mice injected with GATA-4$^{-/-}$ ES cells suggest that the cardiac defect is not cell autonomous.[27,29] In fact, both the *in vivo* and *in vitro* studies suggest that GATA-4 is required for proliferation and/or migration of cardiac cells or for early mesoderm-endoderm interactions. These possibilities are not exclusive as GATA-4 may be involved at more than one developmental stage.

Complementary gain-of-function studies revealed another function for GATA-4 in cardiogenesis. Experiments carried out in P19 cells, where GATA-4

Table 1. Spatial and temporal expression of GATA transcription factors in the embryonic and postnatal heart

	Cardiac progenitors	Embryonic heart		Postnatal heart	
		Myocardium	Endocardium	Myocardium	Endocardium
GATA-4	+	+	+	++	+
GATA-5	+*	+/−	++	−	++
GATA-6	+*	+*	+/−*	+	−

This table is based on results from refs 8,10–13,15–17,19 and from our unpublished results.
*Indicates that the expression was not confirmed at the protein level.

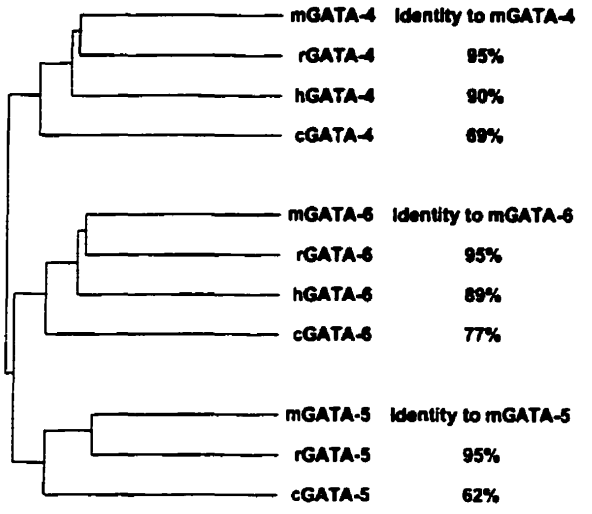

Figure 1. GATA-4, -5, and -6 are highly conserved across species, consistent with a conserved specialized function for these factors. Amino acid identities are shown in percentage relative to the mouse protein. m-, r-, h-, and c-GATA refer to mouse, rat, human, and chicken GATA factors, respectively.

was stably over-expressed, revealed that GATA-4 markedly potentiates cardiogenesis as evidenced by the earlier appearance and persistence of beating cardiac cells.[26] These results suggest that GATA-4 can potentiate cardiogenesis by recruiting more cells to the cardiogenic field. A recent study in zebrafish suggests that early expression of GATA-4 may even serve to maintain precursor cells in a cardiac competent stage.[30]

The role of the other GATA factors in the developing heart is not clear yet. Axis disruption experiments in *Xenopus* showed intimate association between GATA-4, -5, and -6 expression and specification of cardiac progenitors.[11] Moreover, ectopic expression of GATA-4, -5, or -6 was shown to activate transcription of the cardiac α-actin (c. α-actin) and α-myosin heavy chain (α-MHC) genes. Other gain-of-function studies in *Xenopus* revealed that GATA-6 could also be a regulator of the cardiogenic field.[16] Injection of GATA-6 mRNA in gastrulating embryos resulted in a transient block of cardiac differentiation and enhanced proliferation of cardioblasts; after the decay of the injected GATA-6 mRNA, cardiomyocytes resumed differentiation to generate an enlarged heart. This is reminiscent of the proliferative effect of GATA-2 in hemopoietic progenitors[3] and suggests that GATA-6 might regulate proliferation of cardiac progenitor cells. Collectively, these genetic manipulations are consistent with critical roles for GATA factors in the developing heart.

Combinatorial interaction between GATA-4 and Nkx2-5

As stated above, studies in P19 cells indicated that ectopic expression of GATA-4 potentiates cardiogenesis only in 'permissive' cellular environments, suggesting that GATA-4 action requires a co-factor. The expression pattern of GATA-4 overlaps with that of the homeodomain transcription factor Nkx2-5 in the heart-forming region in various species (reviewed in ref 31). Nkx2-5 is one of the mammalian homologs of *tinman*, a gene required for heart development in drosophila.[32,33] Nkx2-5 is also essential for normal heart development as targeted disruption of its gene in mice leads to embryonic death due to cardiac morphogenetic defects.[34] Interestingly, gain-of-function studies in zebrafish and *Xenopus* indicate that ectopic expression of Nkx2-5 results in enhanced myocyte recruitment but is not sufficient to initiate cardiac gene expression or differentiation,[35,36] suggesting that Nkx2-5 acts in concert with other transcription factors to specify the cardiac phenotype. The fact that GATA-4 and Nkx2-5, two of the earliest markers of precardiac cells, are essential for heart formation and that over-expression of either alone cannot initiate cardiogenesis yet enhances recruitment and/or differentiation of committed precursors raised the possibility that these proteins may be mutual co-factors. Because ANF was the only shown transcriptional target for both GATA-4 and Nkx2-5,[8,37] it provided a useful tool to investigate potential functional cooperation between GATA-4 and Nkx2-5. Indeed, we showed that at the level of the ANF promoter, GATA-4 and Nkx2-5 are mutual co-factors as co-expression of GATA-4 and Nkx2-5 resulted in synergistic activation of the ANF promoter in heterologous cells.[18] The synergy, which requires the DNA binding site for both factors, involves physical interaction between Nkx2-5 and GATA-4 as evidenced *in vitro* and *in vivo*. This interaction maps to the carboxy-terminal zinc finger of GATA-4 and a C-terminus extension; similarly, a C-terminally extended homeodomain of Nkx2-5 is required for GATA-4 binding. The structure/function study suggests that binding of GATA-4 to the C-terminus autorepressive domain of Nkx2-5 may induce a conformational change that unmasks Nkx2-5 activation domains. Remarkably, the other myocardial GATA factor, GATA-6, is unable to substitute for GATA-4 in

binding or functional interaction with Nkx2-5. Thus, the molecular interaction, which appears to have been evolutionarily conserved between specific members of the GATA zinc finger family and Nkx2-5, may impart functional specificity to GATA factors and provide cooperative cross-talk between two pathways that are critical for the early events of cardiogenesis. Functional and physical interactions between GATA-4 and Nkx2-5 were also observed on the ANF and c. α-actin promoters by other groups.[38,39]

The GATA-4 / Nkx2-5 interaction: a mediator of BMP signalling?

Since GATA-4 and Nkx2-5 are the earliest markers of the myocardial cell fate, the identification of the upstream regulators of GATA-4 and Nkx2-5 in the precardiac mesoderm should give important insights on the nature of the inducers of the cardiac fate. In *Drosophila*, genetic studies have established that the *tinman* (*tin*) gene, the homologue of Nkx2-5, is directly downstream of the ectodermal *decapentaplegic* (*dpp*) signal.[40,41] This signal is required for cardiac cell formation since null mutations in *dpp* result in an absence of cardiac differentiation. *Dpp* is part of the growing family of BMP/TGF-β-related molecules. Its sequence is most related to that of the vertebrate BMP-2 and BMP-4 proteins and the *dpp* signal transduction pathway seems to be conserved from *Drosophila* to mammals since its receptors (Type I and Type II), its inhibitors (such as *short-of-gastrulation* in flies; noggin or chordin in vertebrates) and its intracellular effectors (such as the Mad proteins) are conserved in mammals (reviewed in ref 42).

Interestingly, molecular conservation of the *dpp/tin* cascade was elegantly demonstrated in chicken since recombinant BMP-2, -4, and -7 can induce Nkx2-5 in anterior lateral mesoderm.[43] Moreover, GATA-4 expression is also robustly induced in anterior mesoderm explants treated with BMP-2 and -4 implying that GATA-4 is also a downstream target of the BMPs. However, when explants are treated with BMP-7, GATA-4 induction is not observed and this correlates with the lesser potency of BMP-7 to induce the expression of terminal cardiac phenotypic markers, such as ventricular myosin heavy chain (vMHC) and the formation of beating explants.[43] These observations are consistent with the gain-of-function studies which demonstrated that neither GATA-4 nor Nkx2-5 could alone initiate cardiogenesis although either protein could potentiate it in committed cells.[26,35,36] The physical and functional interaction between Nkx2-5 and GATA-4 may provide an explanation for the requirement of both proteins in BMP-mediated cardiogenesis. This functional interaction, which potentiates the transcriptional activities of both proteins, would be especially important at low concentrations of GATA-4 and Nkx2-5, a situation that likely occurs in the early moments of cardiac cell fate induction.

Roles of GATA factors in postnatal heart development

Heterotypic interactions between GATA-4 and GATA-6 and the maintenance of cardiac gene expression

Two lines of evidence suggest that GATA-4 may play a critical role in postnatal cardiac transcription: GATA elements were found to be essential for activation of some cardiac promoters in adult myocardium[44-47] and GATA-4 was shown to functionally and physically interact with NF-AT3, which would implicate it as a mediator of the calcineurin-dependent hypertrophic process in the myocardium.[48] Unfortunately, because mice lacking GATA-4 die prior to formation of the primitive heart tube, they are not useful for assessing the role of GATA-4 in postnatal heart development. An alternate model that blocks GATA-4 expression later in development or in specific cells or regions of the heart had to be developed. We have developed an adenovirus-mediated antisense strategy to specifically inhibit GATA-4 or GATA-6 protein production in cardiomyocytes and used this approach to assess the role of these factors in postnatal cardiomyocytes.[49] The results indicate that several endogenous cardiac genes, including ANF, BNP, cTnI, α-MHC, β-myosin heavy chain (β-MHC), and platelet-derived growth factor receptor β (PDGFRβ), are down-regulated in cardiomyocytes lacking either GATA-4 or GATA-6, suggesting that these genes are *bona fide* targets for both GATA-4 and GATA-6. Interestingly, the α- and β-MHC genes appear to be preferential targets for GATA-4. As determined by binding affinity, this is likely due to the higher affinity of GATA-4 for their promoter GATA element and suggests that selectivity at the level of DNA binding may be one mechanism of target gene specificity for GATA factors. This approach also identified for the first time downstream targets for GATA-4 and -6 in the myocardium and revealed that a subset of genes is targeted by both GATA-4 and GATA-6. Remarkably, removal of both GATA proteins had the same effect as removing

either one by itself suggesting that GATA-4 and -6 might be part of the same active transcription complex.[49] Indeed, experiments using the ANF and BNP promoters revealed that GATA-4 and GATA-6 form a heterotypic complex that binds a single GATA element and synergistically activates transcription of cardiac promoters. To confirm the *in vivo* relevance of this mechanism, antibodies specific for the different GATA factors were developed and used for immunohistochemical localization of GATA-4 and -6 proteins in the heart. The data clearly showed that GATA-4 and GATA-6 co-localize in postnatal cardiomyocytes and could therefore potentially interact with each other. Taken together, these results suggest that GATA-4 and GATA-6 can act in concert to regulate expression of a subset of cardiac genes.

A role for GATA factors in hypertrophic development?

Soon after birth, cardiomyocytes lose their ability to proliferate and respond to growth stimulation by increasing their size but not their number, a process known as cardiac hypertrophy. Over the last year, a few groups have accumulated various lines of evidence consistent with a role for GATA factors in cardiac hypertrophy. Using direct injection of DNA into the myocardium, two groups have found that GATA elements present on the promoters of the angiotensin type 1A receptor ($AT1_AR$) and the β-MHC are required for activation of these promoters in response to pressure overload.[46,47] Using a similar approach in the nephrectomized rat, a model of volume overload, we have found that regulation of

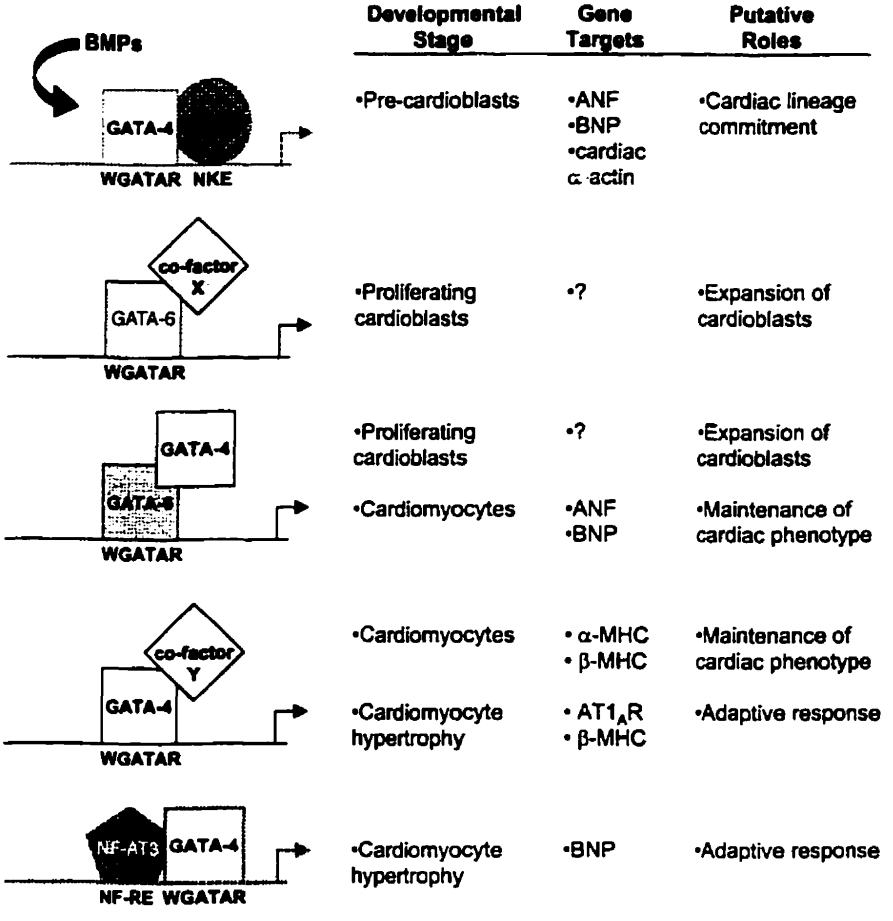

Figure 2. Combinatorial interactions involving GATA-4 or -6 during embryonic and postnatal heart development. Interactions involve the cooperation among GATA factors or with other co-factors, such as Nkx2-5, NF-AT3, or other putative co-factors (co-factor X or Y). WGATAR, NKE, and NF-RE are GATA, Nkx2-5, and NF-AT3 binding sites, respectively.

the BNP promoter in response to hemodynamic stress is also mediated by GATA elements.[50] GATA-4 was also found to functionally and physically interact with transcription factor NF-AT3 that mediates calcineurin-dependent cardiac hypertrophy.[48] Finally, our analysis of genetic and experimental models of hypertrophy, the spontaneously hypertensive rats (SHR) and the one kidney one clip rat model, revealed increased GATA-4 transcripts in association with ventricular hypertrophy (unpublished data). Thus, GATA-4 may play a role in the genetic reprogramming of the hypertrophied heart either through increased expression of the protein or through recruitment of other co-factors (such as NF-AT3).

Conclusions

The establishment and maintenance of the cardiac phenotype require the activation of cardiac-specific genes in a tightly regulated temporal and spatial manner. This process is likely governed by the combinatorial action of cell-restricted as well as ubiquitous transcriptional regulators. Of the few known cardiac-restricted and/or enriched transcription factors, GATA-4 and -6 are receiving increasing attention. Both *in vitro* and *in vivo* studies clearly demonstrated the essential roles of these factors for embryonic and postnatal heart development, although the molecular mechanisms underlying these roles are just beginning to be elucidated. One mechanism by which specificity is achieved likely involves the preferential affinity of certain GATA factors for a subset of GATA elements, as shown for α- and β-MHC gene regulation. Other mechanisms involve the cooperative interaction of GATA factors among themselves, such as observed between GATA-4 and GATA-6 in order to maintain expression of a subset of cardiac genes, and their cooperation with other cardiac-restricted transcription factors, such as observed between GATA-4 and Nkx2-5 in order to possibly mediate BMP signalling. Moreover, recruitment by GATA proteins of various inducible co-factors (such as NF-AT3) may be critical for tissue-specific regulation in response to extracellular stimuli (summarized in Figure 2).

Finally, and in addition to their crucial roles in the embryonic heart and in the maintenance of cardiac gene expression in the postnatal heart, various lines of evidence point to a role of GATA-4 and GATA-6 in the reprogramming of gene expression in the hypertrophic heart and possibly in other adaptive responses of the postnatal myocardium.

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Interactions entre les facteurs MEF2 et GATA dans la différenciation cellulaire

Les facteurs de transcription à boîte MADS (MCM1, *Agamous, Deficiens* et *Serum response factor* – SRF) constituent une famille de protéines incluant les facteurs de transcription métazoaires SRF et MEF2 *(myocyte enhancer factor-2)* qui jouent un rôle clé dans le contrôle de divers processus biologiques. Chez les vertébrés, le facteur de transcription SRF contrôle l'expression des gènes précoces et de gènes exprimés spécifiquement dans les muscles. Pour leur part, les facteurs MEF2 ont été impliqués dans la régulation de l'expression des gènes musculaires, la différenciation des cellules du muscle squelettique, l'induction de l'apoptose dans les lymphocytes T et la survie des neurones.

La boîte MADS est un motif structurellement très conservé de 56 acides aminés présent dans le domaine de liaison à l'ADN des membres de cette famille [1], qui possèdent également des spécificités de liaison à l'ADN très similaires. La capacité de ces protéines de former des dimères corrèle avec la symétrie en dyade de leurs sites de liaison à l'ADN.

Les facteurs de transcription de la famille MEF2

Les membres de la famille de facteurs de transcription MEF2 ont été initialement identifiés par leur activité de liaison aux sites d'ADN spécifiques des muscles, activité qui est observée majoritairement dans les cellules musculaires différenciées (*pour revue voir* [2]). Ces facteurs MEF2 jouent un rôle important dans l'activation transcriptionnelle de plusieurs gènes des muscles cardiaque et squelettiques. Chez les vertébrés, il existe quatre gènes *mef2: mef2a, mef2b, mef2c* et *mef2d*. L'organisation des introns et des exons des gènes *mef2* des vertébrés et de l'unique gène *mef2* de la drosophile *(D-mef2)* est identique dans les régions conservées de ces gènes (boîte MADS et domaine MEF2 ; *figure 1*), suggérant qu'ils proviennent d'un gène *mef2* ancestral commun. En revanche, leur domaine carboxy-terminal est non seulement très divergent, mais il subit aussi un épissage alternatif. Il est fort probable que les mécanismes qu'utilisent ces facteurs pour activer la transcription soient eux aussi divergents car c'est ce domaine carboxy-terminal qui est responsable de la transactivation.

Les protéines MEF2 peuvent former entre elles des homo- et des hétérodimères, mais elles ne peuvent pas former d'hétérodimères avec d'autres facteurs à boîte MADS; cette capacité de dimérisation des protéines MEF2 requiert un domaine de 29 acides aminés situé directement en carboxy-terminal de la boîte MADS et appelé le domaine MEF2 *(figure 1)*. Il contribue également à assurer une affinité de liaison maximale à l'ADN. Ensemble, la boîte MADS et le domaine MEF2 sont nécessaires et suffisants pour permettre une liaison de haute affinité à la séquence consensus (T/C)TA $(A/T)_4$TA(G/A). Des sites de liaison pour les protéines MEF2 ont été identifiés dans les promoteurs de plusieurs gènes des muscles cardiaque et squelettiques (*pour revue voir* [2]).

Expression des facteurs MEF2 au cours du développement

Durant l'embryogenèse des vertébrés, les transcrits *mef2* sont abondamment exprimés dans les cellules musculaires cardiaques, squelettiques et lisses ainsi que dans d'autres types cellulaires, incluant les neurones et les lymphocytes. Chez la souris, les gènes *mef2b* et *mef2c* sont les premiers membres de la famille exprimés, leurs transcrits apparaissant dès le jour embryonaire 7,5 (E7,5) au niveau des précurseurs mésodermiques qui formeront le cœur [2]. Peu après, au jour E8,0, les transcrits des gènes *mef2a* et *mef2d* sont exprimés dans l'ébauche du myocarde. Puis l'expression des quatre gènes *mef2* est aussi détectée dans les cellules musculaires squelettiques et lisses de façon concomitante à l'activation de leur programme de différenciation. Plus spécifiquement, l'expression des gènes *mef2* débute dans le mésoderme somitique pour ensuite gagner le myotome.

Les membres de la famille MEF2 sont également exprimés dans les lymphocytes B [3] et dans le système nerveux central en développement [4]. Temporellement, l'expression des protéines MEF2 dans les neurones est corrélée avec l'activation de la différenciation de ces cellules.

Chez l'adulte, en dépit de l'expression ubiquitaire des ARNm de *mef2*, les protéines MEF2 et leur activité de liaison à l'ADN sont très enrichies dans les muscles, les lymphocytes et le cerveau. Cette disparité entre l'expression des transcrits et des protéines suggère l'existence de mécanismes post-transcriptionnels impliqués dans la régulation de l'expression de ces facteurs.

Analyses génétiques du rôle des facteurs MEF2

Plusieurs évidences génétiques suggèrent que les protéines MEF2 contribuent à l'expression des gènes mus-

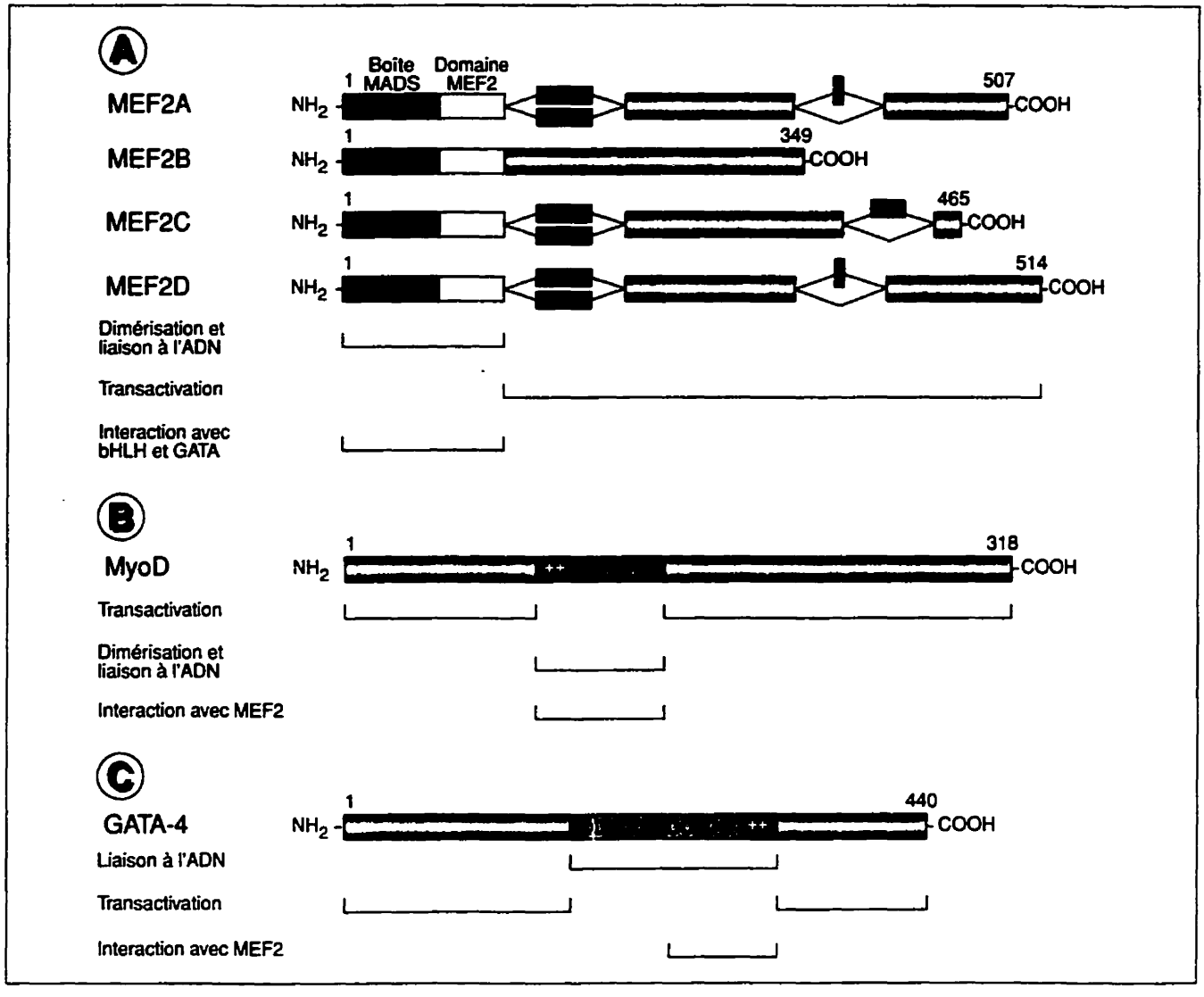

Figure 1. ***Représentation schématique des membres de la famille MEF2, MyoD et GATA. A.*** *Les quatre membres de la famille MEF2: MEF2A, MEF2B, MEF2C et MEF2D sont représentés avec, pour chacun, les épissages alternatifs possibles. Le domaine de dimérisation et de liaison à l'ADN comprend la boîte MADS et le domaine MEF2. La longueur des protéines ainsi que les domaines d'activation de la transcription et d'interaction avec les facteurs bHLH myogéniques et GATA sont également indiqués.* ***B.*** *Représentation schématique de MyoD. Le domaine HLH et la région basique (++), qui permettent la liaison à l'ADN, sont indiqués.* ***C.*** *Représentation de GATA-4. Les doigts de zinc (CC CC) et la région basique (++) sont requis pour la liaison à l'ADN. La structure des autres facteurs GATA est similaire à celle de GATA-4.*

culaires et à la myogenèse. Chez la drosophile, la mutation de *D-mef2* inhibe la différenciation terminale des cellules musculaires squelettiques, cardiaques et viscérales [2]. Cependant, les précurseurs myogéniques de chacune de ces lignées semblent spécifiés normalement. Ces résultats suggèrent un rôle relativement tardif de *D-mef2* dans la différenciation de tous les types musculaires chez la drosophile.

Chez les vertébrés, la fonction des gènes *mef2* commence seulement à être élucidée, notamment grâce aux études d'inactivation de gène chez la souris. Le premier gène *mef2* à avoir été inactivé est *mef2c* (*mef2c*$^{-/-}$) [5]. Les embryons *mef2c*$^{-/-}$ paraissent normaux jusqu'au jour E9,0, où l'on observe un ralentissement généralisé de leur développement ainsi qu'un épanchement péricardique, témoin d'une insuffisance cardiaque. Le tube cardiaque reste droit sans subir la courbure permettant la morphogenèse cardiaque et le futur ventricule droit ne se forme pas. Dans ces embryons mutants, plusieurs transcrits cardiaques, comme ceux de l'ANF *(atrial natriuretic factor)*, l'αMHC *(α-myosin heavy chain)* et l'α-actine cardiaque, ne sont pas exprimés, tandis que d'autres, comme MLC2V *(myosin light chain)* et MLC2A, sont exprimés normalement [5]. Ces résultats confir-

ment donc le rôle essentiel de MEF2 dans la différenciation terminale des cardiomyocytes.
Outre ces défauts de développement du muscle cardiaque, les souris *mef2c*$^{-/-}$ présentent également des défauts vasculaires [6, 7] : les cellules musculaires lisses ne se différencient pas et l'organisation des cellules endothéliales est anormale, suggérant un rôle crucial de MEF2C dans le développement vasculaire.

Interactions entre les facteurs MEF2 et les protéines de la famille bHLH

Les membres de la famille MEF2, comme les autres protéines à boîte MADS, coopèrent avec d'autres facteurs de transcription pour contrôler le programme d'expression génique propre à un tissu. Dans le muscle squelettique, il s'agit des membres de la famille des facteurs myogéniques de type hélice-boucle-hélice (bHLH), MyoD, myogénine, Myf5 et MRF4, qui contrôlent la spécification et la différenciation des cellules musculaires squelettiques ([8] et *m/s 1997, n°10, p.1182*). Ces facteurs myogéniques forment des hétérodimères avec les facteurs de transcription bHLH ubiquitaires, les protéines E, et lient une séquence spécifique appelée boîte E (CANNTG) sur l'ADN de plusieurs promoteurs de gènes spécifiques des muscles. Chacun de ces facteurs myogéniques peut activer le programme de différenciation musculaire lorsqu'il est exprimé de façon ectopique dans des cellules non musculaires. Contrairement aux facteurs myogéniques, les protéines de la famille MEF2 sont incapables d'activer le programme de différenciation musculaire. Cependant, elles peuvent coopérer avec les facteurs myogéniques pour activer de façon synergique les promoteurs des gènes musculaires squelettiques et pour induire la myogenèse [9, 10]. Plusieurs études ont démontré que cette coopération est due à une interaction physique directe entre la boîte MADS des protéines MEF2 et le domaine de liaison à l'ADN bHLH des facteurs myogéniques *(figure 1)*. De plus, le site de liaison à l'ADN d'un seul des deux facteurs est requis. Ces résultats suggèrent que les facteurs MEF2 peuvent moduler la transcription des gènes musculaires selon deux mécanismes différents: par leur liaison directe à leur site sur le promoteur, ou encore en étant recrutés par un membre de la famille myogénique lié à une boîte E sur le promoteur.
Les protéines MEF2 peuvent également interagir avec MASH1 *(mouse achaete scute homolog 1)*, une protéine bHLH de la famille *achaete scute* exprimée dans les neurones [11, 12]. Comme dans le muscle squelettique, la boîte MADS des protéines MEF2 interagit directement avec le domaine bHLH de MASH1 et cette interaction résulte en une activation synergique de la transcription. Bien qu'aucun gène neuronal n'ait été identifié comme cible de l'interaction MEF2/MASH1, ces résultats suggèrent un rôle possible des protéines MEF2 comme co-facteurs des protéines bHLH neurogéniques.

Interactions entre les facteurs MEF2 et les protéines de la famille GATA

• *Mécanismes d'action des facteurs MEF2 dans le muscle cardiaque*
L'inactivation du gène *mef2c* étant responsable d'un arrêt du développement cardiaque et d'une diminution (voire une abolition) de l'expression de nombreux gènes cardiaques comme ceux codant pour l'ANF, αMHC et αCA, on pouvait supposer que les facteurs MEF2 soient impliqués dans la régulation transcriptionnelle de ces gènes cibles. Cependant, la plupart des promoteurs de ces gènes ne contiennent pas de site de liaison, ou seulement des sites de liaison de faible affinité, pour les protéines MEF2. En outre, lorsqu'ils sont exprimés dans des cellules hétérologues, les facteurs MEF2 ne peuvent activer de façon significative ces promoteurs [13]. Ces résultats suggèrent que l'action des facteurs MEF2 dans le muscle cardiaque nécessite la présence d'un co-facteur spécifique de ce tissu.
En accord avec cette hypothèse, nous avons démontré que les facteurs MEF2 sont recrutés au niveau des promoteurs de leurs gènes cibles par le facteur de transcription spécifique du cœur GATA-4 dont ils potentialisent l'activité transcriptionnelle [13]. GATA-4 est, chez les vertébrés, un des six membres (GATA-1 à -6) connus de la famille des facteurs de transcription GATA (*pour revue voir* [14]). Ils possèdent tous un domaine de liaison à l'ADN très conservé de type doigt de zinc qui lie spécifiquement la séquence (A/T)GATA(A/G), et peuvent être divisés en deux groupes selon leur patron d'expression et leur homologie de séquence: l'un comprend GATA-1, GATA-2 et GATA-3, qui sont princi-

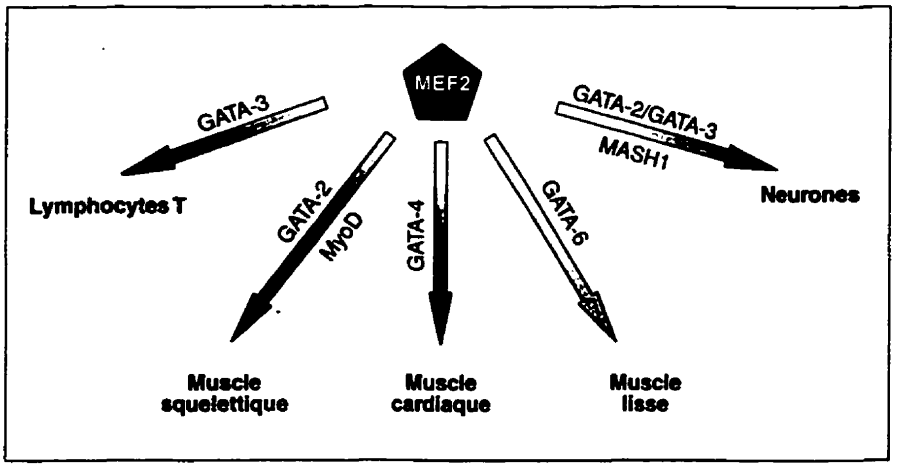

Figure 2. ***Interactions combinatoires entre les facteurs MEF2, bHLH et GATA.*** *Ces interactions, dont le rôle fonctionnel est démontré dans le muscle squelettique et cardiaque, représentent probablement un mécanisme général de contrôle spécifique de la transcription dans certains tissus comme le muscle lisse, les lymphocytes et les neurones.*

palement exprimés dans le système hématopoïétique et le système nerveux, et l'autre comprend GATA-4, GATA-5 et GATA-6, qui le sont principalement dans le système cardiovasculaire. Plus précisément, GATA-4 et GATA-6 sont tous deux exprimés dans les cardiomyocytes [15, 16] dans lesquels ils sont essentiels au maintien du phénotype cardiaque [16], GATA-4 étant en outre requis pour la différenciation et la survie des cardiomyocytes [17, 18].

En coopérant avec GATA-4 et GATA-6, les facteurs MEF2 contribuent ainsi à l'activation de nombreux promoteurs de gènes cardiaques [13]. Cette activation synergique entre les facteurs MEF2 et GATA-4 requiert à la fois une interaction physique entre le domaine de liaison à l'ADN de MEF2 et le doigt de zinc carboxy-terminal de GATA-4, et la présence des domaines d'activation de la transcription de chacun des deux facteurs *(figure 1)*. Si la liaison de GATA sur sa séquence consensus d'ADN est indispensable à cette synergie, celle des facteurs MEF2 ne l'est pas. Ces résultats suggèrent donc l'existence d'un nouveau mécanisme de régulation combinatoire impliquant le recrutement des facteurs MEF2 par les protéines GATA liées à leur site sur le promoteur. Ce mécanisme n'est pas sans rappeler le recrutement des facteurs MEF2 par les facteurs myogéniques et suggère un rôle fondamental des facteurs MEF2 en tant que co-facteurs transcriptionnels essentiels à la myogenèse et à la régulation de l'expression des gènes musculaires cardiaques et squelettiques.

• *Un paradigme pour la régulation spécifique de la transcription dans de nombreux tissus ?*

Les mécanismes moléculaires contrôlant l'expression des gènes spécifiques au muscle lisse demeurent inconnus. Cela est principalement dû au fait que peu de facteurs de transcription spécifiques de ce tissu ont été identifiés à ce jour. De façon intéressante, outre le myocarde, GATA-6 est également exprimé dans le muscle lisse et est capable d'interagir physiquement et fonctionnellement avec les protéines MEF2 [13]. Il est donc possible que le recrutement des facteurs MEF2 par GATA-6 contribue aussi à l'expression des gènes du muscle lisse *(figure 2)*.

L'interaction des facteurs GATA et MEF2 pourrait aussi jouer un rôle dans le muscle squelettique. En effet, des protéines GATA, telle GATA-2, y sont exprimées et contribuent, de concert avec les facteurs NFAT *(nuclear factors of activated T cells)*, à la réponse des cellules aux facteurs de croissance [19]. On peut envisager que les facteurs GATA participent aussi à l'action de MEF2 dans la régulation du programme d'expression génique et la différenciation du muscle squelettique.

Enfin, la co-expression des facteurs GATA et des protéines MEF2 n'est pas limitée au muscle, mais est également observée dans les lymphocytes T (MEF2/GATA-3 ; [3, 20, 21]) et les neurones (MEF2/GATA-2 et GATA-3 ; [22, 23]). Puisque les facteurs GATA contrôlent l'expression de nombreux gènes lymphoïdes et neuronaux, une implication éventuelle des facteurs MEF2 dans ces régulations est proposée.

Les facteurs MEF2 : points de convergence pour de multiples voies de signalisation

L'activité transcriptionnelle des facteurs MEF2 est modulée par trois voies de signalisation : la voie des MAPK *(mitogen-activated protein kinases)* p38 et ERK5/BMK1, la voie de la calcineurine et la voie des CaMK *(calcium/calmodulin-dependent protein kinases)*. Les MAPK p38, en phosphorylant directement les facteurs MEF2 dans leur domaine d'activation de la transcription, augmentent leur activité transcriptionnelle [24]. Inversement, la calcineurine, une phosphatase qui est activée par une augmentation de la concentration intracellulaire du calcium, peut déphosphoryler les facteurs MEF2 dans leur domaine de liaison à l'ADN, augmentant ainsi leur capacité de liaison à l'ADN et, indirectement leur activité transcriptionnelle [25]. L'élévation des niveaux de calcium intracellulaire active également les CaMK, qui sont elles-mêmes capables de stimuler l'activité transcriptionnelle des facteurs MEF2 [26]. Les mécanismes par lesquels ces voies de signalisation modulent l'activité des facteurs MEF2 demeurent largement inconnus. La phosphorylation des facteurs MEF2 par les MAPK p38 et la CaMK se situe au niveau du domaine de transactivation et n'affecte pas leur activité de liaison à l'ADN. Ces résultats suggèrent qu'un gain de charge négative et/ou une modification de la conformation de la protéine module sa capacité à interagir avec la machinerie transcriptionnelle de base ou avec un co-facteur. Dans ce contexte, il serait intéressant de déterminer si ces voies de signalisation peuvent influencer la capacité des facteurs MEF2 à interagir avec les facteurs myogéniques ou les facteurs de la famille GATA et ainsi contribuer à régler de façon fine les mécanismes combinatoires des facteurs MEF2.

Conclusions

L'ensemble de ces données montrent que les interactions combinatoires des facteurs MEF2 avec les différents facteurs GATA et à motif bHLH pourraient représenter un mécanisme général permettant de contrôler la transcription de nombreux gènes de façon spécifique dans certains tissus. Elles pourraient plus généralement être impliquées dans les processus de différenciation, d'apoptose et de survie cellulaire ■

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