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The *Xenopus* GATA-4/5/6 Genes Are Associated with Cardiac Specification and Can Regulate Cardiac-Specific Transcription during Embryogenesis

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The GATA family of nuclear factors has been implicated in the regulation of cell type-specific transcription. We report the isolation of the *Xenopus* GATA-4 and GATA-6 cDNA clones and characterize the expression patterns of the xGATA-4/5/6 genes. By comparing the sequence of the cDNAs with those previously reported from chick and mammalian sources, we conclude that each is conserved across vertebrate evolution as a distinct gene product. Each gene is expressed in differentiated adult heart and gut, but maintains distinct transcript patterns in various other adult tissues. During embryogenesis, each gene displays a similar overlapping distribution of transcripts localized throughout the developing cardiogenic region. The xGATA-4 gene can be detected in dorsal cardiac progenitor rudiments prior to migration. Axis disruption experiments were used to demonstrate that transcription of these genes is intimately associated with the specification of cardiac progenitors. Ectopic expression of each gene is specifically capable of activating during embryogenesis the transcription of the cardiac genes encoding actin and myosin heavy chain α. The data are consistent with a primary role for the GATA-4/5/6 genes in regulating heart development.

INTRODUCTION

The expression of a unique set of genes during terminal differentiation of a specific cell type is regulated in large part by lineage-restricted transcription factors. An important model system for understanding tissue-specific gene expression is cardiac tissue. While certain terminal differentiation genes are specific to cardiomyocytes, other genes are coexpressed in both skeletal and cardiac muscle [see Sartorelli *et al.* (1993), for a review]. Skeletal muscle-specific gene expression is regulated largely by the binding of myogenic bHLH factors to consensus E-box sequences present in promoters and enhancers of muscle-specific genes. Although some evidence for bHLH binding activities in cardiomyocytes has been presented (Litvin *et al.*, 1993; Sartorelli *et al.*, 1992), the myogenic bHLH factors active in skeletal muscle are not present in cardiac cells. This has led to an understanding that E-box-independent transcriptional regulatory pathways function in cardiac muscle (Navankasattusas *et al.*, 1992). Although less well characterized, mechanisms must also exist for the coordinate regulation of nonmuscle genes in the endocardium and epicardium of differentiating heart tissue. Determining how cardiac-specific regulatory factors are themselves regulated would provide insight into the molecular mechanisms of cardiac cell determination during embryogenesis.

Certain members of the GATA factor family of transcription factors, thought to be important for cell lineage-restricted gene expression in vertebrates, have recently been implicated in the regulation of cardiac gene transcription (Grepin *et al.*, 1994; Ip *et al.*, 1994; Molkentin *et al.*, 1994; Thuerauf *et al.*, 1994). Six distinct GATA factors have so far been characterized (Laverriere *et al.*, 1994); each encodes a conserved central DNA-binding domain consisting of two similar but distinct zinc fingers. These proteins bind to similar *cis* elements, including a WGATAR or related sequence, and function as cell-specific transcriptional activators (Or-

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kin, 1992). GATA-1/2/3 are each expressed in hematopoietic lineages (Ness and Engel, 1994); GATA-1 is essential for maturation of erythroid cells (Pevny *et al.*, 1995, 1991), while GATA-2 is required at an early stage of myeloid progenitor cell development (Tsai *et al.*, 1994).

In contrast to the GATA-1/2/3 genes, the mGATA-4 and xGATA-5 genes apparently have no role in blood cell development. Instead, they are expressed during early embryogenesis in the developing heart region. Although both genes were originally designated GATA-4 (Arceci *et al.*, 1993; Kelley *et al.*, 1993), the isolation of distinct chick cDNAs similar to each demonstrated that they represent different genes (Laverriere *et al.*, 1994). A third related chick cDNA, designated GATA-6, was found to have a distinct but overlapping expression profile relative to GATA-4 and GATA-5. Rat homologues of GATA-4 and GATA-6 have also been described (Grepin *et al.*, 1994; Tamura *et al.*, 1993). All three cDNAs are clearly more related to each other than to the GATA-1/2/3 genes.

In the adult, the mGATA-4 and cGATA-4 genes are transcribed predominantly in the heart, small intestine, and gonads (Arceci *et al.*, 1993; Heikinheimo *et al.*, 1994; Laverriere *et al.*, 1994). GATA-5 transcripts are abundant in heart, throughout the gut, and in gut derivatives. While cGATA-6 is also transcribed in heart, stomach, and small intestine, transcripts are in addition relatively abundant in lung, liver, and ovary. Therefore, these genes are each expressed in differentiated heart tissue, while mRNA is absent from skeletal muscle. High-level expression of GATA-5 in the gut is specific to epithelium and has been shown to correlate with its terminal differentiation (Kelley *et al.*, 1993; Laverriere *et al.*, 1994). The expression patterns described for mGATA-4, xGATA-5, and cGATA-5 during early embryogenesis are consistent with a role in regulating heart formation and differentiation. Each has been shown to be transcribed initially within cardiac primordia during early stages of heart formation.

By analogy to the well-characterized erythroid transcription factor GATA-1, it was predicted that the GATA-4/5/6 proteins would interact with WGATAR regulatory *cis* elements of cardiac cell-specific genes (Arceci *et al.*, 1993; Kelley *et al.*, 1993). Recently, several such regulatory elements have been characterized, including those of the rat A- and B-type natriuretic genes (Grepin *et al.*, 1994; Thuerauf *et al.*, 1994), the rat α-myosin heavy chain gene (Molkentin *et al.*, 1994), and the murine cardiac-specific troponin C gene (Ip *et al.*, 1994). The WGATAR *cis* element is critical for cardiac expression of these genes, and GATA-4 has been shown to activate reporter genes through GATA-dependent upstream regulatory elements during transient cotransfection experiments. Potential GATA factor binding sites have been noted in putative regulatory regions of numerous other cardiac-specific genes. In sum, the GATA-4/5/6 genes are excellent candidates for activating the cardiac program during early embryogenesis and within the terminally differentiated state.

Despite this recent progress, several important questions remain concerning the role these factors play during cardiac development. For example, it has been entirely unclear whether each GATA factor has a distinct function or if the GATA-4/5/6 genes represent a family of redundant gene members. It has been unclear whether the transcription of GATA-4/5/6 genes is merely coincident with the precardiac mesoderm or if the expression patterns correlate embryologically with the specification of cardiac lineages. Also, the embryonic transcription pattern of GATA-6 has not been described. Finally, although GATA-4 has been shown to function in cell-culture transfections, it is not known whether these genes can actually activate cardiac-specific genes during development. The *Xenopus* system is advantageous for probing these questions. In addition to analyzing expression patterns, it is possible to alter cardiac specification by axis disruption experiments and to regulate early gene expression in developing embryos by microinjection experiments. Here we report the isolation and initial characterization of the *Xenopus* GATA-4 and GATA-6 homologues and analyze their expression and function together with the previously isolated (Kelley *et al.*, 1993) xGATA-5. Based on the conservation of individual GATA factors, we suggest that distinct functions for each GATA factor is likely. We find that GATA-4/5/6 transcription is intimately associated with cardiac specification and provide novel evidence for the importance of these genes in regulating cardiac-specific gene expression.

MATERIALS AND METHODS

Isolation and Characterization of cDNA Clones

Probes used to isolate cDNA clones encoding xGATA-4 and xGATA-6 were generated by RT/PCR using PCR primers consistent with conserved regions of GATA-4/5/6. The cDNA used for PCR was generated by random primed synthesis of poly(A)$^+$ RNA isolated from adult frog heart or gut (which each gave similar results). Initially, primers TE271 and JB466 (shown on Fig. 1A as primers 1 and 2, respectively) were used to amplify sequences encoding a portion of the highly conserved DNA-binding domain. PCR products were cloned and sequenced. Products were isolated with the predicted structure for each of the GATA-4/5/6 genes. Based on these sequences, primers with an exact match to xGATA-4 (TE283, shown on Fig. 1A as primer 3) or xGATA-6 (TE284, shown on Fig. 1A as primer 4) were used with JB485 (shown on Fig. 1A as primer 5) to isolate fragments upstream of the DNA-binding domain. These partial cDNAs were sequenced and found to be consistent with GATA-4 or GATA-6 by comparison with the chicken and mammalian clones. These fragments were used as probes to screen a stage 13 whole embryo *Xenopus* cDNA library in λgt11 under standard hybridization conditions (5× SSPE, 0.1% SDS, 5× Denhardt's solution, 65°C). In an initial screen of 10^6 recombinant phage, using both probes combined, 70 positive clones were identified. Upon rescreening a subset, 2 clones were positive with the GATA-4 probe, while 13 others were positive with the GATA-6 probe. The *Eco*RI cDNA inserts of 2 clones from each class were

subcloned into the pBluescript(SK) vector and sequenced using the Sequenase kit and protocol (USB). The entire sequence of each was determined on both strands using exonuclease III/mung bean deletions and by synthesizing internal oligomers. We believe that clones were isolated containing the full-length coding sequences based on conservation with the chicken clones; GATA-4 (but not GATA-6) contains an in-frame stop codon upstream of the initial methionine. The sequences of the PCR primers are as follows (N = A/G/C/T, I = inosine): TE271, 5′-TI(A/T) (G/C)IG A(A/G)(A/T) (G/C)I(A/C) GIG A(A/G)T G; JB466, 5′-CC(G/A) TGN A(G/A)(T/C) TTC AT(G/A) TA; TE283, 5′-TAG ACC CAC CCG GCG AGA C; TE284, 5′-CAG TCC AAT TCG TCT TGA T; JB485, 5′-CCN GTN TA(C/T) GTN CCN AC.

RNA Analysis

RNA was prepared from adult frog tissues by homogenization in RNAzol according to the manufacturer (Cinna Biotecx). The mRNA was purified using the polyATtract III system (Promega). RNA derived from staged (Nieuwkoop and Faber, 1967) embryos was extracted by homogenization in 50 m*M* Tris (pH 7.5), 50 m*M* NaCl, 5 m*M* EDTA, 0.5% SDS, and 0.2 mg/ml proteinase K. Samples were incubated at 37°C for 30 min, followed by phenol/chloroform extraction and ethanol precipitation in the presence of 0.3 *M* sodium acetate. RNA was then recovered by precipitating twice with ethanol in the presence of 2 *M* ammonium acetate. RT and PCR reactions were performed as described (Zhang and Evans, 1994). To avoid detecting any contaminating genomic DNA, primers were designed to cross intron/exon boundaries. For each reaction, the number of cycles was optimized to be certain that the signal accumulates within a linear range. The specificity of xGATA-4/5/6 primers was confirmed by demonstrating that the PCR products give a predicted pattern upon restriction enzyme digestion. The parameters for xGATA-5, xGATA-6, fibroblast growth factor receptor (FGFR), cardiac actin, myosin heavy chain α (MHCα), and EF-1α were: 1 min at 94°C, 1 min at 55°C, 1 min at 72°C. Annealing temperature for xGATA-4 was 59°C. The cycle numbers for each pair of primers were: 28 for GATA-4, GATA-5, and GATA-6; 18 for EF-1α; 24 for FGFR and cardiac actin; 38 for MHCα. Trace [α-^{32}P]dCTP was included in each reaction to allow direct quantification.

Primers used for PCR were as follows (F, forward primer; R, reverse primer): xGATA-4, F: 5′-GTG CCA CCT ATG CAA GCC C; R: 5′-TAG ACC CAC CCG GCG AGA C; xGATA-5, F: 5′-AGA CCA CTT ATC AAG CCA CAG; R: 5′-TGG GAT GTG ATG TTG GGT TC; xGATA-6, F: CCG CTC AGC CTC TCC; R: CTG AAG TTG CCT GGG; EF1α, F: 5′-CCT GAA TCA CCC AGG CCA G; R: 5′-GAG GGT AGT CTG AGA AGC TC; FGFR, F: 5′-TTG AAG TCT GAT GCG AGT GA; R: 5′-GGG TTG TAG CAG TAC TCC AT; cardiac actin, F: 5′-TCC CTG TAC GCT TCT GGT CGT A; R: 5′-TCT CAA AGT CCA AAG CCA CAT A; MHCα, F: 5′-CAC GAG CTG GAT GAG GC; R: 5′-TCA TGC TGG TTA ACA GG.

For Northern blotting experiments, 1.5 μg poly(A)$^+$ RNA isolated from adult tissues was electrophoresed through 1% agarose/formaldehyde gels and transferred onto Genescreen plus membrane. Blots containing aliquots derived from the same samples were hybridized according to the manufacturer (DuPont) with probes labeled by random primed synthesis specific for xGATA-4, xGATA-5, xGATA-6, or EF-1α.

Whole mount *in situ* hybridization was performed using albino embryos as described (Harland, 1991), except for the use of a dis-

A

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          1                                                  50
XGATA-4   MYQSIAMATN HGPSGYE.GT GSFMHSATAA TSPVYVPTTR VSSMIHSLPY
XGATA-5   MYPSLALTAN HAQPAYSHDT PNFLHS..TG SPPVYVPTSR MPAMLQSLPY
XGATA-6   MYQTLTITSA QGPLSYDPSP GTFMHS..AA SSPVYVPTSR VGSMLTSISY

          51                                                 100
XGATA-4   LQTSGSSQQG SPVSGHNMWA QAGVESSAYN PGTSHPPVSP RFTFSSSPPI
XGATA-5   LQSCDTAHQG HHLANHPGWA QTA.ESHAFN ..ASSPHTPT GFSYSHSPPV
XGATA-6   LQGTGASQGA HSVNSHWSQA .TS.ESSSFN ..NSSPHTSS RYHYPPSPPM

          101                                                150
XGATA-4   TAPSSREVSY SSPLGISANG REQYS....R GLGATYASPY PAYMSPDMGA
XGATA-5   GNSSARDGGY QSPLIMGGGA RDQYGNTLVR T..GSYPSPY .SYVGADMPP
XGATA-6   HNGSTRDTGY SSSLTVSS.. RDQYT.PLAR SLNGSYGSHY TPYMAPQLTS

          151                                                200
XGATA-4   AWTASPFDSS MLHNLQNR.. AVTSRHPNIE FFDDFS.EGR ECVNCGAMST
XGATA-5   SWAAGHFEGS MLHSLQGR.Q SLSGRRSSLE FLEEFPGEGR ECVNCGAMST
XGATA-6   AWPAGPFDNT MLHSLQSRGA PISVRGAPGD VLDELP.ESR ECVNCGSVQT

          201                                                250
XGATA-4   PLWRRDGTGH YLCNACGLYH KMNGINRPLI KPQRRLSASR RVGLSCANCH
XGATA-5   PLWRRDGTGH YLCNACGLYH KMNGMNRPLI KPQKRLSSSR RAGLCCTNCH
XGATA-6   PLWRRDGTGH FLCNACGLYS KMNGLSRPLI KPQKRVPSSR RIGLACANCH

          251                                                300
XGATA-4   TTTTTLWRRN AEGEPVCNAC GLYMKLHGVP RPLAMKKEGI QTRKRKPKNL
XGATA-5   TSTTTLWRRN SEGEPVCNAC GLYMKLHGVP RPLAMKKESI QTRKRKPKNI
XGATA-6   TSTTTLWRRN TEGEPVCNAC GLYMKLHGVP RPLAMKKEGI QTRKRKPKTL

          301                                                350
XGATA-4   SKSKTLTGQS GSD.....SL TPSTSSTNSM GEEMRPIKIE PGLSPPYDHS
XGATA-5   GKGKTSTGSS TSANN..... .SPSSVTNS. .DPTPVLKTE PNITSQYSGQ
XGATA-6   NKSKSSSSNG NSSHQISMTP TSTTSSTNS. .DDC....IK NGSPSQNTTP

          351                                                400
XGATA-4   ..NSISQASA LSTITSHGSS YYPMPSLKLS PQNH.HSTFN PSPQ....AN
XGATA-5   AIVPVSQGHS QTDDLVNGS. ....HELKFM PDEYTYSPTA LSQQSGLSVP
XGATA-6   VVASSLMSTQ QTESTSPNS. ....NTLKYT GQDGLYSAVS LSSASEVAAS

          401        413
XGATA-4   SKHDSWNNLV LA*
XGATA-5   LRQESWCALA LA*
XGATA-6   VRQDSWCALA LA*
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B

	xG4	xG5	xG6	cG4	cG5	cG6	xG1	xG2	xG3
xG4	----	73/55	69/53	83/76	74/57	72/57			
xG5	73/55	----	68/54	73/57	85/73	68/54			
xG6	69/53	68/54	----	68/56	66/51	80/68	52/40	54/37	54/39

FIG. 1. The GATA-4, GATA-5, and GATA-6 sequences have been conserved during vertebrate evolution as distinct genes. (A) The predicted amino acid sequence of xGATA-4 and xGATA-6 is shown aligned with that of the previously reported (Kelley *et al.*, 1993) xGATA-5a gene. Arrows indicate the sequences used to design PCR primers used for isolating finger region sequences (1 and 2) or for isolating probes specific for xGATA-4 (3 and 5) or xGATA-6 (4 and 5). The highly conserved GATA factor DNA-binding domain containing two similar but distinct zinc fingers is in bold. (B) The amino acid sequences of various GATA factors were compared using the GAP program (GCG, Inc.). The data is given as (% similarity allowing conservative changes)/(% identity). For each set of alignments, the most similar pair is boxed and confirms that genes are conserved across species. The genes are abbreviated, for example: xG4 = *Xenopus* GATA-4, cG5 = chicken GATA-5. (C) The highly conserved DNA-binding domain for all reported GATA-4/5/6 genes is shown. The chicken sequences are from Laverriere *et al.* (1994), mGATA-4 is from Arceci *et al.* (1993), xGATA-5 is from Kelley *et al.* (1993), and rGATA-6 is from Tamura *et al.* (1993). Amino acids that are diagnostic for each individual gene across species are in bold.

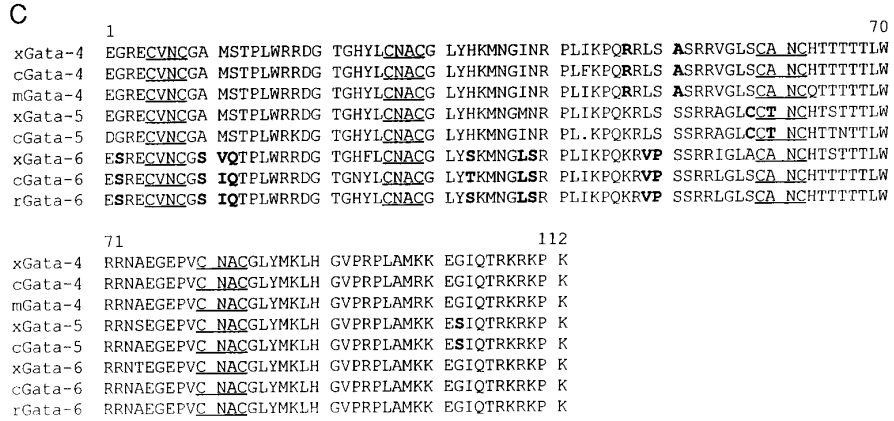

FIG. 1—*Continued*

tinct substrate for alkaline phosphatase (BM purple AP substrate, Boehringer-Mannheim). The specific anti-sense RNA probes (not containing sequence of the conserved DNA-binding domain) were synthesized in the presence of digoxigenin–UTP from linearized plasmids containing cDNA inserts for xGATA-4 (*Eag*I digestion, T3 polymerase), xGATA-5a (*Dra*III digestion, T7 polymerase), or xGATA-6 (*Hin*dIII digestion, T3 polymerase). Control experiments which did not detect any signal used sense strand RNA synthesized from the xGATA-5a plasmid (Kelley *et al.*, 1993). To detect c-actin and MHCα during development, PCR products were subcloned into pSKII- and used to generate anti-sense (or control sense) single-stranded RNA probes.

Embryological Treatments

Eggs were obtained from gonadotropin-induced females and fertilized *in vitro* using minced testes. Embryos were cultured in 0.1× MBS to the desired stage. The UV treatment was performed after fertilization for up to 1 min using a UVL-21 lamp (UV Products, Inc.) from a distance of 2 cm. LiCl treatment was carried out on embryos at the 32-cell stage in 0.3 *M* LiCl/0.1× MBS for 10 min (Kao and Elinson, 1988).

Ectopic Expression

The xGATA-4 and xGATA-6 inserts were subcloned into the *Eco*RI site of the pGEMHE vector and the xGATA-5a insert was inserted into the *Bgl*II site of the pSP64T vector. Capped RNAs were prepared from these constructs by *in vitro* transcription of linearized templates using SP6 or T7 polymerase (Krieg and Melton, 1984). To obtain transcripts containing the full coding region, the following enzymes were used: xGATA-4, *Sph*I and T7 polymerase; xGATA-5a, *Xba*I and SP6 polymerase; xGATA-6, *Nhe*I and T7 polymerase. The xGATA-1a transcript was synthesized as described (Zhang and Evans, 1994). As a further control, RNA was prepared from a mutant xGATA-4 template (pG4M) constructed by deletion of a 167-bp *Msc*I fragment located near the 5′ end of the wild-type clone. This deletion results in a frame shift that excludes the DNA-binding domain and therefore does not encode a functional protein. Each RNA was translated *in vitro* in the presence of [^{35}S]methionine and analyzed by SDS–PAGE and gel mobility-shift experiments to confirm the identity of the expected product. For each clone, 1.5 ng purified RNA was injected into fertilized eggs. During injection, the embryos were cultured in 5% Ficoll/0.1× MBS. The embryos were then cultured in 0.1× MBS until stage 9, when total RNA was extracted.

RESULTS

Conserved Features of the GATA-4/5/6 Genes

Based on our previous identification of three distinct GATA family members expressed in chick heart and gut (Laverriere *et al.*, 1994), we predicted that homologues for GATA-4 and GATA-6 should be present in *Xenopus*. Using degenerate primers consistent with the coding region of the conserved DNA-binding domain, we sought to isolate from adult heart or gut RNA RT/PCR products encoding these GATA factors. For this purpose, degenerate primers were designed to hybridize to sequences conserved in all known vertebrate GATA factors. RT/PCR products of the predicted size were identified using either heart or gut RNA as a source, and these were cloned and sequenced. Based on the presence in the potential open reading frames of specific diagnostic amino acids (see below), the cDNA products are consistent with the predicted sequence of DNA-binding domains for xGATA-4, xGATA-5, or xGATA-6.

Because the highly conserved finger region might cross-hybridize to other GATA factors, we used the sequence of the cDNA fragments to design exact sequence reverse PCR primers that would be specific for either xGATA-4 or xGATA-6. These were used in subsequent RT/PCR reactions using a degenerate forward primer consistent with a highly conserved stretch of amino acids in the N-terminal region of all known GATA-4/5/6 genes (PVYVPT, primer 5 in Fig. 1A); the sequences between these primer pairs would

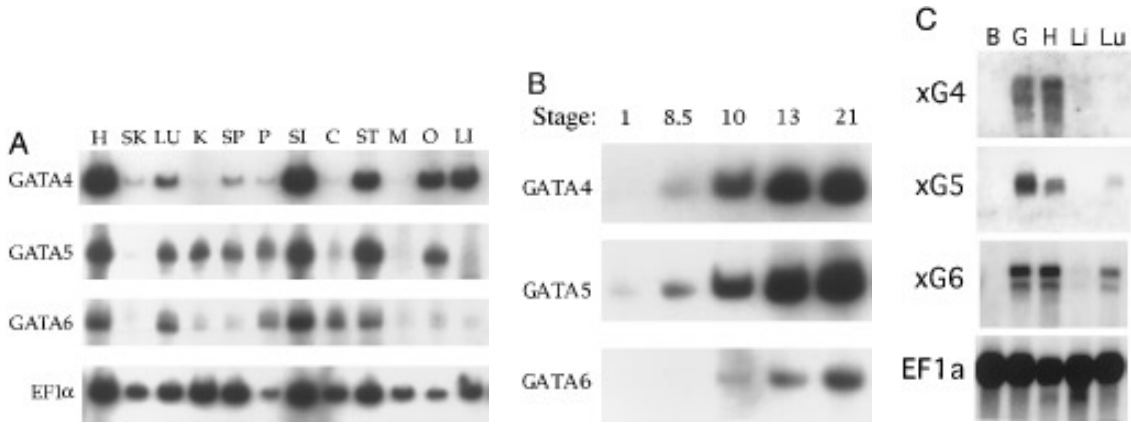

FIG. 2. Transcription patterns for the xGATA-4/5/6 genes. (A) A semiquantitative RT/PCR assay was used to analyze transcript levels in various tissues of the adult frog. Each gene is most highly expressed in heart and small intestine; note that levels cannot be analyzed between the genes, but only among tissues for a given gene. Lanes are abbreviated: H, heart; SK, skin; LU, lung; K, kidney; SP, spleen; P, pancreas; SI, small intestine; C, colon; ST, stomach; M, skeletal muscle; O, ovary; LI, liver. Additional reactions used primers to detect levels of EF-1α, to demonstrate the presence of cDNA in each reaction. (B) RNA from the indicated stages of development was analyzed by RT/PCR for the relative abundance of each gene product. (C) Northern blots were performed using RNA isolated from adult blood (B), gut (G), heart (H), liver (Li), or lung (Lu). Blots were analyzed using gene-specific probes derived from the xGATA-4/5/6 genes, or the EF-1α gene, as indicated. Transcript sizes (GATA-4: 5.7 and 4.3 kb, GATA-5: 1.9 kb, GATA-6: 4.4 and 3.8 kb) were estimated using RNA molecular weight standards (not shown). The GATA-4 and GATA-5 blots were exposed 4 days, the GATA-6 blot was exposed for 2 days, and the EF-1α blot was exposed for 12 hr.

not be expected to be conserved among distinct GATA factors. RT/PCR products of the expected size were generated from heart or gut RNA; these were cloned and used as probes to screen a library derived from stage 13 *Xenopus* gastrula RNA. As predicted, these probes hybridized to distinct cDNA clones; inserts specific for each were isolated and characterized by DNA sequencing.

The predicted amino acid sequence encoded by the novel xGATA-4 and xGATA-6 cDNA clones are compared in Fig. 1A to that of the previously reported xGATA-5. All three cDNAs encode proteins of approximately 390 amino acids, arranged similarly with respect to the central conserved finger region. Based on a comparison of the three chicken genes (Laverriere *et al.*, 1994), we expected to find that GATA-5 and GATA-6 are more similar to each other than to GATA-4, and there are significant stretches of similarity outside of the finger region (for example at the very carboxyl terminus). To further analyze the relationship of the three genes, we compared the predicted sequences of each gene with each other, as well as to the three chicken genes. Surprisingly, we found that xGATA-4 is not significantly diverged from xGATA-5/6 (Fig. 1B). In fact, in the case of frogs, the GATA-4/5 genes have a slightly higher degree of overall similarity relative to other pairs (73% vs 69 or 68%).

Most importantly, the sequence comparison shows that individual GATA factors are more highly conserved between species than they are among each other within a given species. Therefore, the xGATA-4 gene is 83% similar to the cGATA-4 gene, but only 73 or 69% similar to xGATA-5 and xGATA-6, respectively. The statistics are similar for each of the GATA-4/5/6 genes and indicate that the unique sequence of each family member has been highly conserved among vertebrates. This implies that each family member provides a distinct function that has been maintained during evolution. The xGATA-4/5/6 genes are only 52–54% similar to the xGATA-1/2/3 genes, representing predominantly the DNA-binding domain. We analyzed further the relationship among known GATA-4/5/6 genes by comparing the sequences encoding the DNA-binding domain, which is highly conserved among all known GATA factors (Fig. 1C). Interestingly, specific amino acids have been conserved for each member from amphibians to mammals, even within this region of global similarity. These amino acids represent diagnostic ''signature'' sequences that distinguish a factor as GATA-4, GATA-5, or GATA-6. This is most evident within the first finger of GATA-6, although examples are found for each of the genes.

Expression Patterns of the xGATA-4/5/6 Genes

We have used an RT/PCR assay to analyze the expression of the xGATA-4/5/6 genes in differentiated adult tissues. PCR primers were determined in preliminary experiments to generate a product which is specific for each gene, based on size and restriction pattern (not shown). As shown in Fig. 2A, each of the three genes is expressed in adult heart, as demonstrated previously in the case of the chicken (Laverriere *et al.*, 1994). GATA-4 mRNA is also abundant in

RNA derived from small intestine, stomach, ovary, and liver. Although the RT/PCR assay should not be used to accurately determine relative levels of message in various tissues, the sensitivity of the RT/PCR assay allows us to distinguish lower RNA levels in numerous tissues; much lower signals are detected in lung, spleen, pancreas, and skin. As shown previously (Kelley *et al.*, 1993), xGATA-5 RNA is also detected in small intestine, stomach, and at lower levels in lung, kidney, spleen, pancreas, and ovary. GATA-6 is similarly transcribed in small intestine and stomach. Lower GATA-6 RNA levels are found in lung, pancreas, and colon.

In general, these expression patterns are quite similar to those reported for the chicken genes and mGATA-4. Most strikingly, all three genes are transcribed in heart and gut, but not in skeletal muscle. However, there are some notable differences in transcription patterns between species. The relatively abundant GATA-4 transcripts detected in frog lung and liver were not seen in the chick or mouse. Also, although cGATA-5 and cGATA-6 are expressed transiently in the large intestine during chick embryogenesis, xGATA-6 transcripts are maintained in the adult frog colon. Each of the xGATA-4/5/6 genes is expressed during embryogenesis and up-regulated during gastrulation (Fig. 2B). Using the sensitive RT/PCR assay, we detect low levels of maternal xGATA-5 RNA present in the egg, which was not found in Northern blotting experiments (Kelley *et al.*, 1993).

The RT/PCR assay is less useful regarding message complexity or relative abundance of mRNA for different genes. Therefore, we performed Northern blots using probes derived from regions outside of the conserved DNA-binding region. Specifically, we were interested in determining whether similar transcripts are present in RNA derived from heart and gut. Different mRNA patterns might indicate that distinct regulatory mechanisms function for the activation of these genes in disparate lineages. As shown in Fig. 2C, we see no differences for each of the three genes in RNA derived from different tissues. There are two predominant GATA-4 messages of 5.7 and 4.3 kb, one GATA-5 message of 1.9 kb, and two messages that hybridize to the GATA-6 probe; a major transcript of 4.4 kb and a minor mRNA of 3.8 kb. The Northern blotting experiments support in general the data derived by RT/PCR, demonstrating, for example, low levels of mRNA in liver and lung.

GATA-4/5/6 Expression Patterns during Cardiogenesis

In *Xenopus*, the cardiac progenitors are derived from paired dorsolateral mesoderm located deep in the marginal zone, originating on either side of the dorsal blastopore lip [see Jacobson and Sater (1988), for a review]. Following gastrulation, the precardiac mesoderm migrates to the ventral midline and fuses to form the heart primordium, around stages 28–31. Previously, we found that xGATA-5 is transcribed in presumptive cardiac mesoderm at the time bilateral progenitors fuse. We have used the whole mount *in situ* hybridization technique to compare early expression patterns of the xGATA-4/5/6 genes during early heart development.

As shown in Fig. 3A, each of the GATA-4/5/6 genes is transcribed in stage 22 embryos in a broad region that includes the precardiac mesoderm. Transcript levels are highest at the ventral midline, although RNA is present in the lateral plate with a roughly symmetric bilateral distribution. The patterns overlap extensively, although they are not entirely identical. For example, xGATA-5 is consistently detected along more posterior regions of the ventral midline, relative to the other two genes. Also, xGATA-4 and xGATA-6 expression is enhanced in the dorsal precardiac lateral plate, relative to xGATA-5. Therefore, at the tailbud stage of development, the domains of xGATA-4/5/6 transcription correlate well with the cardiogenic field.

At the time a beating heart structure has formed in young stage 35/36 tadpoles, transcription of each of these genes is localized to cardiac tissue (Fig. 3B). Again, the expression patterns for xGATA-4 and xGATA-6 are very similar to that of xGATA-5. Transcripts are not restricted specifically to differentiating heart muscle; we detect RNA in the myocardium, endocardium, and in what appear to be the developing great vessels. Levels appear highest in endocardium, although this might simply reflect differential accessibility to probe. The antibody staining patterns that reflect RNA distribution are diffuse and heterogeneous compared to a muscle-specific marker such as cardiac actin (see below) or the MF-20 antigen (not shown).

Although all three genes are expressed (as assayed by Northern blotting experiments) earlier in development (for example, prior to migration of the dorsal cardiac rudiments), the level of detection by *in situ* hybridization prevents us from drawing firm conclusions regarding earlier patterns. However, weak staining of xGATA-4 is clearly present around one half of the marginal zone of a stage 10 embryo (Fig. 3C, left). This is apparently the DMZ, based on experiments to follow, although the use of albino embryos makes the distinction difficult. By late gastrulation, xGATA-4 RNA is detected in two paired rudiments, consistent with the predicted position of migrating cardiac primordia (Fig. 3C, middle). By the time the heart progenitors have fused at the ventral midline, xGATA-4 RNA is localized exclusively to developing heart tissue (Fig. 3C, right).

GATA-4/5/6 Expression Correlates with Specification of Cardiac Progenitors

The specification of cardiac progenitors occurs during gastrulation in response to mesoderm patterning signals that generate dorsolateral mesoderm (Sater and Jacobson, 1989). It is well established that disruption of axial patterning has a corresponding influence on heart formation (Jacobson and Sater, 1988; Kao and Elinson, 1988). Therefore, treatment of fertilized eggs with UV light causes ventralized embryos that lack

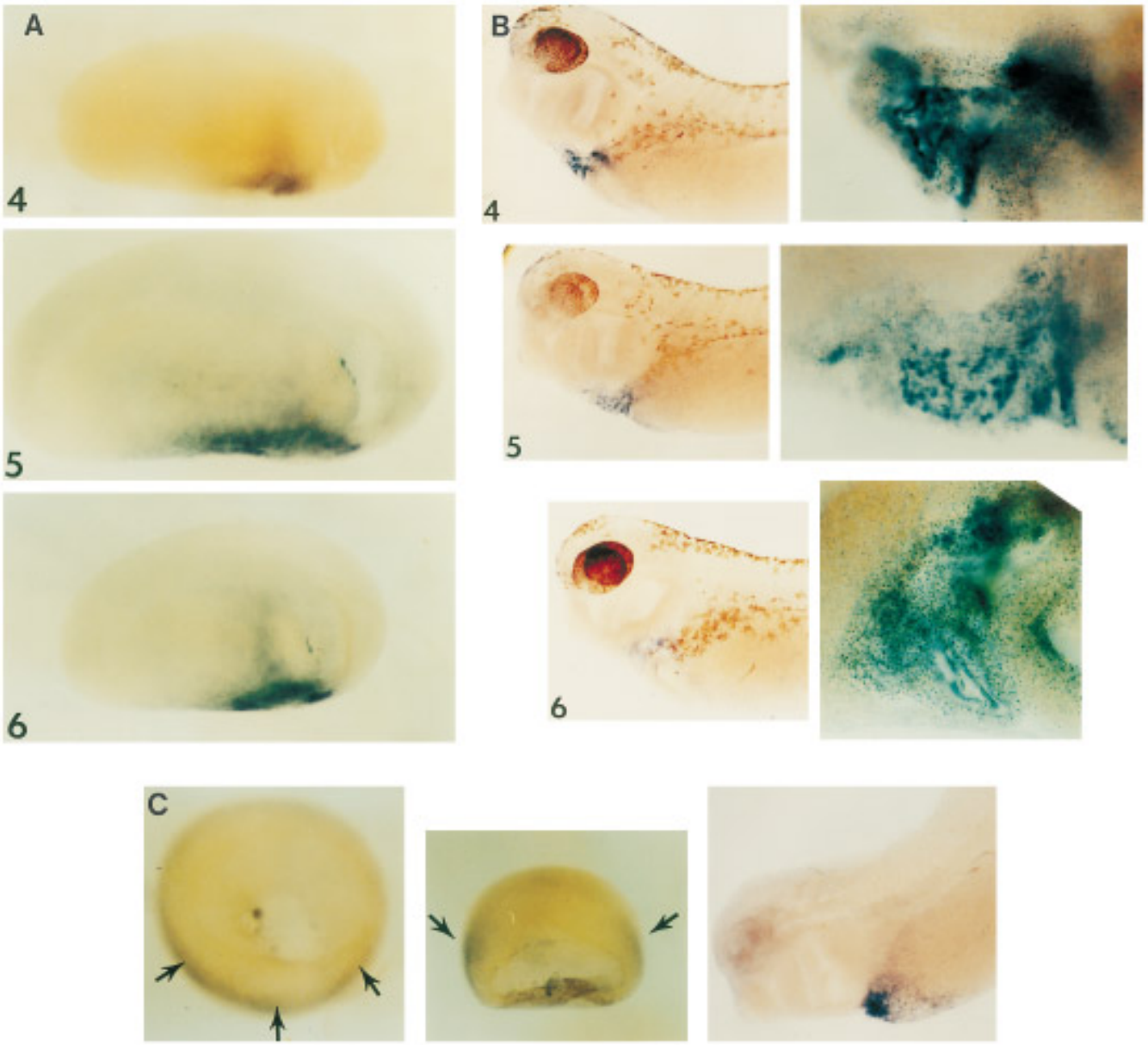

FIG. 3. The xGATA-4/5/6 genes are expressed specifically in the developing cardiogenic region during embryogenesis. Whole mount *in situ* hybridization assays were performed on albino embryos to analyze the early transcript patterns. (A) Stage 22 embryos show transcripts localized to the developing cardiovascular region at the ventral midline. (B) By stage 35/36, transcripts are localized to the beating heart. High-magnification views (right) show that staining is diffuse and throughout the heart region, including endocardium, myocardium, and great vessel endothelium. (C) Early staining pattern detecting xGATA-4 transcripts. At stage 10 (left) RNA is detected along one half of the marginal zone. At stage 12 (center) two distinct rudiments located on opposite sides of the blastopore (bottom) express xGATA-4 transcripts. By stage 30 (right), the cardiac progenitors have migrated and transcripts are localized to the developing heart region at the ventral midline.

heart structures. Likewise, incubating blastula embryos with LiCl enhances dorsal structures and leads to large radial hearts. We have disrupted axial patterning by these methods and analyzed by whole mount *in situ* hybridization how this affects the expression patterns of GATA-4/5/6 genes.

As shown in Fig. 4A, LiCl treatment leads to an alteration in expression patterns, so that by an equivalent of stage 22, RNA is detected in a wide radial band that encircles the dorsalized embryos. Therefore, xGATA-4/5/6 transcription correlates precisely with the predicted position of specified

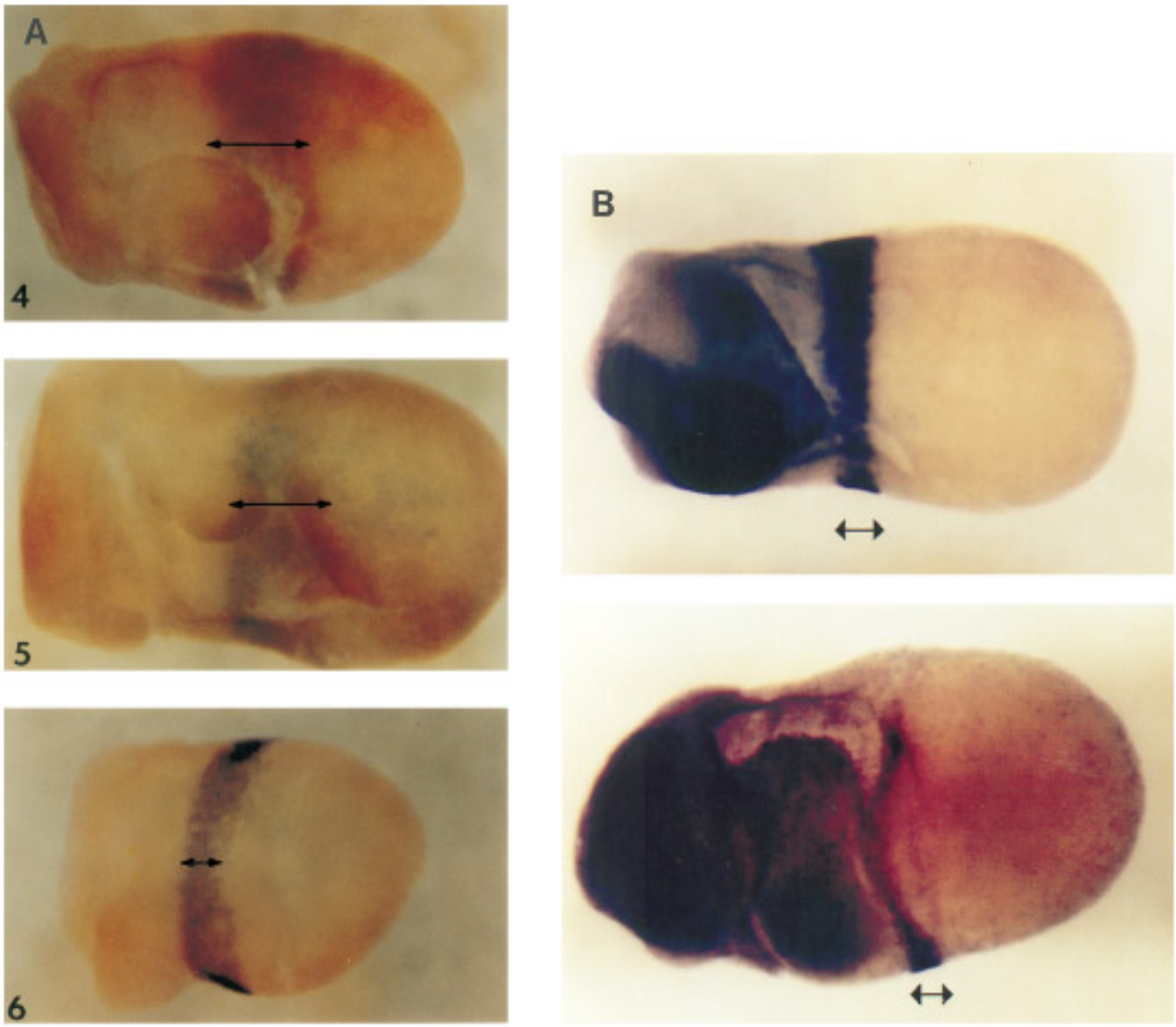

FIG. 4. LiCl treatment results in hyperdorsalized embryos and a concomitant expansion of GATA-4/5/6 expression reflecting the radial distribution of cardiac progenitors. (A) Embryos were treated with LiCl at the 32-cell stage and allowed to develop until untreated siblings had reached stage 22, before being analyzed for GATA-4/5/6 transcripts by whole mount *in situ* hybridization. Arrows indicate the width (along the A/P axis) of the band of cells expressing the genes around the radius of the embryo. (B) The analysis was performed on similarly treated and staged embryos, using probes specific for cardiac actin (top) or MHCα (bottom). Note that, compared to (A), a relatively "thin" band of cells (most similar to the xGATA-6 pattern) marks the presumptive cardiac skeletal muscle progenitors (arrows), while both of these genes are in addition expressed in a much broader region reflecting presumptive embryonic dorsal mesoderm (left side of each embryo).

cardiogenic precursors. The patterns are again similar but not identical; the xGATA-6 gene is transcribed in a more restricted band of cells relative to the other two genes. Embryos completely ventralized by UV treatment do not transcribe any of the three genes; less extensive UV treatment, which leads to a partial loss of dorsal structures, results in a corresponding decrease in the domains of xGATA-4/5/6 mRNA (not shown). Therefore, these three genes coordinately respond to axial disruption in a manner that supports the hypothesis that their expression is intimately associated with the specification of cardiogenic precursors.

A relatively thin radial band of cells (most similar to the

xGATA-6 pattern) is shown upon LiCl treatment to transcribe the control cardiac genes for c-actin and MHCα (Fig. 4B). In each case, extensive additional regions corresponding to presumptive dorsal muscle also transcribe the gene in a radial manner, as these genes are also expressed in embryonic skeletal muscle. These control experiments emphasize that the cells expressing GATA-4/5/6 during embryogenesis are restricted to heart and not muscle, yet appear to mark a more extensive population of cells relative to the cardiac muscle genes. We speculate that these additional cells might represent at least the presumptive endocardial lineage, perhaps including cells of the presumptive great vessel endothelium and/or epicardium.

Regulation of Cardiac-Specific Genes by xGATA-4/5/6

The expression patterns of xGATA-4/5/6 are consistent with the notion that these proteins regulate the transcription of cardiac-specific genes during embryogenesis. The *Xenopus* system allows us to test this hypothesis directly by ectopic expression experiments. The endogenous GATA-4/5/6 transcripts do not accumulate until gastrulation, so that expression of ectopic factors might precociously activate transcription of target genes. RNA encoding each of the three GATA factors was synthesized *in vitro* and injected into fertilized eggs. Embryos were isolated at stage 9 and RNA was prepared and analyzed for cardiac-specific transcripts by quantitative RT/PCR. As shown in Fig. 5A, injection of each of the three transcripts activates the genes encoding MHCα and (to a lesser extent) cardiac-specific actin. In contrast, RNA levels for the FGFR, which is expressed ubiquitously, remain unchanged. As controls, the anti-sense RNA of xGATA-5, or a xGATA-4 transcript derived from a cDNA which was mutated to generate a translation frame-shift and therefore nonfunctional protein, do not affect levels of cardiac transcripts when injected into developing embryos. In addition, RNA encoding xGATA-1 does not activate expression of the cardiac actin or MHCα genes, although it was previously shown to trans-activate a globin promoter (Zhang and Evans, 1994). Therefore, we conclude that the GATA-4/5/6 genes are capable of regulating transcription of at least some cardiac-specific genes.

Given the presumptive role that the GATA-4/5/6 genes play during cardiac embryogenesis, it is of interest to determine whether ectopic expression of these factors could influence the development of cardiogenic progenitors. Embryos injected with each RNA were allowed to develop further until cardiac differentiation markers are normally expressed. Embryos were then analyzed by whole mount *in situ* hybridization to determine whether the expression domains of c-actin or MHCα were diminished or expanded (Fig. 5B). However, in no case did we find any recognizable differences in embryos injected with GATA-4/5/6 RNA, either in heart development or in spatial expression of differentiation markers at these later stages. We conclude that while ectopic GATA-4/5/6 expression during early embryogenesis can influence (perhaps transiently) expression of cardiac genes, it is insufficient to dramatically alter cardiac development.

DISCUSSION

By analyzing DNA elements important for transcription of cardiac genes, several factors have recently been identified as potential cardiac gene regulators. Primary attention has focused on proteins that bind to CArG elements or to an A/T rich region (Argentin *et al.*, 1994; Edmondson *et al.*, 1994; Goswami *et al.*, 1994; Yu *et al.*, 1992; Zhu *et al.*, 1993). The former are members of the SRF family, while the latter are classified as MEF-2 factors, which encode MADS-box DNA-binding proteins also structurally related to SRF. While certain members of these families are likely to play important roles in regulating cardiac genes, the issue has been complicated because members also regulate skeletal muscle gene expression. Other factors shown to interact with cardiac regulatory elements include TEF-1 (Farrance *et al.*, 1992) and fos/jun (McBride *et al.*, 1993).

An alternative approach has been to identify transcription factors that are expressed specifically in cardiac lineages, without a prior knowledge of potential target genes. This has led to the successful identification of two regulatory families that are unique to cardiac (relative to skeletal) muscle cells. The homologue of the *Drosophila* Nkx-2.5 gene (tinman) has been isolated from mouse (Lints *et al.*, 1993) and *Xenopus* (Tonissen *et al.*, 1994) sources. The gene is first expressed in early heart primordia and in primitive pharyngeal endoderm, which is thought to be required for heart induction. The Nkx-2.5 gene is also expressed in lingual myoblasts, but not in any other skeletal muscle lineages. The expression pattern of Nkx-2.5 in cardiac mesoderm and then later in primitive gut is remarkably similar to that of the GATA-4/5/6 genes, which represent the second known cardiac-specific transcription factor family.

The GATA-4/5/6 genes were discovered due to their similarity in the DNA-binding domain with the myeloid regulatory factor GATA-1 (Arceci *et al.*, 1993; Kelley *et al.*, 1993; Laverriere *et al.*, 1994). The initial characterization of xGATA-5 and mGATA-4 expression patterns indicated a potential role in regulating cardiac development during embryogenesis. Although the genes are eventually expressed in numerous tissues, particularly those related to differentiated endoderm-derived epithelium, they are each transcribed in both precardiac progenitors and differentiated heart tissue. Recently, a number of cardiac gene regulatory elements have been confirmed to bind GATA factors. The ability to isolate all three genes initially from chick (Laverriere *et al.*, 1994), and now from frog, allows

us to draw several previously uncertain conclusions regarding the role of these genes during cardiac development:

1. Each of the three cardiac GATA factors has been conserved as a unique gene. Therefore, the GATA-4 genes, for example, are more similar to each other across vertebrate species than they are to family members within a species. This implies that each gene is likely to have some unique function(s), which has been under selective pressure. For instance, this might reflect target gene specificity, which could be directed through the highly conserved DNA-binding domain. It is interesting to note that certain amino acids within the DNA-binding domain have been conserved for each of the three genes. Distinct amino acids within this domain could theoretically provide subtle specificity for the action of each gene product. A similar conservation of gene-specific DNA-binding domain residues was also noted for the GATA-1/2/3 factors (Zon *et al.*, 1991). Therefore, each of the known factors could have significantly different interactions with DNA *in vivo*, although target site specificity has not been dramatic when tested *in vitro* (Ko and Engel, 1993; Merika and Orkin, 1993; Whyatt *et al.*, 1993). Of course, specificity could also be achieved by interaction with distinct factors through amino acid sequences, which are not as highly conserved, outside of the DNA-binding domain.

2. Each of the three genes has a similar expression pattern during early embryogenesis which is specific for the developing heart. xGATA-4/5/6 RNA is found specifically in cardiac progenitors, long before differentiation of heart tissue. Interestingly, the GATA-4/5/6 genes are the only known regulatory proteins found throughout the heart, including endocardium, myocardium, and endothelium of the great vessels. The overlapping expression patterns of GATA-1/2/3 genes in developing blood appears to play a significant role in defining distinct cell lineages within this tissue (Ness and Engel, 1994). Therefore, for example, differentiated erythroid cells contain high levels of GATA-1, while T-cells express predominantly GATA-3. By analogy, it is possible that the precise balance of GATA-4/5/6 expression is involved in specifying specific cell lineages of the cardiovascular system. Higher-resolution comparative mapping of expression domains and/or misexpression experiments may shed light on this speculation. Although other members of the GATA family may yet be characterized, it appears unlikely, based on RT/PCR cloning experiments, that additional cardiac GATA factors are present, unless they differ significantly from GATA-4/5/6 (unpublished data and J. B. E. Burch, personal communication). We note that, as yet, no mammalian homologue of GATA-5 has been described.

3. The GATA-4/5/6 genes are likely to play an early role in the specification of cardiac lineages. The transcription of these genes correlates precisely with the position of dorsal mesoderm heart progenitors, as demonstrated by axis disruption experiments. These genes are therefore likely to be targets of heart induction. Explant experiments have demonstrated roles in *Xenopus* for both Spemann organizer tissue (Sater and Jacobson, 1990), which is flanked by the early cardiac rudiments, and for deep endoderm (Nascone and Mercola, 1995) in providing signals for a multistep induction process. At least for GATA-4, we can detect low levels of transcripts in the early dorsal rudiments, prior to migration. Because dorsal/ventral axis formation affects profoundly expression of GATA-4/5/6, these genes may be direct or indirect targets of the inductive signals. Although these signals are unknown, high levels of activin can induce expression of cardiac MHCα (Logan and Mohun, 1993). We have shown that GATA-4/5/6 can activate expression of MHCα *in vivo*, providing evidence that heart induction is mediated at least in part by these transcription factors. However, we were not been able to demonstrate a direct activation of xGATA-5 by activin in animal cap explant assays (Kelley *et al.*, 1993).

4. The GATA-4/5/6 genes are able to function during early development to regulate cardiac-specific gene expression. By ectopically expressing the genes in early embryos (prior to accumulation of endogenous factors), we showed activation of two cardiac genes, cardiac actin, and MHCα. Each of the three GATA factors function similarly in this assay. While this might indicate a lack of specificity (at least for these two target genes), it is quite possible that ectopic expression levels do not reflect normal regulation. However, taking advantage of the *Xenopus* system, we have documented for the first time the activation of endogenous GATA-4/5/6 target genes in the context of a developing embryo (other experiments have employed transient transfection experiments using exogenous reporter genes in cell culture). The experiment demonstrates specificity as xGATA-1 was unable to activate the cardiac genes. We have been unable to detect activation of two other potential targets of GATA-4/5/6: tropomyosin, and troponin I. However, we find that these genes are normally expressed later in development relative to cardiac actin and MHCα (not shown). Therefore, they may be inaccessible to the action of GATA-4/5/6 at the early stages we have assayed or might require the presence of additional regulatory factors.

The inability of RNA encoding these GATA factors to alter grossly cardiac development is perhaps not surprising; similar results were shown regarding MyoD (Hopwood and Gurdon, 1990), which nevertheless plays a critical role in skeletal muscle development. On the other hand, ectopic expression of MyoD at a particular time and place is able to alter developmental fate during embryogenesis (Miner *et al.*, 1992). Likewise, it is possible that one or more of the GATA-4/5/6 genes might only be capable of influencing progenitor specification when expressed at a certain time of development and/or in association with other cardiac regulatory proteins. By analogy

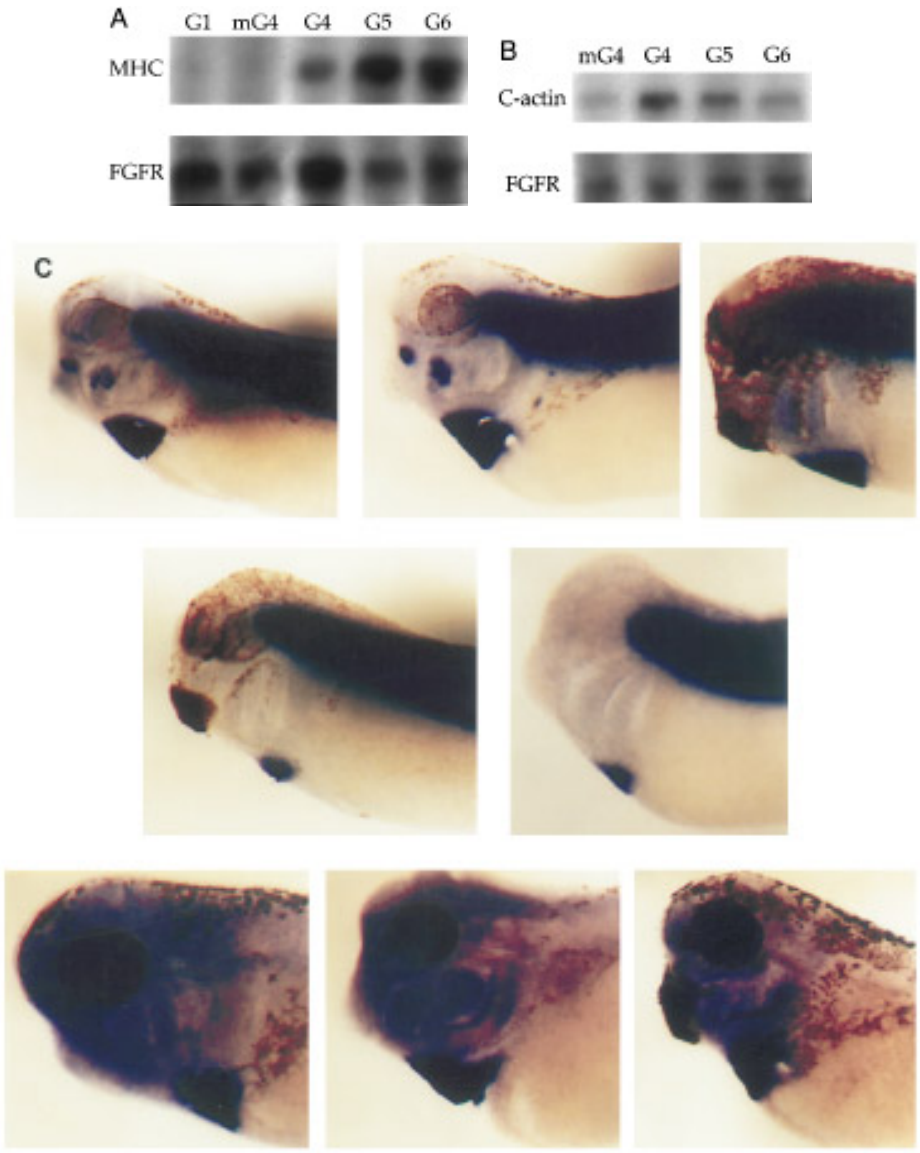

FIG. 5. Ectopic expression of xGATA-4/5/6 genes specifically activates the transcription of cardiac-specific genes but does not disrupt heart development. (A) Quantitative RT/PCR was used to measure transcript levels of the MHCα gene, in RNA derived from stage 9 embryos. Embryos were injected at the one-cell stage with RNA encoding xGATA-1 (G1), a nonfunctional mutant form of xGATA-4 (mG4), xGATA-4 (G4), xGATA-5 (G5), xGATA-6 (G6), or the anti-sense strand of xGATA-5 (not shown; this gave identical results to mG4). (B) In the same manner, transcript levels were analyzed for the cardiac actin gene. Although these data do not show a significant activation by GATA-6, in multiple experiments we have not detected a significant difference in the ability of each GATA-4/5/6 gene to activate transcription of the target genes. (C) Whole mount *in situ* hybridization experiments were used to analyze expression patterns of cardiac actin and MHCα in untreated and injected embryos. Top row (c-actin, stage 32) left to right: uninjected, xGATA-4 injected, xGATA-5 injected. Middle row (c-actin, stage 25) left to right: uninjected, xGATA-4 injected. Bottom row (MHCα, stage 32) left to right: uninjected, xGATA-4 injected, xGATA-5 injected. Similar results were obtained for xGATA-6 injected embryos (not shown). Embryos were either wild-type (pigmented) or albino.

to GATA-1, we hypothesize that the GATA-4/5/6 genes will be important, in cooperation with other cardiac transcription factors, for the expression of most or all cardiac-specific terminal differentiation genes.

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REFERENCES

Arceci, R. J., King, A. A. J., Simon, M. C., Orkin, S. H., and Wilson, D. B. (1993). Mouse GATA-4: A retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Mol. Cell Biol.* 13, 2235–2246.

Argentin, S., Ardati, A., Tremblay, S., Lihrmann, I., Robitaille, L., Drouin, J., and Nemer, M. (1994). Developmental stage-specific regulation of atrial natriuretic factor gene transcription in cardiac cells. *Mol. Cell Biol.* 14, 777–790.

Edmondson, D. G., Lyons, G. E., Martin, J. F., and Olson, E. N. (1994). Mef2 gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis. *Development* 120, 1251–1263.

Farrance, I. K. G., Mar, J. H., and Ordahl, C. P. (1992). M-CAT binding factor is related to the SV40 enhancer binding factor, TEF-1. *J. Biol. Chem.* 267, 17234–17240.

Goswami, S., Qasba, P., Ghatpande, S., Carleton, S., Deshpande, A. K., Baig, M., and Siddiqui, M. A. Q. (1994). Differential expression of the myocyte enhancer 2 family of transcription factors in development: The cardiac factor BBF-1 is an early marker f or cardiogenesis. *Mol. Cell Biol.* 14, 5130–5138.

Grepin, C., Dagnino, L., Robitaille, L., Haberstroh, L., Antakly, T., and Nemer, M. (1994). A hormone-encoding gene identifies a pathway for cardiac but not skeletal muscle gene transcription. *Mol. Cell Biol.* 14, 3115–3129.

Harland, R. M. (1991). In situ hybridization: An improved whole-mount method for *Xenopus* embryos. *Methods Cell. Biol.* 36, 685–695.

Heikinheimo, M., Scandrett, J. M., and Wilson, D. B. (1994). Localization of transcription factor GATA-4 to regions of the mouse embryo involved in cardiac development. *Dev. Biol.* 164, 361–373.

Hopwood, N. D., and Gurdon, J. B. (1990). Activation of muscle genes without myogenesis by ectopic expression of MyoD in frog embryo cells. *Nature* 347, 197–200.

Ip, H. S., Wilson, D. B., Heikinheimo, M., Tang, Z., Ting, C.-N., Simon, M. C., Leiden, J. M., and Parmacek, M. S. (1994). The GATA-4 transcription factor transactivates the cardiac muscle-specific troponin C promoter-enhancer in nonmuscle cells. *Mol. Cell Biol.* 14, 7517–7526.

Jacobson, A. G., and Sater, A. K. (1988). Features of embryonic induction. *Development* 104, 341–359.

Kao, K. R., and Elinson, R. P. (1988). The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced *Xenopus laevis* embryos. *Dev. Biol.* 127, 64–77.

Kelley, C., Blumberg, H., Zon, L. I., and Evans, T. (1993). GATA-4 is a novel transcription factor expressed in endocardium of the developing heart. *Development* 118, 817–827.

Ko, L. J., and Engel, J. D. (1993). DNA-binding specificities of the GATA transcription factor family. *Mol. Cell Biol.* 13, 4011–4022.

Krieg, P. A., and Melton, D. A. (1984). Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. *Nucleic Acids Res.* 12, 7057–7070.

Laverriere, A. C., NacNeill, C., Mueller, C., Poelmann, R. E., Burch, J. B. E., and Evans, T. (1994). GATA-4/5/6: A subfamily of three transcription factors expressed in developing heart and gut. *J. Biol. Chem.* 269, 23177–23184.

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

Litvin, J., Montgomery, M. O., Goldhamer, D. J., Emerson, C. P. J., and Bader, D. M. (1993). Identification of DNA-binding proteins in the developing heart. *Dev. Biol.* 156, 409–417.

Logan, M., and Mohun, T. (1993). Induction of cardiac muscle differentiation in isolated animal pole explants of *Xenopus laevis* embryos. *Development* 118, 865–875.

McBride, K., Robitaille, L., Tremblay, S., Argentin, S., and Nemer, M. (1993). Fos/jun repression of cardiac-specific transcription in quiescent and growth-stimulated myocytes is targeted at a tissue-specific cis element. *Mol. Cell Biol.* 13, 600–612.

Merika, M., and Orkin, S. H. (1993). DNA-binding specificity of GATA family transcription factors. *Mol. Cell Biol.* 13, 3999–4010.

Miner, J. H., Miller, J. B., and Wold, B. J. (1992). Skeletal muscle phenotypes initiated by ectopic MyoD in transgenic mouse heart. *Development* 114, 853–860.

Molkentin, J. D., Kalvakolanu, D. V., and Markham, B. E. (1994). Transcription factor GATA-4 regulates cardiac muscle-specific expression of the α-myosin heavy-chain gene. *Mol. Cell Biol.* 14, 4947–4957.

Nascone, N., and Mercola, M. (1995). An inductive role for the endoderm in *Xenopus* cardiogenesis. *Development* 121, 515–523.

Navankasattusas, S., Zhu, H., Garcia, A. V., Evans, S. M., and Chien, K. R. (1992). A ubiquitous factor (HF-1a) and a distinct muscle factor (HF-1b/MEF-2) form an E-box-independent pathway for cardiac muscle gene expression. *Mol. Cell Biol.* 12, 1469–1479.

Ness, S. A., and Engel, J. D. (1994). Vintage reds and whites: Combinatorial transcription factor utilization in hematopoietic differentiation. *Curr. Opin. Genet. Dev.* 4, 718–724.

Nieuwkoop, P. D., and Faber, J. (1967). "Normal Table of *Xenopus laevis* (Daudin)." North-Holland, Amsterdam.

Orkin, S. H. (1992). GATA-binding transcription factors in hematopoietic cells. *Blood* 80, 575–581.

Pevny, L., Lin, C.-S., D'Agati, V. D., Simon, M. C., and Orkin, S. H. (1995). Development of hematopoietic cells lacking transcription factor GATA-1. *Development* 121, 163–172.

Pevny, L., Simon, M. C., Robertson, E., Klein, W. H., Tsai, S.-F., D'Agati, V., Orkin, S. H., and Costantini, F. (1991). Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* 349, 257–260.

Sartorelli, V., Hong, N. A., Bishopric, N. H., and Kedes, L. (1992). Myocardial activation of the human α-actin promoter by helix-loop-helix proteins. *Proc. Natl. Acad. Sci. USA* 89, 4047–4051.

Sartorelli, V., Kurabayashi, M., and Kedes, L. (1993). Muscle-specific gene expression. A comparison of cardiac and skeletal muscle transcription strategies. *Circ. Res.* 72, 925–931.

Sater, A. K., and Jacobson, A. G. (1989). The specification of heart mesoderm occurs during gastrulation in Xenopus laevis. *Development* 105, 821–830.

Sater, A. K., and Jacobson, A. G. (1990). The role of the dorsal lip in the induction of heart mesoderm in *Xenopus laevis*. *Development* 108, 461–470.

Tamura, S., Wang, X.-H., Maeda, M., and Futai, M. (1993). Gastric DNA-binding proteins recognize upstream sequence motifs of parietal cell-specific genes. *Proc. Natl. Acad. Sci. USA* 90, 10876–10880.

Thuerauf, D. J., Hanford, D. S., and Glembotski, C. C. (1994). Regulation of rat brain natriuretic peptide transcription. *J. Biol. Chem.* 269, 17772–17775.

Tonissen, K. F., Drysdale, T. A., Lints, T., Harvey, R. P., and Krieg, P. A. (1994). Xnkx-2.5, a *Xenopus* gene related to Nkx-2.5 and *tinman:* Evidence for a conserved role in cardiac development. *Dev. Biol.* 162, 325–328.

Tsai, F.-Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W., and Orkin, S. H. (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* 371, 221–226.

Whyatt, D. J., deBoer, E., and Grosveld, F. (1993). The two zinc finger-like domains of GATA-1 have different DNA-binding specificities. *EMBO J.* 12, 4993–5005.

Yu, Y.-T., Breitbart, R. E., Smoot, L. B., Lee, Y., Mahdavi, V., and Nadal-Ginard, B. (1992). Human myocyte-specific enhancer factor 2 comprises a group of tissue-restricted MADS box transcription factors. *Genes Dev.* 6, 1783–1798.

Zhang, C., and Evans, T. (1994). Differential regulation of the two xGATA-1 genes during *Xenopus* development. *J. Biol. Chem.* 269, 478–484.

Zhu, H., Nguyen, V. T. B., Brown, A. B., Pourhosseini, A., Garcia, A. V., van Bilsen, M., and Chien, K. (1993). A novel, tissue-restricted zinc finger protein (HF-1b) binds to the cardiac regulatory element (HF-1b/MEF-2) in the rat myosin light-chain 2 gene. *Mol. Cell Biol.* 13, 4432–4444.

Zon, L. I., Mather, C., Burgess, S., Bolce, M. E., Harland, R. M., and Orkin, S. H. (1991). Expression of GATA-binding proteins during embryonic development in *Xenopus laevis*. *Proc. Natl. Acad. Sci. USA* 88, 10642–10646.

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