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GATA Transcription Factors and Cardiac Development

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I. Introduction

The development of the mammalian heart is a complex process that involves the specification of multiple cell lineages, including endocardial cells, cardiomyocytes, and cells of the coronary vasculature, and their subsequent precisely orchestrated assembly into the mature four-chambered cardiac structure. Despite its relative complexity, the major steps of cardiac development take place over a remarkably short period of early embryonic development, beginning at approximately Embryonic Day (E) 7.5 and culminating by E11 in the mouse (Fishman and Chien, 1997; Kuo *et al.*, 1997; Olson and Srivastava, 1996). The correct assembly of a beating heart and the concomitant establishment of the fetal circulation is required for the viability of the early mouse embryo. Defects in cardiovascular morphogenesis result in embryonic lethality between E8.5 and 12 or, if less severe, in congenital heart disease.

For descriptive purposes, cardiac development can be divided into three temporally and spatially distinct stages (Kuo *et al.*, 1997). The first stage, which begins at approximately E7.0 in the mouse, involves the specification of the cardiomyocyte lineage(s) from cells of the splanchnic mesoderm (see Chapter 1). At E7.0, the for-

mation of the intraembryonic coelom splits the embryonic mesoderm into splanchnic and somatic components (Fig. 1, top). In response to signals from the underlying endoderm, a crescent-shaped portion of the splanchnic mesoderm located in the anterior and dorso-lateral region of the embryo then differentiates into cuboidal precardiac mesoderm or procardiomyocytes. These cells express contractile proteins but fail to assemble these proteins into myofibrils and therefore do not contract. The second stage of cardiac development begins at approximately E8.0 and involves the migration of the specified procardiomyocytes from the dorsal and anterior regions of the embryo to the ventral midline to form a linear heart tube (Fig. 1, middle). This procardiomyocyte migration is intimately linked to a complex series of morphogenic events that together form many of the ventral structures of the developing em-

bryo, including the anterior intestinal portal and foregut, the pericardial cavity and heart tube, and the ventral closure of the yolk sac (Fig. 1, bottom). Conceptually, this process can be viewed as involving two folding events: (i) the rostral-to-caudal folding of head and adjacent precardiac mesoderm which positions the precardiac mesoderm caudal to the head fold, and (ii) the lateral-to-ventral folding and subsequent midline fusion of the precardiac splanchnic mesoderm which positions the heart tube in the ventral midline of the developing thoracic cavity (Kuo *et al.*, 1997). By E8.5, the linear heart tube becomes lined by endocardial cells and attached to the dorsal wall of the pericardial cavity by the dorsal mesocardium. The third stage of cardiac development occurs between E8.5 and 11 as the linear heart tube undergoes looping and septation to generate the mature four-chambered cardiac structure.

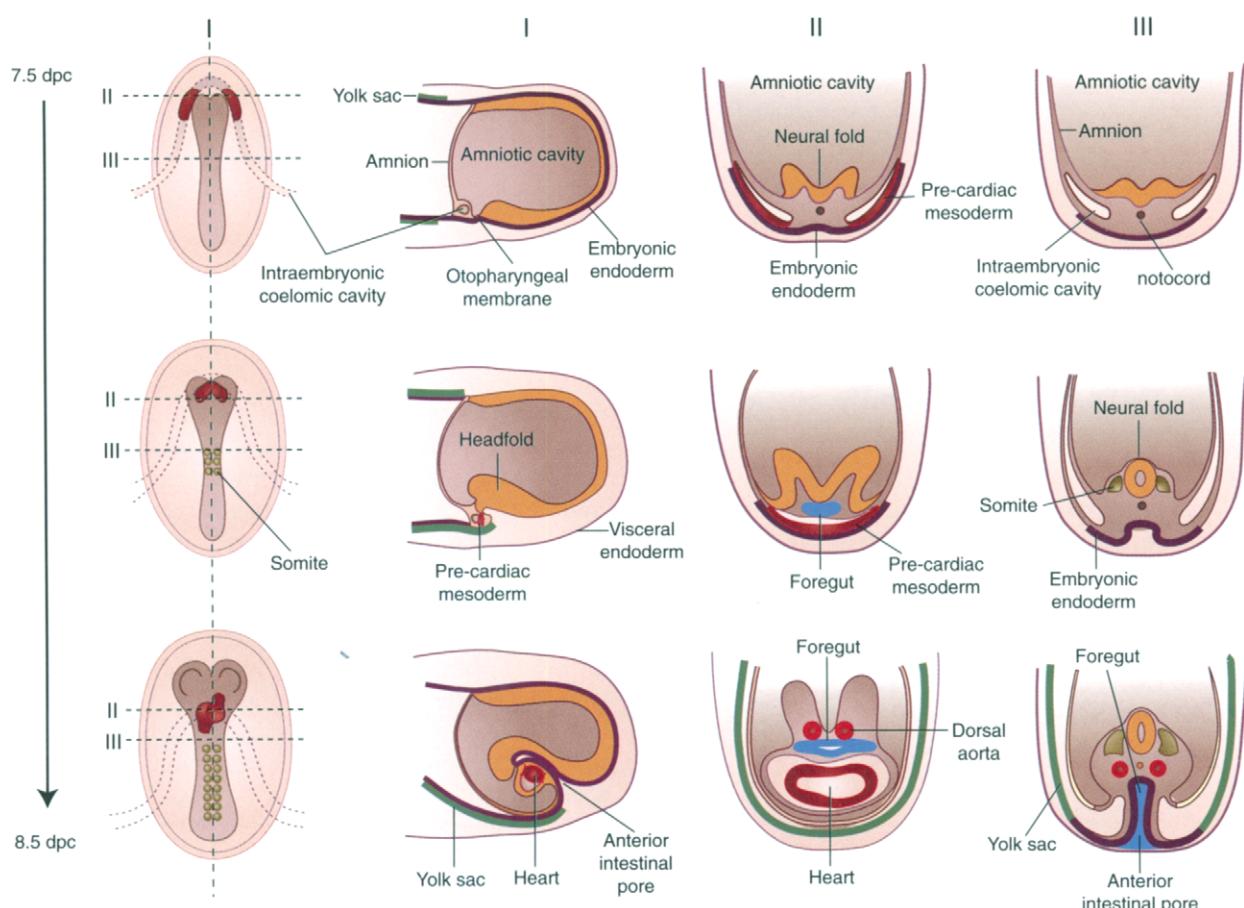


Figure 1 Schematic representation of heart tube formation during murine embryogenesis. (Left) Dorsal view of the developing mouse embryo between 7.5 (top) and 8.5 (bottom) days pc. I, midline sagittal sections of the same embryos; II, transverse sections through the same embryos at the level of the developing heart; III, transverse sections through the same embryos at the level of the developing foregut. The precardiac mesoderm and linear heart tube are shown in red and the somites are shown in solid black. The neural tube is shown by the vertical blue shading. The arrows indicate the direction of embryo folding.

By analogy with other developmental processes, it was reasonable to assume that specific sets of nuclear transcription factors regulate each stage of cardiac development. During the past 5 years, several different approaches have been used to identify such transcription factors. First, a number of groups isolated and characterized a series of cardiac-specific transcriptional regulatory elements and used these elements to identify cardiac-restricted DNA-binding proteins (Amacher *et al.*, 1993; Argentin *et al.*, 1994; Biben *et al.*, 1994, 1996; Cserjesi *et al.*, 1994; Donoviel *et al.*, 1996; Grepin *et al.*, 1994; Iannello *et al.*, 1991; Ip *et al.*, 1994; Knotts *et al.*, 1994; Larkin *et al.*, 1996; Lee *et al.*, 1994; Molkentin *et al.*, 1994, 1996; Navankasattusas *et al.*, 1992; Parmacek *et al.*, 1992; Sartorelli *et al.*, 1990; Seidman *et al.*, 1988; Subramaniam *et al.*, 1991; Wanker *et al.*, 1996; Zhu *et al.*, 1991, 1993). In a complementary approach, novel cardiac transcription factors were isolated on the basis of their sequence similarities to proteins that were known to play important roles in muscle development in mammals, reptiles, and insects (Arceci *et al.*, 1993; Bodmer, 1993; Kelley *et al.*, 1993; Komuro and Izumo, 1993; Laverriere *et al.*, 1994; Lints *et al.*, 1993; Morrisey *et al.*, 1996; Srivastava *et al.*, 1997; Tonissen *et al.*, 1994). Together, these studies have identified several families of transcription factors as potential regulators of cardiac development, including the GATA zinc finger proteins (Arceci *et al.*, 1993; Kelley *et al.*, 1993; Laverriere *et al.*, 1994; Morrisey *et al.*, 1996), the MEF2 and SRF family of MADS box proteins (Cserjesi *et al.*, 1994; Gossett *et al.*, 1989; Lee *et al.*, 1994; Pollock and Treisman, 1991; Zhu *et al.*, 1991; 1993), the bHLH proteins, dHAND and eHAND (Srivastava *et al.*, 1997), homeodomain proteins of the Nkx family (Bodmer, 1993; Komuro and Izumo, 1993; Lints *et al.*, 1993; Tonissen *et al.*, 1994), and TEA domain proteins such as TEF-1 (Azakie *et al.*, 1996; Farrance and Ordahl, 1996; Mar and Ordahl, 1990; Stewart *et al.*, 1994; Xiao *et al.*, 1991). Each of these proteins has been shown to bind to one or more cardiac-specific transcriptional elements and to be expressed in the developing mammalian heart.

An understanding of the precise role of each of these transcription factors in regulating cardiac development has only recently started to emerge from the results of gene targeting studies in flies and mice. Thus, for example, we now know that proper looping and septation of the linear heart tube to form a four-chambered heart requires Nkx2.5, dHAND, and eHAND (Biben and Harvey, 1997; see Chapters 7 and 9). However, these proteins are not required for either cardiac myocyte determination or the assembly of the linear heart tube because both processes occur normally in mice containing targeted mutations of the *Nkx2.5*, *eHAND*, and

dHAND genes. Similarly, *MEF2C* appears to be required for looping morphogenesis of the embryonic heart and/or formation of the right ventricle (Lin *et al.*, 1997; Ross *et al.*, 1996; see Chapter 8).

In this chapter, we summarize the current understanding of the role of three GATA family transcription factors, GATA-4, -5, and -6, in cardiovascular development. Although each of these proteins is expressed in the developing cardiovascular system, definitive evidence concerning their roles in cardiac development, as determined from gene targeting studies, is available only for GATA-4 and GATA-5. Moreover, the question of potential redundancies between the proteins has not yet been definitively addressed by producing mice lacking two or more GATA family members. Thus, much remains to be learned about the role of these transcription factors in cardiovascular development and this review should, accordingly, be viewed as a work in progress rather than as a completed story. Many investigators have contributed to our understanding of the role of GATA proteins in mammalian development. In some cases it has not been possible to include all the relevant citations. We apologize for any inadvertent omissions of specific references.

II. The GATA Family of Zinc Finger Transcription Factors

Members of the GATA family of zinc finger transcription factors play key roles in transducing nuclear events that modulate cell lineage differentiation during vertebrate development (Orkin, 1992; Simon, 1995; Weiss and Orkin, 1995). To date, six related vertebrate GATA proteins have been identified, each of which is expressed in a developmentally regulated lineage-restricted fashion (Arceci *et al.*, 1993; Dorfman *et al.*, 1992; Evans and Felsenfeld, 1988; Ho *et al.*, 1991; Kelley *et al.*, 1993; Ko *et al.*, 1991; Laverriere *et al.*, 1994; Morrisey *et al.*, 1996; 1997a; Tsai *et al.*, 1989; Wilson *et al.*, 1990). All GATA proteins contain a conserved Cys-X₂-Cys-X₁₇-Cys-X₂-Cys type IV zinc finger DNA-binding domain that recognizes and binds to the consensus motif (A/T-G-A-T-A/A/G) (Ko and Engel, 1993; Mericka and Orkin, 1993; Omichinski *et al.*, 1993; Yang *et al.*, 1994). Previous studies have demonstrated that the C-terminal zinc finger and adjacent basic domain of GATA-1 are required for sequence-specific DNA-binding activity, whereas the N-terminal finger increases the affinity of GATA-1 for its cognate binding motif (Martin *et al.*, 1990; Trainor *et al.*, 1996). The zinc fingers of GATA-1 also mediate homo- and heterodimerization with other transcriptional activators, including Sp1,

EKLF, and RBTN2 (Crossley *et al.*, 1995; Merika and Orkin, 1995; Osada *et al.*, 1995; Yang and Evans, 1995).

GATA-1, -2, and -3 are expressed in overlapping subsets of hematopoietic cells and their precursors as well as in several nonhematopoietic tissues (Table I). GATA-1 is expressed in mature erythrocytes, multipotent hematopoietic progenitor cells, megakaryocytes, mast cells, and the Sertoli cells of the testes (Martin *et al.*, 1990; Romeo *et al.*, 1990; Yamamoto *et al.*, 1990). GATA-2 is expressed in hematopoietic stem cells and progenitors, immature erythroid cells, mast cells, megakaryocytes, endothelial cells, and the developing brain (Dorfman *et al.*, 1992; Zon *et al.*, 1993). GATA-3 is expressed in T lymphocytes and in specific regions of the central and peripheral nervous systems (Ho *et al.*, 1991). Functionally important GATA sites have been identified in transcriptional regulatory elements that control the expression of erythroid, lymphoid, myeloid, and endothelial cell-specific genes (Weiss and Orkin, 1995).

Much has been learned about the function of GATA-1, -2, and -3, respectively, in controlling differentiation of the hematopoietic cell lineages from pluripotent stem cells through gene targeting experiments (Simon, 1995; Weiss and Orkin, 1995). GATA-1 is required for

normal erythroid development (Pevny *et al.*, 1991; Simon *et al.*, 1992). Primitive erythroid precursors are not produced in *GATA-1*^{-/-} mice (Pevny *et al.*, 1991) and *GATA-1*^{-/-} embryonic stem (ES) cells do not contribute to the mature erythroid compartment of chimeric mice (Weiss *et al.*, 1994). Disruption of the *GATA-2* locus results in a global defect in hematopoiesis with severe quantitative defects in both primitive and definitive erythropoiesis as well as defects in myelopoiesis and lymphopoiesis, suggesting that GATA-2 plays a critical role in differentiation of an early hematopoietic progenitor or stem cell (Tsai *et al.*, 1994). GATA-3 is required for the development of the T cell lineage and also appears to control *Th2* cytokine gene expression (Ting *et al.*, 1996; Zheng and Flavell, 1997). In addition, *GATA-3*^{-/-} embryos exhibit severe deformities of the spinal cord and brain (Pandolfi *et al.*, 1995). Taken together, these studies demonstrated that although GATA-1, -2, and -3 are developmentally coexpressed in overlapping subsets of hematopoietic cells, each protein subserves a unique function in the developing embryo. These functions include (i) activation of lineage-specific target genes, (ii) distinct steps in restricting the developmental potential of multipotent hematopoietic stem cells, and (iii) direct or indirect regulation of programmed cell death during hematopoietic development. Of note, these studies also suggest that GATA-1, -2, and -3 may be functionally redundant with respect to the activation of lineage-specific genes in cells that coexpress more than one family member.

Table I Cellular Distribution of GATA Family Members

Family member	Tissue distribution
GATA-1	Erythroid, mast, megakaryocytic lineage Hematopoietic progenitor cells Testes
GATA-2	Mast, megakaryocytic lineages Early erythroid cells, endothelial cells Hematopoietic progenitor cells Embryonic brain
GATA-3	T lymphocytes, endothelial cells Embryonic brain and adult central and peripheral nervous systems Placenta, kidney, adrenal gland
GATA-4	Embryonic and adult heart Gut epithelium, embryonic liver Testes, ovaries
GATA-5	Embryonic heart Gut epithelium Embryonic lung (mesenchyme and bronchial SMCs) Embryonic urogenital ridge and bladder SMCs
GATA-6	Embryonic and adult heart Gut epithelium Vascular SMCs Embryonic lung (bronchial epithelium) Embryonic urogenital ridge and bladder SMCs

III. The GATA-4/5/6 Subfamily of Zinc Finger Transcription Factors

Following the isolation and characterization of GATA-1, -2, and -3, cDNA cross-hybridization studies identified three previously undescribed vertebrate GATA factors that were named GATA-4, -5, and -6 (Arceci *et al.*, 1993; Heikinheimo *et al.*, 1994; Kelley *et al.*, 1993; Laverriere *et al.*, 1994; Morrissey *et al.*, 1996, 1997a; Tamura *et al.*, 1993). Each of these proteins contains two type IV zinc fingers that are related closely to those of GATA-1, -2, and -3. Moreover, GATA-4, -5, and -6, but not GATA-1, -2, and -3, share low-level amino acid sequence identity across regions located within their N termini (Morrissey *et al.*, 1997b). Preliminary characterization revealed that the genes encoding GATA-4, -5, and -6 are expressed in an overlapping pattern within the precardiac mesoderm, heart, and gut epithelium (Arceci *et al.*, 1993; Kelley *et al.*, 1993; Laverriere *et al.*, 1994) (Table I). Based on their amino acid sequence identities, and their overlapping patterns of expression, the *GATA-4*, -5, and -6 genes have been sub-

classified as a separate subfamily of GATA factors (Laverriere *et al.*, 1994).

Detailed analyses of the temporal and spatial patterns of expression of *GATA-4*, -5, and -6 gene expression in staged murine embryos revealed that each gene displays a unique pattern of expression during vertebrate development (Heikinheimo *et al.*, 1994; Morrisey *et al.*, 1996, 1997a). In the early mouse embryo, *GATA-6* transcripts are first detectable within the visceral endoderm at the advanced egg cylinder stage (E6.5) (E. Morrisey and M. Parmacek, unpublished observation). At E7.5, the *GATA-4* and -6 genes are both expressed in the primitive streak mesoderm, the mesoderm subjacent to the headfold region (which gives rise to the cardiogenic plate), and the extraembryonic visceral and parietal endoderm. In contrast, between E7.0 and 8.0,

the *GATA-5* gene is expressed in a more spatially restricted fashion within the precardiac mesoderm that is limited to the cardiogenic plate (Heikinheimo *et al.*, 1994; Morrisey *et al.*, 1996, 1997a).

At E9.5, concomitant with the onset of regular beating of the primitive heart and connection of the embryonic and yolk sac vasculatures, the *GATA-4*, -5, and -6 genes are expressed in both endocardium and myocardium of the primitive atria, ventricle, and truncus arteriosus, or cardiac outflow tract (Figs. 2A–2C). In addition, all three genes are expressed in the underlying septum transversum, which gives rise to cardiac myocytes and cells of the embryonic liver (Figs. 2A–2C). At midgestation (E12.5), the *GATA-4* and -6 genes continue to be expressed throughout the embryonic heart (Figs. 2D and 2F). In contrast, *GATA-5* mRNA is ob-

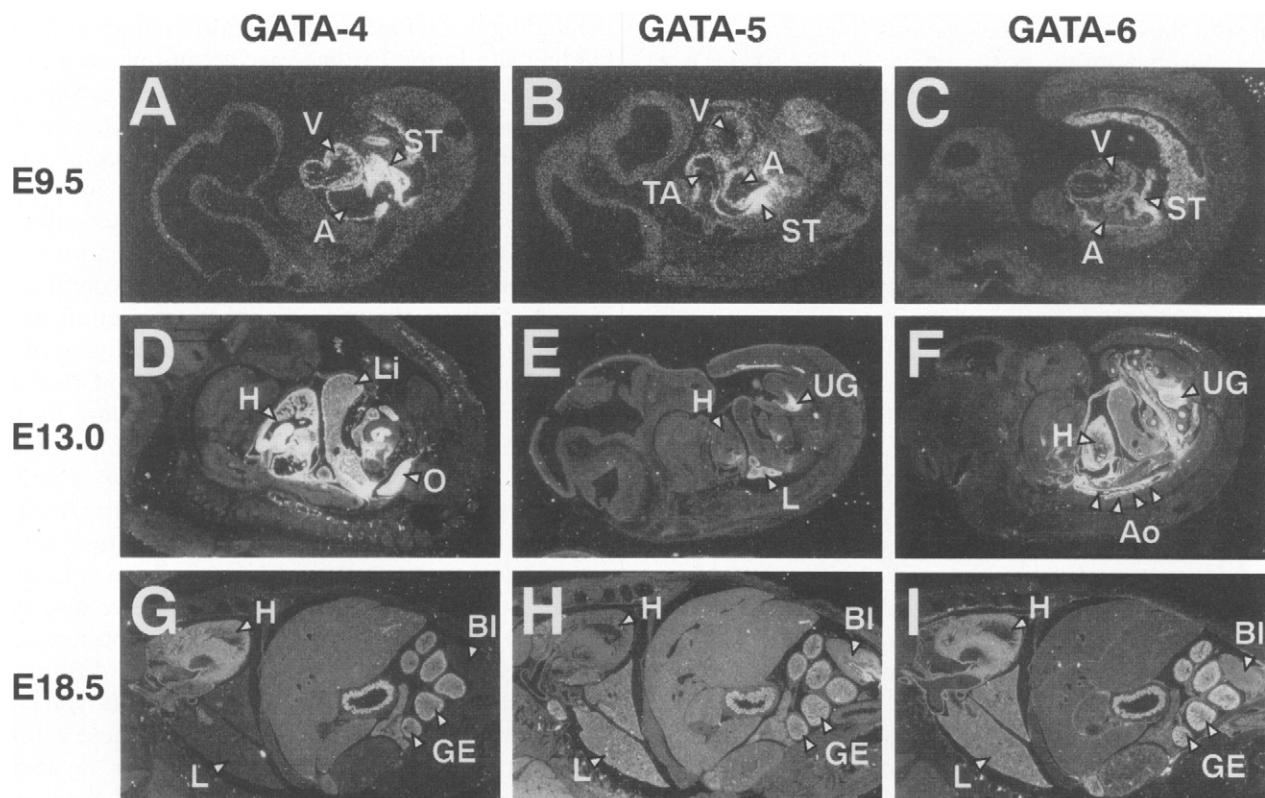


Figure 2 The temporal and spatial patterns of *GATA-4*, -5, and -6 gene expression during murine embryonic development. *In situ* hybridization analyses were performed using the control GATA-4 antisense riboprobe (A, D, G), the GATA-5 antisense riboprobe (B, E, H), or the GATA-6 antisense riboprobe (C, F, I) on staged E9.5 (A–C), E13.0 (D–F), and E18.5 (G and H) embryos. (A–C) In E9.5 embryos, the GATA-4 (A), -5 (B), and -6 (C) riboprobes hybridized (white staining) to the atria (A) and ventricle (V), and truncus arteriosus (TA) of the two-chambered heart, the septum transversum (ST), and the cells lining the midgut and hindgut regions. Magnification = $\times 12.5$. (D–F) In E13.0 embryos, both the GATA-4 (D) and GATA-6 (F) riboprobes hybridized to the heart (H). In contrast, the GATA-5 riboprobe hybridized predominantly to the atrial endocardium (E). All three riboprobes hybridized to the gut epithelium, whereas the GATA-4 riboprobe hybridized to the embryonic liver (Li) and ovary (O), and the GATA-5 and -6 riboprobes hybridized to the urogenital ridge (UG) and lung bud (L) (D). Only the GATA-6 riboprobe hybridized to the dorsal aorta (Ao; F). Magnification = $\times 3.1$ – $\times 3.5$. (G–I) In E18.5 embryos, the GATA-4 (G) and -6 (I) riboprobe hybridized to the heart (H) and stomach and intestinal epithelium (GE). In contrast, *GATA-5* mRNA was not detected within the late fetal heart (H). Only the GATA-6 riboprobe hybridized to the aorta, small arteries, and veins (I and data not shown), whereas the GATA-5 and -6 riboprobes hybridized to the bladder (BI) (H and I). Magnification = $\times 3.1$.

served exclusively within the atria and becomes restricted primarily to endocardial cells lining the atria and endocardial cushions (Fig. 2E). Shortly thereafter (E16.5), *GATA-5* gene expression within the heart is extinguished (Fig. 2H and data not shown). Thus, the *GATA-4* and -6 genes are expressed throughout the precardiac mesoderm and embryonic heart, and these genes continue to be expressed throughout pre and postnatal development. In contrast, the *GATA-5* gene is expressed transiently within the primitive heart, becomes restricted to the atrial endocardium by E12.5, and is extinguished by E16.5.

Each member of the *GATA-4/5/6* subfamily is also coexpressed in the epithelial cells lining the primitive and postnatal gastrointestinal tract (Figs. 2G–2I; Morrisey *et al.*, 1996, 1997a). However, outside of the gastrointestinal tract, each gene has a unique temporally and spatially restricted pattern of expression. Coincident with the onset of vasculogenesis (Fig. 2F), and continuing throughout the postnatal period, *GATA-6* is expressed in arterial and venous smooth muscle (Fig. 2E; Morrisey *et al.*, 1996; Narita *et al.*, 1998; Suzuki *et al.*, 1996). *GATA-6* is also expressed transiently within the embryonic bronchial epithelium as well as in the urogenital ridge and the smooth muscle cells (SMCs) of the bladder wall (Figs. 2F and 2I). In contrast, at midgestation (E13.0), the *GATA-5* gene is expressed most abundantly within the primitive lung bud and urogenital ridge (Fig. 2E). Within the embryonic lung, the *GATA-5* gene is expressed initially in the pulmonary mesenchyme (at E13.0) and subsequently within bronchiolar SMCs (at E18.5). In addition, *GATA-5* is coexpressed with *GATA-6* in SMCs of the bladder wall (Figs. 2H and 2I). Thus, *GATA-5* and -6 are expressed in overlapping, but distinct, tissue-restricted subsets of SMCs. This finding suggests that previously unrecognized transcriptional programs may distinguish SMC sublineages and that *GATA-5* and -6 may play important roles in controlling the specification and/or development of these different SMC lineage(s).

In summary, *GATA-4*, -5, and -6 are three of the earliest markers of the cardiac myocyte lineage(s). Each protein is expressed in the precardiac mesoderm and primitive heart tube at least 6–12 hr prior to the expression of genes encoding cardiac-specific contractile protein isoforms. Moreover, *GATA-4*, -5, and -6 are expressed at least as early as the cardiac-specific homeobox gene *Nkx2.5* (Biben and Harvey, 1997; Harvey, 1996; Komuro and Izumo, 1993; Lints *et al.*, 1993) and prior to the bHLH proteins dHAND and eHAND (Srivastava *et al.*, 1997). Interestingly, *GATA-4* and -6 are also expressed in the visceral endoderm, a tissue that plays an inductive role in cardiac myocyte specifi-

cation within the subjacent lateral plate mesoderm. *GATA-4* and -6 continue to be expressed throughout the myocardium and endocardium throughout the life of the organism, whereas *GATA-5* gene expression within the heart is extinguished by E16.5. Finally, in extracardiac tissues, *GATA-4*, -5, and -6 are each expressed in unique temporal and spatial patterns in higher vertebrates.

IV. Multiple Cardiac-Specific Transcriptional Regulatory Elements Contain Functionally Important GATA-Binding Sites

The first evidence that GATA factors might play an important role in cardiac myocyte differentiation was the finding that a functionally important nuclear protein binding site in the cardiac-specific murine *cardiac troponin C* (*cTnC*) promoter, designated CEF-1, contained an embedded GATA motif (WGATAR), and that oligonucleotides corresponding to CEF-1 bound at least one cardiac lineage-restricted nuclear protein complex (Parmacek *et al.*, 1992). Molecular characterization of CEF-1-binding activities revealed that the cardiac muscle cell lineage-restricted nuclear protein complex did in fact contain the zinc finger transcription factor *GATA-4* (Ip *et al.*, 1994). Functional analyses of the *cTnC* promoter revealed that a mutation that abolished *GATA-4* binding resulted in a 90% reduction in transcriptional activity (Ip *et al.*, 1994). Most important, as shown in Fig. 3, forced expression of *GATA-4* in NIH 3T3 cells could transactivate the 124-base pair *cTnC* promoter by 40- to 50-fold and this transactivation was dependent on a functional GATA binding site in CEF-1.

Functionally important GATA sites have also been identified in other cardiac-specific transcriptional regulatory elements including the cardiac α - and β -*myosin heavy chain* promoters and the *ANP* and *B-type natriuretic peptide* (*BNP*) promoters. Each of these cardiac-specific transcriptional regulatory elements can also be transactivated by forced expression of *GATA-4* in non-cardiac muscle cells (Grepin *et al.*, 1994; Huang *et al.*, 1995; Molkentin *et al.*, 1994). Although not functionally tested, consensus GATA binding sites (WGATAR) are also present in regulatory elements controlling the expression of other cardiac-specific genes, including the mouse *cardiac troponin I* promoter, the chicken *cardiac troponin T* promoter, and the rat *phospholamban* promoter. Recent studies demonstrated that forced expression of *GATA-5* or *GATA-6* also transactivates the cardiac-specific *cTnC* promoter-enhancer in noncardiac

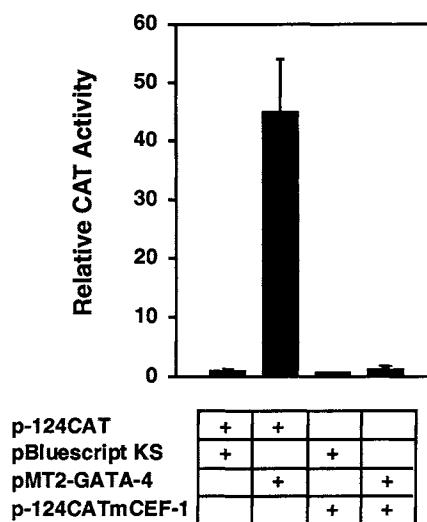


Figure 3 GATA-4-modulated transactivation of the cTnC promoter-enhancer in NIH 3T3 cells. NIH 3T3 cells were transfected with 24 μ g of the p-124CAT reporter plasmid containing the wild-type cTnC promoter-enhancer (p-124CAT+) or the p-124CATmCEF-1 reporter plasmid containing a five-nucleotide substitution in the CEF-1, GATA-4 binding site (p-124CATmCEF-1 +), and either 6 μ g of the control plasmid pBluescript KS (pBluescript +) or 6 μ g of the GATA-4 expression plasmid pMT2-GATA-4 (p-124CATmCEF-1 +). All transfections also contained 5 μ g of the pMSV β gal reference plasmid. Forty-eight hours after transfection, CAT and β -galactosidase activities were determined. CAT activities, corrected for differences in transfection efficiencies, were normalized to the CAT activity obtained following transfection of the p-124CAT plasmid with the pBluescript KS control plasmid, which produced 0.1% acetylation. The data are presented as relative CAT activities \pm SEM.

muscle cells (Morrisey *et al.*, 1996; 1997a), suggesting that at least *in vitro* the activation of cardiac-specific target genes by GATA-4, -5, and -6 is redundant. Taken together, these data strongly suggested that members of the GATA-4/5/6 subfamily of transcription factors play important roles in controlling the expression of multiple cardiac-specific genes.

This model has gained additional support from both *in vitro* loss-of-function and *in vivo* gain-of-function analyses. For example, expression of antisense GATA-4 transcripts in pluripotent P19 embryonal carcinoma cells blocked retinoic acid-inducible expression of genes encoding cardiac-specific contractile proteins (Grepin *et al.*, 1995). In addition, forced expression of GATA-4 in P19 cells accelerated cardiogenesis and increased the number of cardiac myocytes during *in vitro* differentiation of P19 cells (Grepin *et al.*, 1997). Moreover, injection of GATA-4 mRNA into *Xenopus* oocytes resulted in the premature expression of genes encoding *cardiac* α -actin and α -myosin heavy chain (Jiang and Evans, 1996). Together, these data strongly suggested that

GATA-4 and/or other GATA factors function (directly or indirectly) to regulate tissue-specific gene expression during vertebrate cardiac development.

V. GATA-4 Activates Transcription via Two Novel Domains That Are Conserved within the GATA-4/5/6 Subfamily

Although genetic studies have identified important roles for the GATA proteins in vertebrate development, relatively little is understood about the molecular mechanisms that control the functional activity of each GATA factor. Consistent with previous analyses of the GATA-1, -2, and -3 proteins, the conserved C-terminal zinc finger and adjacent basic domain [amino acids (aa) 251–324] of the murine GATA-4 protein are necessary and sufficient for sequence-specific DNA-binding activity (Morrisey *et al.*, 1997b). In addition, this domain contains a nuclear localization signal (Morrisey *et al.*, 1997b). Taken together, these data demonstrate that the GATA-4 C-terminal zinc finger and basic domain are bifunctional, modulating both DNA-binding and nuclear targeting activities. Both these domains are conserved in yeast single-finger GATA proteins, suggesting that this important bifunctional domain has been conserved through ancient evolution (Cunningham and Cooper, 1991; Fu and Marzluf, 1990).

Structure–function analyses of the murine GATA-4 protein led to the identification of two independent transcriptional activation domains within the N terminus of the protein (Morrisey *et al.*, 1997b). Activation domain I (ADI) (aa 1–74) is a neutral proline-rich motif (Fig. 4A), whereas ADII (aa 130–177) has a pI of 6.20 and contains three proline, five serine, and four tyrosine residues (Fig. 4B). Both ADI and ADII are conserved from *Xenopus laevis* to humans. Interestingly, highly related activation domains are also present in the *Xenopus* and chicken GATA-5 and -6 proteins (Figs. 4A and 4B). Functional analyses confirmed that these regions of GATA-5 and -6 function as *bona fide* transcriptional activators *in vivo* (Morrisey *et al.*, 1997b). In contrast, amino acid sequence identity was not observed between ADI or ADII and the previously identified transcriptional activation domains in the GATA-1 and -3 proteins (Martin and Orkin, 1990; Yang and Evans, 1992; Yang *et al.*, 1994). Interestingly, both ADI and ADII contain conserved tyrosine and serine residues, suggesting that posttranslational modifications of GATA-4, -5, and -6 may play an important role in regulating their functional activity (Figs. 4A and 4B (arrowheads)).



Figure 4 Amino acid sequence alignment of activation domains I (top) and II (bottom) in members of the GATA-4/5/6 subfamily. The amino acid sequences of the deduced human, murine, *Xenopus*, and chicken GATA-4, -5, and -6 proteins were aligned using the multiple sequence alignment protocol computer algorithm. Only the conserved regions of the protein are shown in this alignment. Subdomains that are conserved across each family member and across species are boxed in gray. Tyrosine residues that are conserved within each activation domain are indicated with arrowheads.

VI. GATA-4 Is Required for Ventral Morphogenesis and Heart Tube Formation

As described previously *in vitro* studies had suggested an important role for one or more GATA proteins in regulating the transcription of multiple cardiac genes and by inference the processes of cardiomyocyte specification and/or cardiac morphogenesis. To more precisely determine the role of the GATA proteins in cardiovascular development *in vivo*, targeted mutations of the GATA-4, -5, and -6 genes were created in ES cells and these ES cells were used to produce mice harboring null mutations in each of the GATA genes (Kuo *et al.*, 1997; Molkentin *et al.*, 1997). The phenotypes of these mice are summarized below.

GATA-4-deficient mice produced using two independent gene targeting strategies displayed identical phenotypes (Kuo *et al.*, 1997; Molkentin *et al.*, 1997). Heterozygous *GATA-4^{+/−}* mice were viable, fertile, and displayed normal cardiovascular development and function. In contrast, homozygous deficient (*GATA-4^{−/−}*) embryos died between E8.5 and 10.5 and displayed severe defects in both rostral-to-caudal and lateral-to-ventral folding which were reflected in a generalized disruption of the ventral body pattern (schematically depicted in Kuo *et al.*, 1997). Perhaps most strikingly, these embryos displayed markedly

aberrant cardiac morphogenesis. The initial stage of cardiac development, specification of cardiac myocytes from splanchnic mesoderm, was normal in the *GATA-4^{−/−}* embryos. Like wild-type littermates, the *GATA-4^{−/−}* embryos developed primitive cardiac myocytes in the dorsolateral region of the embryos that expressed the normal array of genes encoding contractile proteins including *myosin heavy chain*, *myosin light chains 2A and 2B*, *cardiac troponin C*, *cardiac troponin I*, and *atrial natriuretic factor* (Fig. 5 and data not shown). In contrast, the GATA-4-deficient embryos displayed severe abnormalities of the second stage of cardiac development. Specified procardiomyocytes failed to migrate from the anterior and dorsal region of the embryo to the ventral midline to form the linear heart tube. Instead, those *GATA-4^{−/−}* embryos that survived until E10.5 formed one or two aberrant cardiac structures in the dorsolateral and anterior regions of the embryo, anterior to the head fold (Fig. 6 and data not shown). These aberrant cardiac structures expressed the full array of contractile proteins but failed to form a functional circulatory system resulting in embryonic lethality between E8.0 and 10.5. Taken together, these results demonstrated that GATA-4 is not required for the specification of the cardiac myocyte lineage, but instead appears to regulate the second stage of cardiac morphogenesis—the rostral-to-caudal and lateral-to-ventral migration of procardiomyocytes to form the ventral heart tube.

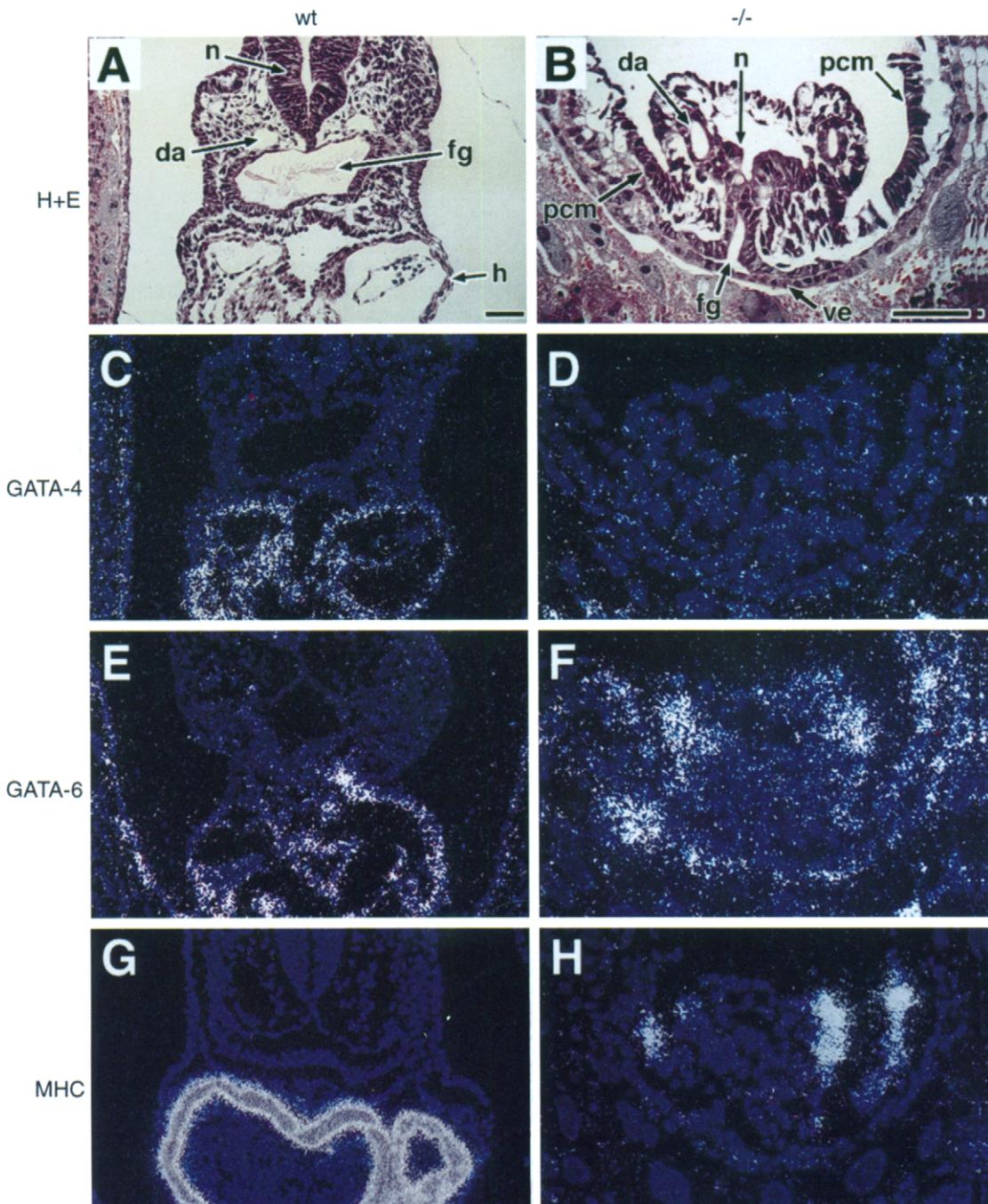
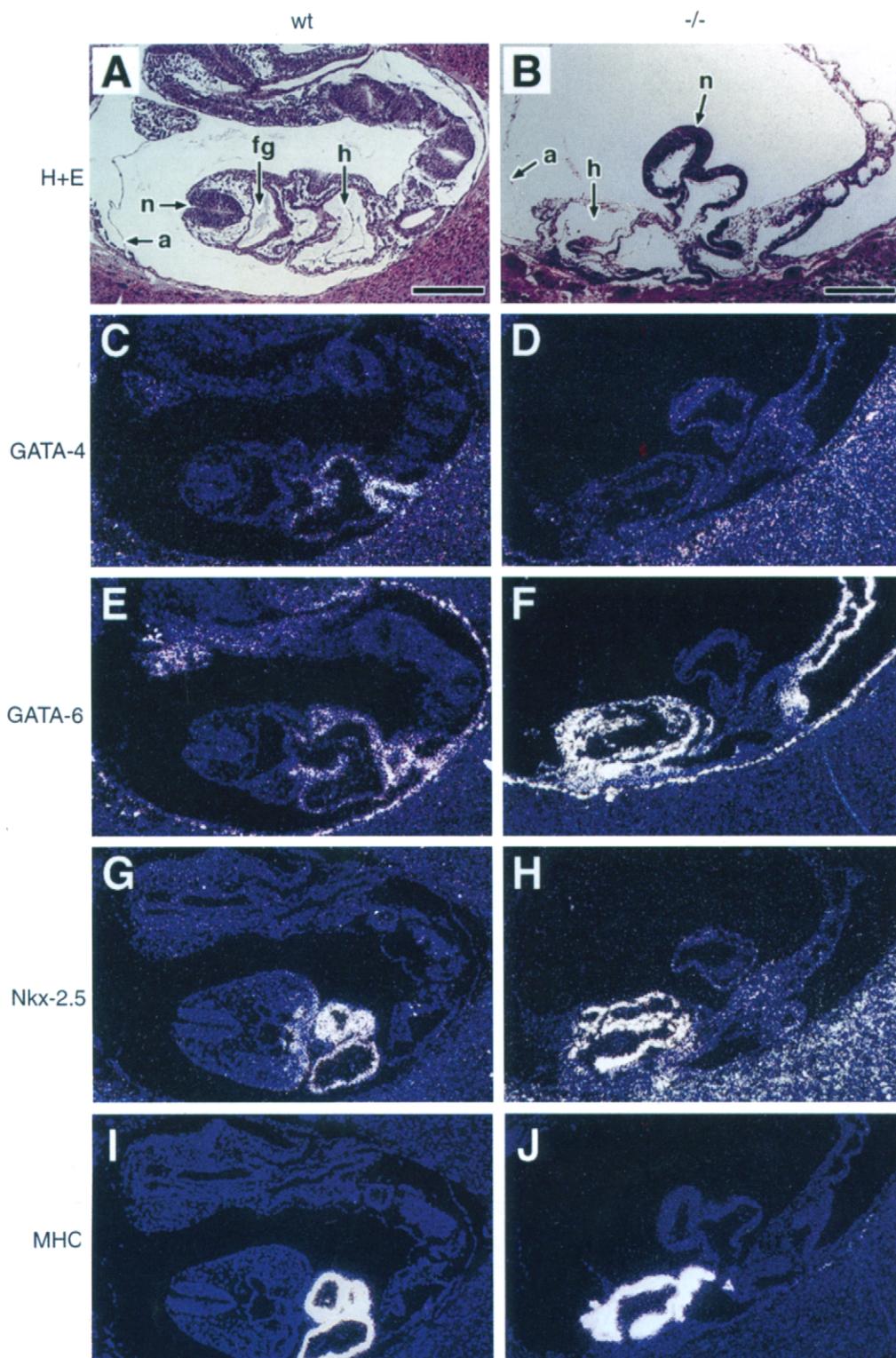


Figure 5 Cardiac defects in E8.5 *GATA-4*^{-/-} embryos. Histological (A, B) and *in situ* hybridization (C–H) analyses of E8.5 wild-type (wt) and *GATA-4*^{-/-} (^{-/-}) embryos. (A) Hematoxylin and eosin (H & E) stained transverse section of an E8.5 wild-type embryo. Note the dorsally located neural tube (n), dorsal aortas (da), foregut (fg), and the ventrally located heart (h). (B) H & E stained transverse section of an E8.5 *GATA-4*^{-/-} embryo. Note the dorsally located neural tube (n), dorsal aortas (da), ventrally located invaginating foregut (fg), visceral endoderm (ve), and laterally located precardiac mesoderm (pcm). (C, D) *In situ* hybridizations using a *GATA-4* antisense cRNA probe. Note the *GATA-4* expression in the developing heart tube of an E8.5 wild-type embryo and the lack of *GATA-4* expression in the *GATA-4*^{-/-} embryo. (E, F) *In situ* hybridizations using a *GATA-6* antisense cRNA probe. *GATA-6* is expressed in an overlapping pattern with *GATA-4* in the developing heart tube and in the visceral endoderm of the wild-type embryo. In the *GATA-4*^{-/-} embryo, *GATA-6* is expressed in visceral endoderm, precardiac mesoderm, and in regions surrounding the dorsal aortas lateral to the neural fold. (G, H) *In situ* hybridizations using an MHC antisense cRNA probe. MHC is expressed in the heart tube of the wild-type embryo and in regions lateral to the neural fold as well as in the precardiac mesoderm of the *GATA-4*^{-/-} embryo. Different magnifications and photographic exposures of the wt and *GATA-4*^{-/-} embryos were used to ensure adequate visualization of each of the probes in both embryos. Scale bars = 33 μ M.



Consistent with the hypothesis that the cardiac abnormalities observed in the *GATA-4*^{-/-} embryos reflected a more generalized defect in ventral morphogenesis, these embryos also displayed severe abnormalities of other ventral structures (Kuo *et al.*, 1997; Molkentin *et al.*, 1997). For example, they lacked a ventral pericardial cavity and displayed defective formation of the anterior intestinal portal and foregut which appeared as a thin fragmented slit in the *GATA-4*^{-/-} embryos compared to the normal tube-like structure seen in wild-type E9–10.5 embryos. Despite these defects, the *GATA-4*^{-/-} embryos contained differentiated foregut epithelium as measured by expression of *HNF3α* and *HNF3β*. Although *in vitro* evidence suggested that GATA-4 is required for visceral endoderm and yolk sac formation (Bielinska *et al.*, 1996; Soudais *et al.*, 1995), the GATA-4-deficient embryos contained intact visceral endoderm that expressed *GATA-6* and yolk sacs that expressed high levels of both *AFP* and *HNF4* (Kuo *et al.*, 1997). However, both the yolk sac and the amnion failed to close around the ventral portion of the *GATA-4*^{-/-} embryos (Kuo *et al.*, 1997; Molkentin *et al.*, 1997). Ventral closure of these membranes is tightly linked to the same lateral-to-ventral folding process that is responsible for heart tube and foregut formation, suggesting that this defect also reflected abnormal ventral morphogenesis.

One important question raised by the cardiac developmental phenotype of the *GATA-4*^{-/-} embryos was whether this phenotype reflected an inability of the GATA-4-deficient cardiomyocytes to respond to ventral morphogenic signals or, alternatively, defects in the morphogenic signals themselves. To address this question directly, β-galactosidase-expressing *GATA-4*^{-/-} ES cells were injected into wild-type C57BL/6 blastocysts to produce chimeric mice. Histological analyses of such chimeric embryos demonstrated contribution of the *GATA-4*^{-/-} ES cells to the pericardium, endocardium, and myocardium (Fig. 7). This finding suggested that the defect in cardiac morphogenesis seen in the absence of GATA-4 represented a defective morphogenic signal rather than an intrinsic failure of cardiomyocyte re-

sponsiveness. The identity of the aberrant morphogenic signal in the *GATA-4*^{-/-} embryos is unknown. However, possibilities include a soluble morphogen, an extracellular matrix molecule, or a protease that is required for normal cardiomyocyte migration. Such a molecule might be produced by procardiomyocytes themselves or by the visceral endoderm or splanchnic mesoderm, the three tissues that are known to express GATA-4 during early embryogenesis. These possibilities are currently under investigation.

In contrast to the results observed in the *GATA-4*^{-/-} animals, GATA-5-deficient mice do not display a cardiovascular phenotype (J. D. Molkentin and E. N. Olson, unpublished observation). Thus, despite the fact that *GATA-5* is expressed in the early developing heart, and that it can bind to and transactivate cardiac specific promoters, it is not required for cardiovascular development. As discussed later, however, this does not imply that GATA-5 is not involved in cardiac development because it is possible that GATA-4 and/or GATA-6 may serve redundant functions that can rescue the cardiovascular phenotype of the GATA-5-deficient animals. This possibility is currently being investigated by crossing the *GATA-5*^{+/-} mice with *GATA-4*^{+/-} and *GATA-6*^{+/-} mice to obtain *GATA-5*^{-/-}-*GATA-4*^{+/-}, *GATA-5*^{-/-}-*GATA-4*^{-/-}, *GATA-5*^{-/-}-*GATA-6*^{+/-}, and *GATA-5*^{-/-}-*GATA-6*^{-/-} mice. Preliminary analyses of mice carrying null mutations of the *GATA-6* gene demonstrated that homozygous *GATA-6*^{-/-} embryos die before E7.5 (E. Morrisey and M. Parmacek, unpublished observation). The cause of their lethality and their cardiovascular phenotype is currently under investigation.

VII. Potential Functional Redundancy and Cross Talk between GATA-4 and GATA-6

GATA-4, -5, and -6 are each expressed in the early developing heart and can each bind to and transactivate multiple cardiac-specific transcriptional regulatory ele-

Figure 6 Cardiac defects in E10.5 *GATA-4*^{-/-} embryos. Histological (A, B) and *in situ* hybridization (C–J) analyses of E9.0 wild-type (wt) and E10.5 *GATA-4*^{-/-} (^{-/-}) embryos. (A, B) Hematoxylin and eosin (H & E)-stained sagittal sections. a, amnion; n, neural fold; fg, foregut; h, heart. The *GATA-4*^{-/-} embryo is partially outside of the amniotic cavity and the *GATA-4*^{-/-} heart is located anterior to the head fold. (C, D) *In situ* hybridizations using a GATA-4 antisense cRNA probe. *GATA-4* is expressed in the heart tube and outflow track of 9.0-day pc wild-type embryo but not in the *GATA-4*^{-/-} embryo. (E, F) *In situ* hybridizations using a GATA-6 antisense cRNA probe. *GATA-6* is expressed in developing heart tube and outflow track, the visceral endoderm of the wild-type embryo, and in the visceral endoderm, mutant heart, and regions posterior to the neural fold in the *GATA-4*^{-/-} embryo. Different photographic exposures of the wt and *GATA-4*^{-/-} embryos were used to ensure adequate visualization of GATA-6 expression in both embryos. (G, H) *In situ* hybridizations using a *Nkx-2.5* antisense cRNA probe. *Nkx-2.5* is expressed in the heart tube of the wild-type embryo and in the mutant heart of the *GATA-4*^{-/-} embryo. (I, J) *In situ* hybridizations using a *MHC* antisense cRNA probe. *MHC* is expressed in the heart tube of the wild-type embryo and in the mutant heart of the *GATA-4*^{-/-} embryo. Scale bars = 100 μM.

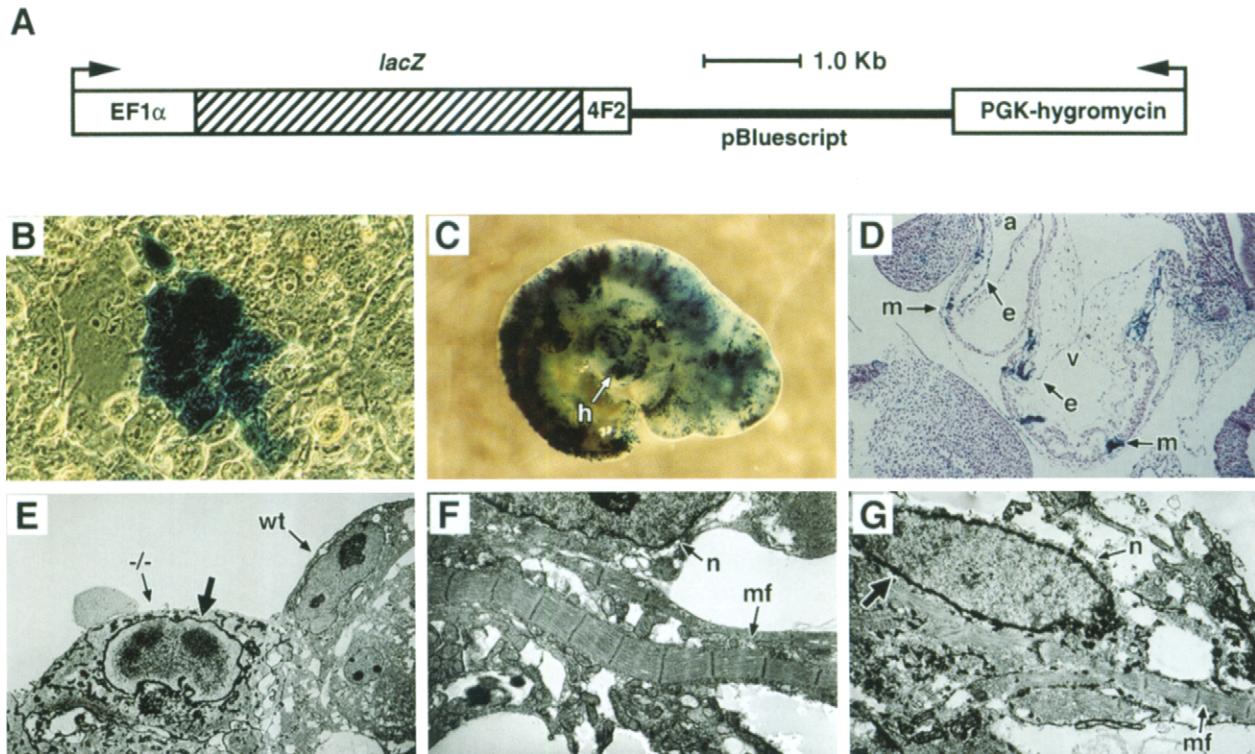


Figure 7 Contribution of β -gal $^{+/-}$ -GATA-4 $^{-/-}$ ES cells to the hearts of GATA-4 $^{-/-}$ -C57BL/6 chimeric embryos. (A) Schematic representation of the lacZ expression vector which contains the EF1 α promoter (EF1 α) and 4F2 heavy chain first intron enhancer (4F2) driving transcription of lacZ as well as a PGK-hygromycin cassette. (B) X-gal staining of GATA-4 $^{-/-}$ ES cells stably transfected with the lacZ expression vector. Note the blue ES cell colony growing on a monolayer of nontransfected fibroblasts. (C) X-gal staining of an E10.5 β -gal $^{+/-}$ -GATA-4 $^{-/-}$ chimeric embryo showing blue cells in all areas of the embryo including the developing heart (h). (D) Histological analysis of β -gal $^{+/-}$ -GATA-4 $^{-/-}$ chimeric embryo. β -gal $^{+/-}$ -GATA-4 $^{-/-}$ cardiac myocytes (m) and endocardial cells (e) are present in both the atrium (a) and the ventricle (v). (E–G) Electron microscopic analysis of myocardium in a β -gal $^{+/-}$ -GATA-4 $^{-/-}$ chimeric embryo. (E) Note the wild-type cardiac myocyte (wt) next to a β -gal $^{+/-}$ -GATA-4 $^{-/-}$ cardiac myocyte (−/−). (F, G) Note the cardiac contractile myofibers (mf) in both wild-type (F) and β -gal $^{+/-}$ -GATA-4 $^{-/-}$ (G) cardiac myocytes. The large arrows point to the electron-dense cytoskeletal X-gal reaction products surrounding the nuclei (n) in the β -gal $^{+/-}$ -GATA-4 $^{-/-}$ cardiac myocytes.

ments. Thus, it was of interest to study the patterns of GATA-5 and -6 expression in the GATA-4 $^{-/-}$ embryos. By *in situ* hybridization, the expression of GATA-5 appeared to be both qualitatively and quantitatively normal in the GATA-4-deficient mice. In contrast, the E 8.0–10.5 GATA-4 $^{-/-}$ embryos expressed significantly higher levels of GATA-6 in both cardiac myocytes and extraembryonic membranes compared to wild-type age-matched embryos. Moreover, the GATA-4 $^{-/-}$ embryos displayed high levels of GATA-6 expression in the body wall posterior to the head fold, a region of the embryo which does not normally express detectable GATA-6. Finally, GATA-6 expression was also significantly elevated in embryoid bodies differentiated *in vitro* from GATA-4 $^{-/-}$ ES cells. Taken together, these findings suggested that GATA-4 and GATA-6 may belong to a common developmental pathway in which

GATA-4 normally downregulates the expression of GATA-6. Interestingly, similar relationships have recently been described between GATA-2 and GATA-1, which regulate the development of the hematopoietic stem cell and erythroid lineages, respectively, and between the myogenic bHLH proteins that regulate skeletal myogenesis. These findings also raised the possibility that GATA-6 overexpression may partially rescue the phenotype of the GATA-4-deficient mice in those tissues that coexpress both proteins (i.e., heart and gut) and/or that overexpression of GATA-6 was itself responsible for at least some of the developmental defects observed in the GATA-4-deficient mice. In this regard, it will be of interest to study GATA-4 expression in the GATA-6 $^{-/-}$ embryos and to produce mice with targeted mutations in both the GATA-4 and GATA-6 genes. Such studies are currently in progress.

VIII. Summary and Future Directions

During the past 5 years, much has been learned about the role of GATA transcription factors in cardiovascular development. We now know that there are three closely related GATA family members (GATA-4, -5, and -6) expressed in temporally distinct patterns in the developing mammalian heart. In addition, *GATA-6* is expressed in both arterial and venous SMCs, suggesting a potential role for this protein in the development of the vasculature. GATA-4, -5, and -6 can each bind to the transcriptional regulatory regions of multiple cardiac promoters and enhancers and transactivate these transcriptional regulatory elements in nonmuscle cells. Each of these factors share two unique and independent transcriptional activation domains. Gene targeting experiments have demonstrated a necessary role for GATA-4 in regulating the second stage of cardiac development—the migration of specified procardiomyocytes from the dorsolateral region of the embryo to form the ventral linear heart tube. Interestingly, this defect appears to reflect a generalized requirement for GATA-4 in ventral morphogenesis rather than a cardiac-restricted phenotype. Chimera experiments suggest that GATA-4 is required to initiate and/or maintain the ventral morphogenic signal rather than to act as a regulator of cardiomyocyte responsiveness to this signal. GATA-6 is also required for early embryonic viability and its precise role in cardiovascular development is currently under investigation. In contrast, GATA-5 does not appear to be required for the development of the heart and vasculature. Finally, preliminary evidence suggests that GATA-4 and GATA-6 may belong to a common developmental pathway in which GATA-4 normally downregulates the expression of GATA-6 in the heart and extraembryonic membranes.

As is often the case, recent progress in understanding the role of GATA proteins in cardiac development has raised as many (or more) questions than it has answered. It is likely that there are significant functional redundancies between GATA-4, -5, and -6 which can only be fully understood by producing animals lacking two or more GATA proteins. The regulation of GATA-4, -5, and -6 expression during early heart development, including potential cross talk between the different proteins, is of great interest. However, to date it has been difficult to identify and characterize the promoter regions of the *GATA-4*, -5, and -6 genes. This difficulty may reflect the existence of distant and complex transcriptional regulatory elements in these genes. Although the GATA proteins can each bind to many cardiac promoters and enhancers, we currently know relatively little about the *bona fide* transcriptional tar-

gets for each of the proteins *in vivo*. Subtractive hybridization techniques using embryos and embryoid bodies deficient in each of the proteins may be useful in this regard. GATA-4, -5, and -6 each contain conserved transcriptional activation domains, suggesting that they may interact with common transcriptional regulatory proteins. The identities of these proteins remain unknown. However, recent studies have suggested that GATA-4 can interact with MEF-2 and Nkx-2.5. Other interacting partners may be identified using yeast two-hybrid approaches. Given the importance of GATA proteins in mammalian development and the recent significant progress in this area, the next several years should lead to a much more detailed molecular understanding of the role of these interesting proteins in cardiovascular development.

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