

GATA-4 is a novel transcription factor expressed in endocardium of the developing heart

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SUMMARY

We have isolated and characterized *Xenopus* cDNA clones for a new transcription factor that represents an early marker for the developing heart. The cDNAs encode a protein that we have designated GATA-4; it contains the highly conserved DNA-binding domain that characterizes this family of cell-type restricted transcriptional activators. Whole-embryo in situ analysis of *Xenopus* embryos demonstrates that the GATA-4 gene is transcribed in presumptive cardiac ventral mesoderm at the time that bilateral progenitors fuse and form the cardiac tube. GATA-4 is therefore the earliest molecular marker of cardiogenesis yet characterized. By stage 30, the GATA-4 mRNA is expressed in the developing atria and ventricles; at stage 38, cross-sections reveal that the gene is active in the endocardial layer, but not in myocardium. By stage 40, GATA-4 message is detected in the great vessels. In the adult frog, the GATA-4 gene is highly transcribed in heart and gut; lower levels of

message are detected in various endoderm-derived tissues and gonads. Expression in the stomach is largely confined to the epithelium. The GATA-4 gene is first activated at stage 11; mRNA is initially present throughout the marginal zone of explants and later partially localized to the ventral marginal zone. GATA-4 mRNA is also detected at high levels in cultured endodermal explants derived from the vegetal region of early embryos. In mesoderm induction experiments, GATA-4 transcription is not induced in animal caps treated with activin or bFGF. The GATA-4 gene may provide a new early marker for studying the inductive processes that lead to the formation of the cardiovascular system and for the specification of the endocardial lineage.

Key words: GATA-binding proteins, transcription factor, *Xenopus laevis*, cardiovascular, endocardium, cardiac mesoderm, endoderm

INTRODUCTION

The formation of cardiac tissue has provided an excellent developmental system to study inductive processes and specification of cell lineages during early vertebrate embryogenesis. Heart formation involves the definition of specific tissues during embryogenesis: the endocardium, myocardium and epicardium. The endocardium consists of endothelial cells with specialized functions including cardiac jelly production, valve assembly and blood-heart interface. Endocardial cells may also direct some processes in cardiac morphogenesis. The myocardium functions as contractile muscle which drives systemic blood flow. The epicardium defines an outer layer, which bounds other mediastinal structures and may have protective or directive functions during embryogenesis. Certain descriptive aspects of heart formation have been clearly demonstrated by embryological techniques (Hirakow et al., 1987; Icardo, 1988). Further progress in the field has been largely hampered by a relative lack of early markers specific for committed cardiac lineages. Consequently, very little is

presently understood concerning the molecular regulation of cardiovascular development and induction.

The presumptive cardiac tissue is derived from two bilateral regions of the inner marginal zone mesoderm (for a review, see Jacobson and Sater, 1988). By vital dye staining, the cardiac progenitors have been localized to the upper flanks on each side of the mesodermal mantle adjoining the neural folds (Wilens, 1955; Keller, 1976). The progenitors migrate and meet at midline during the early tailbud tadpole stage. The rudiment of the endocardium is formed as presumptive endocardial cells accumulate in the midline to form a long thin-walled vascular tube. Ultimately, the parietal layer of the fused mesodermal mantle persists as the pericardium (the epithelial wall of the pericardial cavity) while the visceral layer adheres to the endocardial tube, differentiates into muscle and forms the myocardium. By stage 30 of *Xenopus* development [(Nieuwkoop and Faber, 1967), approximately 36 hours after fertilization] the tube starts to develop a bend to the right. At stage 34, the vascular tree has developed, cardiac heart beating starts and circulation begins.

It appears that both early and late inductive events are required for the specification of heart tissue from presumptive cardiac mesoderm in the marginal zone. As predicted by the three-signal model for mesoderm induction in *Xenopus*, a predominant ventral vegetal signal of endodermal origin induces overlying cells to become mesoderm (Smith, 1989). A dorsal signal induces mesoderm in the marginal zone to acquire organizer activity, which is involved in further regionalizing the presumptive mesoderm. The organizer region appears to participate in the induction of the cardiac program (Jacobson and Sater, 1988). In addition, many observations regarding the timing and tissue interactions required for heart formation in Urodeles have led to the conclusion that the anterior neural plate and fold suppress cardiac development, while pharyngeal endoderm induces cardiac formation. Results from explant experiments indicate that pharyngeal endoderm may not be required for heart induction in *Xenopus* (Jacobson and Sater, 1988).

Numerous experimental manipulations that are known to disrupt the establishment of the dorsal-ventral axis during mesoderm induction are capable of altering normal amphibian heart formation. For example, UV irradiation ventralizes the embryo and leads to decreased size or absence of the heart (Scharf and Gerhart, 1980). LiCl treatment, which dorsalizes the mesoderm, leads to formation of a large radial heart (Kao and Elinson, 1988). Recently much progress has been made in identifying specific growth factors involved in mesoderm formation (Kimelman et al., 1992), although efforts to identify induction of cardiac mesoderm have not progressed due to the lack of early lineage markers.

There is also considerable evidence for the role of specific growth factors in the later inductive interactions during cardiovascular development (Muslin, 1992). For example, Williams and colleagues have shown that both TGF- and PDGF increase the frequency of beating heart formation in hanging-drop explant assays, while bFGF may mimic an endogenous inhibitor of induction by decreasing formation of cardiac tissue (Muslin and Williams, 1991). Clearly, early markers for cardiac induction will be extremely useful in understanding the interactions leading to heart formation.

We present data demonstrating the identification of a gene transcribed in cells of the developing embryonic heart. We have isolated cDNA clones for a new member of the GATA factor family of DNA-binding transcription factors. This protein, which we designate GATA-4, is highly conserved in the characteristic GATA factor DNA-binding domain and specifically recognizes a GATA *cis*-element. Transcription of the GATA-4 gene is restricted to a distinct subset of tissues in the adult frog; RNA levels are particularly high in heart and in endoderm-derived tissues such as the stomach and small intestine. GATA-4 RNA is first detected in cells of the presumptive cardiac tube; transcription appears to be largely restricted to endocardial cells within the heart. This new transcription factor therefore demonstrates at the molecular level a heterogeneity of the vascular system and may provide a useful means to investigate the inductive processes that lead to the formation of the cardiovascular system during embryogenesis.

MATERIALS AND METHODS

Isolation and characterization of cDNAs

A *Xenopus* tadpole liver cDNA library in the zapII vector (Stratagene) was screened under standard conditions (Maniatis et al., 1982) using as probe the full-length chicken GATA-1 cDNA (Evans and Felsenfeld, 1989). Inserts from several purified positive clones were rescued into phagemids and sequenced. Although most clones were clearly related to cGATA-1, one partial clone (desig-

Xgata1a			MDYTTLLTQ	DPDPNYTESG	LASTSEDSQF
Xgata1b			MDYTTLLTQ	DPLQNYTESA	LASTSEDSQF
Xgata2	MEVATDQPRW	MA...HHAVL	NGQHPDSSHHP	GLAHNYMEPT	QLLPPDEVVDV
Xgata3	MEVSABQPRW	VSHPHHPALL	NGQHTDSSHQ	..VHNYMDSF	QYPPPEMDMV
1					
Xgata4a	MYPSSLALTAN	HAQPAYSHDT	PNFLHSTGSP	PVYVPTSRM...	PAMLQS...
Xgata4b	MYPSSLALTAN	HAQPAYSHDT	PNFLHSTGSP	PVYVPTSRM...	PAMLQS...
Xgata1a	LY...GLGGE	SSPGHYGGAV	SSRAVGGFR...	HSPVQTFP
Xgata1b	LY...GLGGE	NSPGHYGGIV	SSRAVGGFR...	HSPVQTFP
Xgata2	FFNHLDSCQN	...PYANSA	HARARVYSQ	AHARLHSGCM	CRPHLLHSPG
Xgata3	LPN.IDAQGN	HVPSYYSNSV	RTVPR..YYP	PH...HGSLV	CHPSILQS...
45					
46					
Xgata4a	LPYLQSCDTA	HQGHHLANHPGWAQT	A.ESHAFNAS	SPHTPTGFSY
Xgata4b	LPYLQSCDTA	HQGHHLANHPGWAQT	ANESHAFNAS	SPHTPTGFSY
Xgata1a	LHWPETS...AGIPS	NLTAYGR...	...STGTLSF	YPSAASALGP
Xgata1b	LHWPETS...AGIPQ	SULTYGR...	...SPGTLPL	YPSAASALGS
Xgata2	LPWLESGRKA	LSAAHHNPW	TVSPFGKAPL	HPAARGG.SL	YPGTSSACPL
Xgata3	...WTDGRKS	LGGPHAAASAW	NL.PFTKPSI	HNNSPGGLSV	YP.PASSASL
89					
90					
Xgata4a	SHSPFVGNSS	ARDGGYQSP	IMGGGARDQY	GNTLVRTGSY	PSFYSYVGD
Xgata4b	SHSPFVGNSS	ARDGGYQSP	IMGGGARDQY	GNTLVRTGSY	PSFYSYVGD
Xgata1a	ITSPPLYSAT	SFLLGSAPPA	EREGSP...K
Xgata1b	ITSPPLYSAT	PFLGGSAPLA	ERDGS...K
Xgata2	SSSH...SS	PHLFGFPPPT	PKDVSPDP..	GPASPPSSSR	LEDKDSI..K
Xgata3	ATGH...SS	PHLFTFPPT	PKDVSPDPSI	STSGTSSSR	HEEKECVVK
139					
140					
Xgata4a	MPPSWAAGHF	EGSMLHSLQG	R.QSLSGRRS	SLEFLEEFPG
Xgata4b	MPPSWATGHF	EGSMLHSLQG	R.QYLPGRIS	SLEFLEEFHG
Xgata1a	FL...ETL	KTERASPLTS	DLLPLEPRSP	SILQVGYIGG	..GGQEF..
Xgata1b	FL...ETL	KTERTPSPLTS	DILSLPRSP	SLLQVGYLGG	..GGQEF..
Xgata2	YQMSLSE.GM	KMEGGSPLR	SLAPMGTQCS	THHPIPTYP	YVPAADHYSS
Xgata3	YQVSLGDTGM	KLESLHP.RN	SMGIGG.VS	AHHPIITYP	Y.....YGA
178					
179					
Xgata4a	EG	RECVNCGAMS	TPLWRRDGTG
Xgata4b	EG	RECVNCGAMS	TPLWRRDGTG
Xgata1a	SLFQ.....	STED	RECVNCGATV	TPLWRRDMSG
Xgata1b	SLFQ.....	STED	RECVNCGATV	TPLWRRDMSG
Xgata2	GLPHPGSLLG	GPASSFTPKQ	RSKRSRCSSE	RECVNCGATA	TPLWRRDGTG
Xgata3	GLFPQGSILD	GSPTHFSSNA	RPKTRSTEG	RECVNCGATS	TPLWRRDGTG
200					
201					
Xgata4a	HYLCNACGLY	HKMNGMNRPL	IKPKRRLSSS	RRAGLCTCNC	HTSTTTLWRR
Xgata4b	HYLCNACGLY	HKMNGINRPL	IKPKRRLSSS	RRAGLCTCNC	HTSTTTLWRR
Xgata1a	HYLCNACGLY	HKMNGQNRPL	IRPKRLIVS	KRAGTQCSNC	HTSTTTLWRR
Xgata1b	HYLCNACGLY	HKMNGQNRPL	IRPKRLIIS	KRAGTQCSNC	HTSTTTLWRR
Xgata2	HYLCNACGLY	HKMNGQNRPL	IKPKRRLSAA	RRAGTCCANC	QTSTTTLWRR
Xgata3	HYLCNACGLY	HKMNGQNRPL	IKPKRRLSAA	RRAGTCCANC	QTSTTTLWRR
250					
251					
Xgata4a	NSEGEFVCNA	CGLYMKLHGV	PRPLAMKES	IQTRKRKPKN	IGKGRKSTGCS
Xgata4b	NSEGEFVCNA	CGLYMKLHGV	PRPLAMKES	IQTRKRKPKN	IGKGRKSTGCS
Xgata1a	NASGDFVCNA	CGLYKHLHNV	NRPLTMKKEG	IQTRNRKVS	..RSKRRKQL
Xgata1b	NAGGDFVCNA	CGLYKHLHNV	NRPLTMKKEG	IQTRNRKVS	..RSKRRKQL
Xgata2	NANGDFVCNA	CGLYKHLHNV	NRPLTMKKEG	IQTRNRKMSN	..KSKRKNKG
Xgata3	NANGDFVCNA	CGLYKHLHNI	NRPLTMKKEG	IQTRNRKMSJ	..KSKRKNKG
300					
301					
Xgata4a	STSAANSPSS	VTNSDPTPVL	KTEPNITSQY	SGQAIVPVSQ	GHSQTDDLNV
Xgata4b	STSAANSPSS	VTNTDPTPVL	KTEPNITSHY	PGQAIVPVSQ	GQSQTDDLNV
Xgata1a	DNPFEPKAG	VEEPSYP..F	GPLLFHGQMP	PMGHM..LNP
Xgata1b	ENPFEPKAG	VEEPSYPQ..F	GPLLFHGQMP	TMGHM..LSP
Xgata2	SECFEELSRC	MQEKSS.P..F	SAAALASHMA	PMGHAPFSH
Xgata3	HDSLEDYP..	...KSS.S..F	SPAALSRHMS	SLSHI..PFSS
350					
351					
Xgata4a	GSHELKMPD	EYTSPTALS	QQSGLSVPLR	QESWCALALA	
Xgata4b	GSHELKMPD	EYTSPTALS	QQ...SVPLR	QESWCALALA	
Xgata1a	PHHFLQ...	...SPRISH	SAPAVSY..R	QAASGVTPP	
Xgata1b	PHHFLQ...	...SPRMSH	STPAVSY..R	YSASGVTPPE	LA
Xgata2	SGHILQ...	...TPTPIH	PSSLSFGPH	HSSSMVTAMG	
Xgata3	SSHMLT...	...TPTPMH	PSSLSFGPH	HSSSMVTAMG	

Fig. 1. The amino acid sequence of *Xenopus* GATA-4 as predicted from the cDNA clones is shown compared to the other known *Xenopus* GATA factors. Numbering refers to amino acids and is relative to xGATA-4a. Brackets indicate the highly conserved DNA-binding domain containing the two characteristic related fingers. Genbank nucleotide accession numbers are xGATA-4a: L13701, xGATA-4b: L13702.

nated Ex11) was found to differ considerably, yet the predicted amino acid sequence was 90% identical in the characteristic GATA factor DNA-binding domain. This partial cDNA was used as a probe to screen a stage 13 *Xenopus* embryonic gt10 cDNA library. Multiple positive clones were purified and inserts were found to belong to two subclasses. One class was identical to Ex11 and is designated xGATA-4a; the other class is greater than 90% identical at the amino acid level but differs substantially at wobble nucleotide bases and is designated xGATA-4b. The largest insert representing each class (clone 35 for xGATA-4a and clone 23 for xGATA-4b) was subcloned into the pBluescript II SK- vector and sequenced on both strands using the Sequenase kit (USB). We employed exoIII deletion subclones and in some cases synthesized specific oligomer sequencing primers. Regions of additional clones were also sequenced to be certain that the primary structure for both strands of each cDNA was confirmed on at least two independent isolates.

Functional analysis of recombinant GATA-4

A 480 bp fragment of the clone 35 insert encompassing amino acids 191-350 (Fig. 1) was subcloned in frame with the glutathione

S-transferase coding sequence in the pGEX-3 vector (Smith and Johnson, 1988). The resulting GST/GATA-4 fusion protein includes most of the N-terminal finger, all of the DNA-binding C-terminal finger and most of the 3' coding region of GATA-4a. Expression of fusion protein was induced with IPTG; after 5 hours bacterial cells were pelleted and lysed in SDS sample buffer. Lysates were electrophoresed in SDS-PAGE gels and the induced recombinant protein localized by brief Coomassie blue staining in H₂O. After crushing the gel slice, protein was eluted for 3 hours into 50 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 0.1 mM EDTA, 5 mM DTT. Gel debris was pelleted and protein concentrated using Centricon filters. The resulting soluble protein was used as antigen to generate rabbit polyclonal antisera or to initiate an immune response in mice. Mouse spleen cells were subsequently used to generate hybridomas and a GATA-4-specific monoclonal antibody-secreting clone was identified. In some experiments, the fusion protein was TCA precipitated, denatured in guanidine, allowed to renature by dialysis and used in gel mobility-shift assays essentially as described (Evans and Felsenfeld, 1989).

For expressing GATA-4 in cell culture, the full-length GATA-4a insert was cloned into the pXM mammalian expression vector

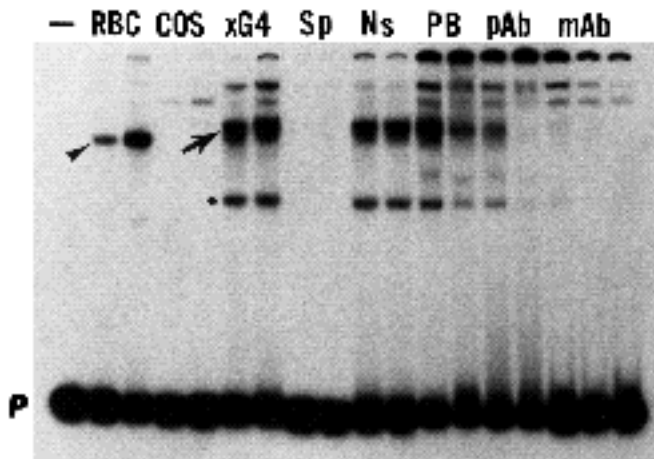


Fig. 2. GATA-4 binds specifically to a GATA *cis*-element. Gel mobility-shift assays were performed using a labeled oligomer probe (P) containing a GATA *cis*-element from the chicken β -globin promoter [TE72/73 (Evans and Felsenfeld, 1991)]. The probe was incubated in the absence of added extract (-) or with 1 or 2 μ l (left to right) of nuclear extract prepared from chicken erythroid cells containing cGATA-1 (RBC), COS cells transfected with the pXM expression vector without an insert (COS), or COS cells transfected with the xGATA-4 expression plasmid (xG4). All other samples included 2 μ l of the xG4 nuclear extract in addition to competitor. Competitors were a 30- or 60-fold excess (left to right) of unlabeled TE72/73 oligomer as a specific DNA competitor (Sp), the same amounts of unlabeled TE78/79 (Evans and Felsenfeld, 1991), which is identical to TE72/73 except for a mutation of the GATA motif, as a non-specific DNA competitor (Ns), 1 or 2 μ l of pre-immunized rabbit serum (pb), 1 or 2 μ l of polyclonal rabbit anti-GATA-4 serum (pAb), 1, 2 or 4 μ l of protein A affinity-purified monoclonal antibody directed against GATA-4 (mAb). Note that the complex formed by xGATA-4 (arrow) is of slightly higher mobility compared to that formed with cGATA-1 (arrowhead), consistent with the larger molecular weight of xGATA-4. The asterisk indicates a complex formed to a partial degradation product of GATA-4, which retains binding activity.

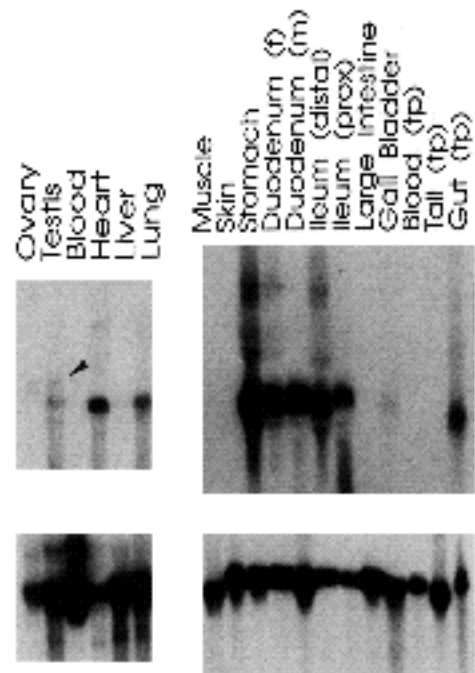


Fig. 3. The GATA-4 gene is transcribed in heart, gonads and endodermal derivatives. Northern blots containing 10 μ g of total cellular RNA were hybridized first to a GATA-4 cDNA probe. After exposure, the blots were stripped and reprobed using an EF-1 cDNA probe to demonstrate RNA integrity. RNA standards (not shown) were used to determine an approximate size of the GATA-4 message as 1.8 kb. The arrow indicates a faint but reproducible slightly larger message found in ovary and testis. Additional minor higher molecular weight RNA species are evident in tissues that express high levels of GATA-4. Small intestine was divided into duodenum (f, female; m, male) and a distal or proximal (prox) segment of the ileum. Additional tissues were derived from 2-week-old tadpoles (tp). In the experiment shown, transcription in the lung was unusually high. Also, although not obvious in the reproduction, the GATA-4 message was consistently detected in liver.

and co-transfected into COS cells by the DEAE-dextran method as previously described (Tsai et al., 1989). After 48-60 hours, lysates were prepared (Andrews and Faller, 1991) and analyzed for GATA-binding activity by the gel mobility-shift assay using the TE72/73 oligomer probe as described (Yang and Evans, 1992). In competition studies, specific oligomers or antibodies were added to the gel mobility-shift reaction prior to addition of COS cell nuclear extract. Western blots demonstrated that the monoclonal antibody does not recognize GATA-1, -2, or -3 in lysates prepared from COS cells expressing these proteins.

Quantitative PCR

Assays were performed as described (Rupp and Weintraub, 1991; Zon et al., 1991) using randomly primed RNA isolated from

specific stages, tissues or explants. To avoid amplification of genomic DNA contamination, PCR primers were designed to encompass an exon boundary of the GATA-4 genes based on the known structure of the chicken, murine and human GATA-1 genes (Tsai et al., 1991; Hannon et al., 1991). For each experiment, the number of cycles was optimized to be certain that the amplification occurs in a linear manner. PCR reactions included trace ^{-32}P dCTP to allow direct quantification.

PCR primers used in this study were as follows:

GATA-4: 5'-AGACCACTTATCAAGCCACAG (F, TE163)

5'-TGGGATGTGATGTTGGGTTTC (R, TE165); note that these primers detect both GATA-4a and GATA-4b cDNA, corresponding to the region encoding amino acids 218-329 of Fig. 1.

EF-1 : 5'-CCTGAATCACCCAGGCCAGATTGGTG (F);

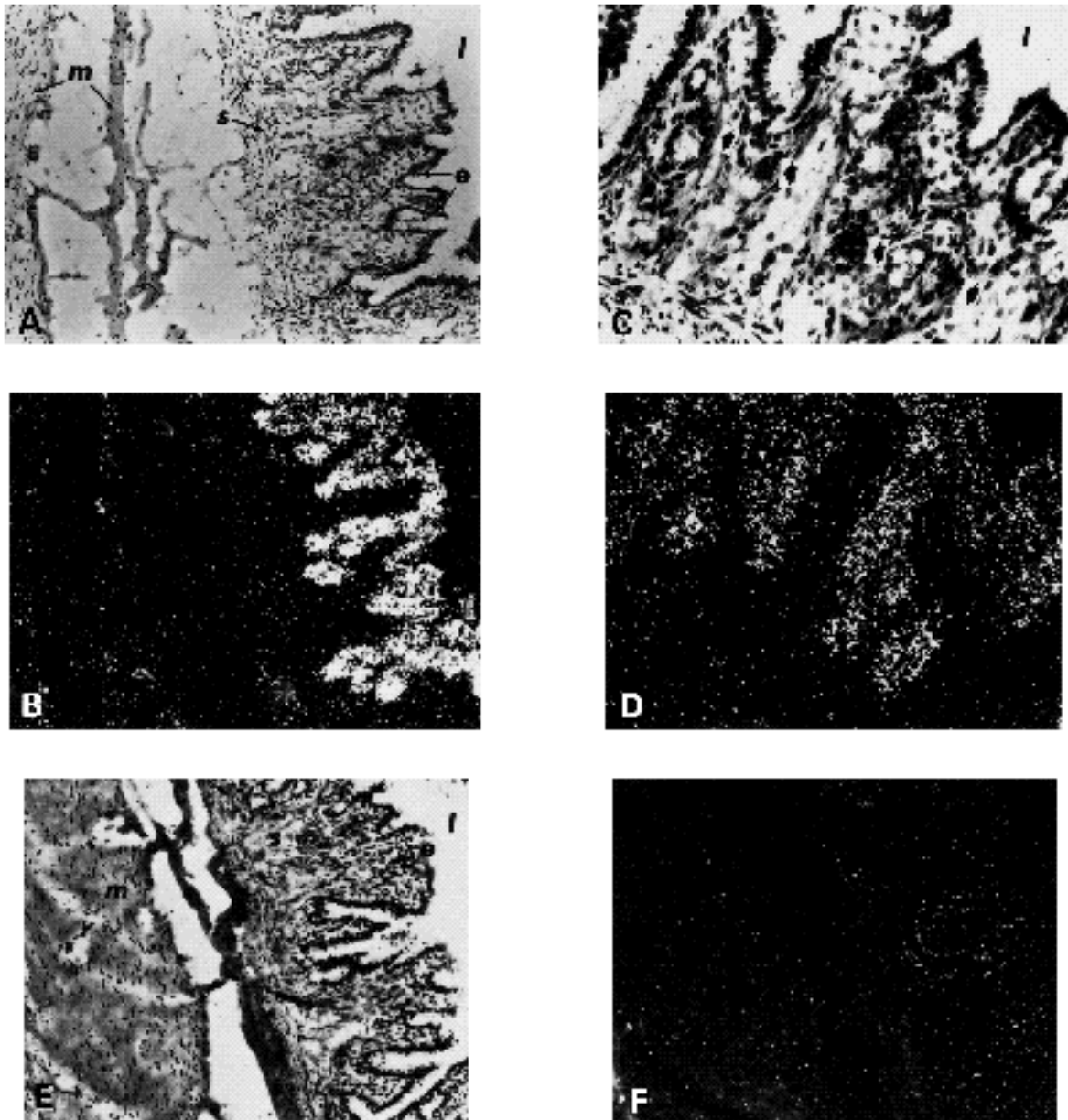


Fig. 4. GATA-4 is highly transcribed in gut epithelium. Sections of adult *Xenopus* stomach were hybridized in situ to antisense (A-D) or control sense (E,F) ^{35}S -labeled RNA probes derived from the GATA-4a cDNA. (A,C,E) Bright-field photographs; (B,D,F) corresponding dark-field views. Indicated in A are the lumen (l), epithelium (e), submucosa (s) and smooth muscle (m). C is a higher magnification view of the section shown in A. The arrows in C indicate regions of the lumenal crypts with particularly high levels of GATA-4 RNA.

5'-GAGGGTAGTCTGAGAAGCTCTCCACG (R); nucleotide positions 1088-1311 (Krieg et al., 1989).

ODC: 5'-CACATGTCAAGCCAGTTC (F)

5'-GCCTATACATTGATGCTG (R); corresponds to nucleotides 1310-1691 (Isaacs et al., 1992).

Xenopus embryos and induction assays

Female frogs were induced to lay eggs by gonadotropin injection. The eggs were fertilized in water using macerated testes and allowed to develop to the desired stage (Nieuwkoop and Faber, 1967) in $0.1 \times$ MBS. For explant assays, animal caps, vegetal pole regions, presumptive mesoderm, or marginal zone regions were isolated from staged embryos using eyebrow hair knives and incubated in $0.1 \times$ MBS.

RNA analysis and in situ hybridizations

For northern blots, RNA was prepared from staged embryos, tadpoles, or adult frog tissues by homogenization in RNazol according to the manufacturer (Cinna Biotech), followed by two ethanol precipitations. Agarose/formaldehyde gels were blotted onto Genescreen plus and hybridized (Church and Gilbert, 1984) to probes radiolabeled by random priming. The same blots were washed of GATA-4 probe and rehybridized to labeled EF-1 cDNA probe (a gift from D. Melton).

Frozen ($8 \mu\text{m}$) sections of adult *Xenopus* heart or stomach were cut on a microtome and fixed onto slides. Following pronase digestion, re-fixing and acetylation essentially as described (Awgulewitsch et al., 1986), sections were hybridized under coverslips to antisense or sense ^{35}S -labeled RNA probes. Probes were made from linearized templates containing a 581 bp insert of the GATA-4A cDNA (corresponding to the region encoding amino acids 157-352 of Fig. 1) using appropriate bacteriophage polymerases (T3 or T7). After purifying the RNA probes by DNaseI digestion, phenol extraction and ethanol precipitation, they were partially hydrolyzed at 60°C for 20 minutes in pH 10.4 carbonate buffer. Hybridizations were performed under standard (50% formamide) conditions at 55°C for 14 hours. Following RNaseA digestion, sections were washed extensively (overnight) at 50°C . After dehydration, slides were dipped in emulsion and exposed for 4 days at 4°C . Sections were counter stained in haematoxylin and eosin prior to photography.

Whole-mount hybridizations were performed on staged albino embryos as described (Harland, 1991). The GATA-4 anti-sense or control sense-strand RNA probes were generated from linearized plasmids containing full-length cDNA inserts. The probes were synthesized to incorporate digoxigenin-11 UTP using bacteriophage RNA polymerases (T3 or T7). The hybridization signal was detected by a secondary antibody against digoxigenin coupled to alkaline phosphatase, followed by the phosphatase reaction. For staining analysis, whole embryos were cleared using a 2:1 solution of BB/BA (Harland, 1991). For some experiments, stained embryos were fixed in Bouin's, embedded in paraplast and cut into $8 \mu\text{m}$ sections.

To confirm the specificity of probes used in experiments analyzing GATA-4 RNA, complementary experiments have been performed using probes derived from GATA-1, -2, or -3 cDNA clones; we find that DNA or anti-sense RNA probes do not cross-hybridize to related GATA factor messages in northern blotting or in situ hybridization experiments. For example, on northern blots, GATA-1, -2, or -3 cDNA probes (even containing the sequences of the conserved DNA-binding domain) do not detect any signal in RNA prepared from heart samples and a full-length GATA-4 cDNA probe does not detect the GATA-1 message present in RNA from erythroid cells. Likewise, entirely distinct patterns of hybridization are detected using the whole-mount in situ hybridization assay (C.K. and L.I.Z., unpublished results).

RESULTS

Identification of a novel member of the GATA-binding transcription factor family

In order to isolate *Xenopus* cDNA clones related to the GATA family of transcription factors, we screened a tadpole liver library using as probe the chicken GATA-1 cDNA. In addition to identifying primarily xGATA-1 clones, a distinct cDNA was discovered; the predicted amino acid sequence contained the conserved central DNA-binding domain characteristic of the GATA factor family (Orkin, 1990). The sequence of this clone diverged significantly from GATA-1 outside of the finger region and appeared likely to be truncated at the 5' border of this conserved domain. This initial clone was used to identify full-length cDNAs from a stage 13 *Xenopus* whole embryo library. Multiple clones were isolated of two distinct versions which are approximately 90% identical at the nucleotide level. We presume that the two versions represent duplicated homologues of the same gene, due to *Xenopus* tetraploidy. Based on the presence of the conserved DNA-binding domain, we have designated the predicted protein xGATA-4 and refer to the two cDNA subtypes as xGATA-4a and xGATA-4b; the predicted primary structure of the xGATA-4 proteins is shown in Fig. 1, compared to the sequence of the other *Xenopus* GATA factors.

The xGATA-4a and xGATA-4b clones encode open reading frames of 390 and 387 amino acids, respectively. The length of the largest cDNAs (about 1.8 kb) are consistent with the size of the major RNA species identified on northern blots (see below). The sequences of the cDNA clones include

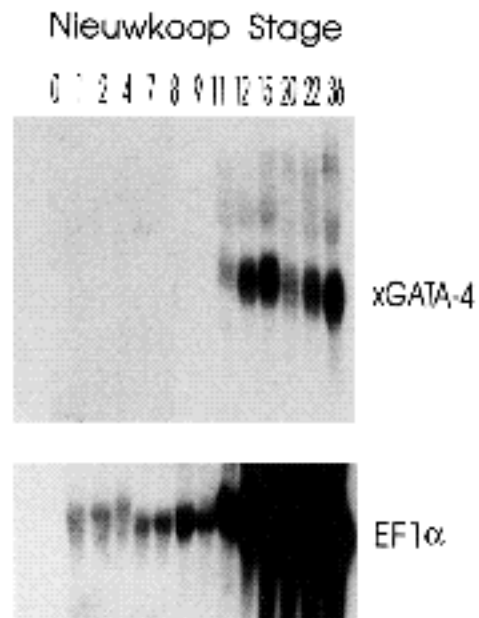
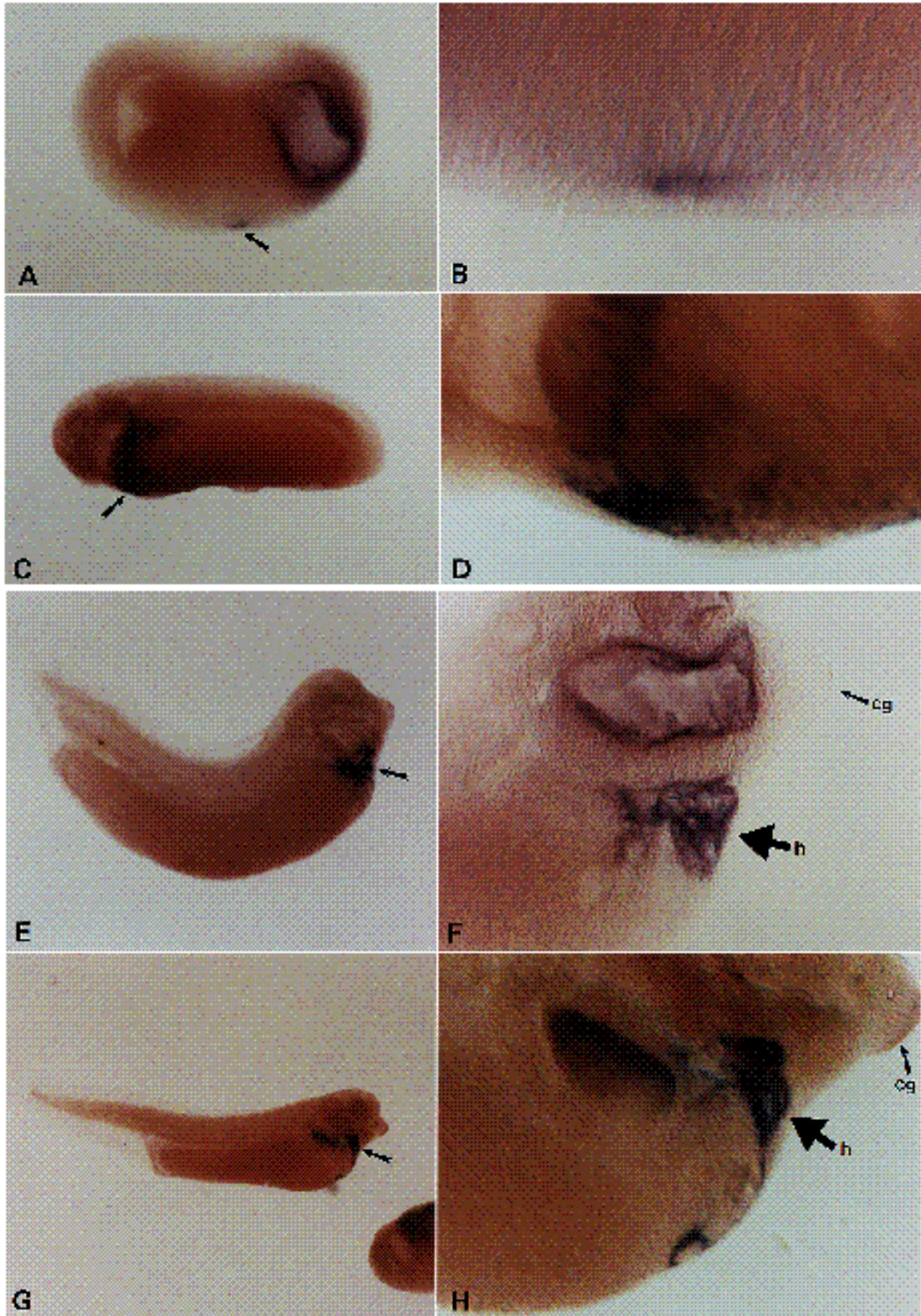


Fig. 5. Activation of the GATA-4 gene during development. Northern blots were used to detect RNA from whole embryos taken at the indicated stage of development. Each lane contained $20 \mu\text{g}$ of total RNA. The blot was reprobbed for EF-1 message. Note that zygotic EF-1 begins to accumulate at stage 8; GATA-4 is first detected around stage 11.



in-frame stop codons in both the 3' region and 5' to the presumptive ATG initiation codon. Within the conserved finger/basic region (brackets in Fig. 1) the GATA-4 proteins are identical and differ from all other GATA factors at only 16 of 112 residues. Outside of this region, there are no obvious stretches of significant similarity between GATA-4 and the other factors, or, by searching the database, between GATA-4 and other known proteins.

GATA-4 functions as a DNA-binding factor

A fragment of the xGATA-4a cDNA (encoding 160 amino acids including the DNA-binding domain and most of the C-terminus) was cloned into a pGEX vector and used to express a recombinant GST-GATA-4 fusion protein in *E. coli*. Following induction to express the recombinant protein, cell lysates were electrophoresed through an SDS-polyacrylamide gel; the recombinant protein was identified by staining and eluted from the gel slice. Following denaturation in Gn-HCl, the protein was allowed to renature by dialysis and shown to bind specifically to a GATA *cis*-element derived from the chicken β -globin promoter (not shown), confirming that the cDNA encodes a functional GATA factor with regard to DNA-binding. The full-length cDNA was also inserted into a mammalian expression vector; transfection of the resulting plasmid into COS cells generates GATA-4 protein, which interacts specifically with the GATA *cis*-element, as shown by the gel mobility-shift assay (Fig. 2). This GATA-4/DNA complex is specifically competed by unlabeled GATA-containing oligomer, but not by an oligomer which is identical except that the GATA motif has been mutated. Polyclonal serum or a monoclonal antibody generated against the GST-GATA-4 fusion protein specifically interferes with the DNA-binding activity expressed in the COS cell system. These results indicate that GATA-4 protein should be capable of productive interactions with putative target genes containing GATA *cis*-elements.

GATA-4 is restricted to distinct tissue types

Northern blots demonstrate that GATA-4 transcription is regulated in different tissue types and the patterns are distinct from other GATA factors (Fig. 3). A single predominant 1.8 kb message is detected at highest levels in RNA prepared from adult *Xenopus* heart or gut (stomach or throughout the small intestine). Much lower levels are found in lung, gall bladder and liver RNA. The GATA-4 message

Fig. 6. GATA-4 is an early marker for the developing heart. Embryos were analyzed by whole-mount in situ hybridization for GATA-4 RNA. Stages are as follows: 18 (A,B); 26 (C,D); 34 (E,F); 40 (G,H). The photographs shown in B,D,F and H are high magnification views of the embryos shown in A,C,E and G, respectively. Arrows indicate detection of GATA-4 message by alkaline phosphatase staining in the region of the developing heart; non-specific staining around the residual blastocoel (for example, in A) is seen with control sense-strand probes. Note that by stage 40, GATA-4 message is detected in other organs where transcription is high in adults, such as the stomach. In all cases, dorsal is towards the top. In C and D, anterior is to the left, otherwise anterior is to the right. Large arrows in F and H indicate staining in the developing heart (h). For reference, cg indicates the position of the cement gland.

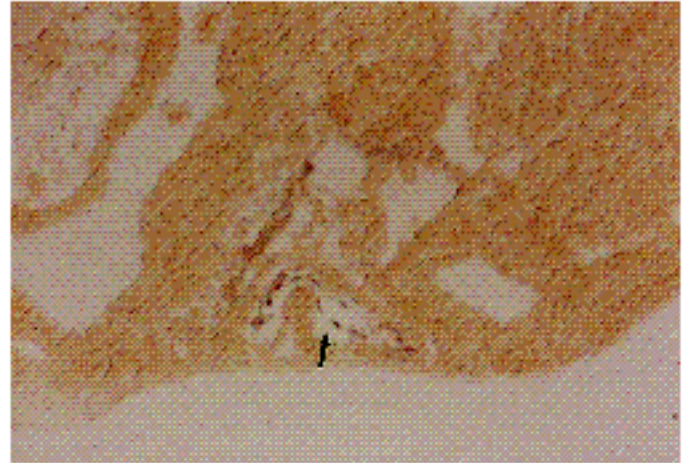


Fig. 7. The GATA-4 gene is activated in the endocardium of the developing heart. Shown is a sagittal section of a stained stage 38 embryo which has undergone the whole-mount in situ hybridization procedure. Ventral is towards the bottom and anterior is to the left. Note that only the inner layer (endocardium) of the developing heart is stained. In this sample, the endocardial cells have separated from the overlying primitive myocardium in the region consisting of cardiac jelly (arrow).

was not detected by northern blot in RNA from whole blood, muscle, skin, or large intestine. Very low levels of the transcript are found in testis, while an additional slightly larger RNA is found at low abundance in both ovary and testis. Similar results were obtained using RNA from different stages of tadpoles: the GATA-4 message is most abundant in heart and gut. It is not detected in tadpole RNA isolated from blood, tails (muscle and skin), or eyes (Fig. 3 and data not shown). Because of the presence of two GATA-4 genes in the frog, we have analyzed whether the two genes are differentially transcribed in distinct tissues. Following amplification of randomly primed RNA from various tissues, the GATA-4 cDNA was digested with restriction enzymes which are polymorphic for either the xGATA-4a or xGATA-4b cDNA. We find (data not shown) that the two GATA-4 genes contribute equally to the RNA levels detected by northern blot, in both heart and gut.

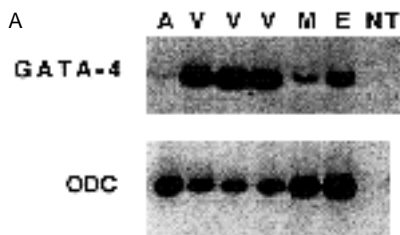
GATA-4 is localized within stomach tissue to the epithelium

We have attempted to determine more precisely the cellular distribution of xGATA-4 message in the heart and stomach by in situ hybridization of anti-sense RNA probes to frozen adult tissue sections. Control sections were hybridized to sense-strand probes. Examples of such experiments using sectioned adult stomach tissue are shown in Fig. 4. Strikingly, the signal is localized to the layer of epithelial cells lining the lumen. Particularly high levels of message appear to be localized in small patches of cells at the base of the luminal crypts.

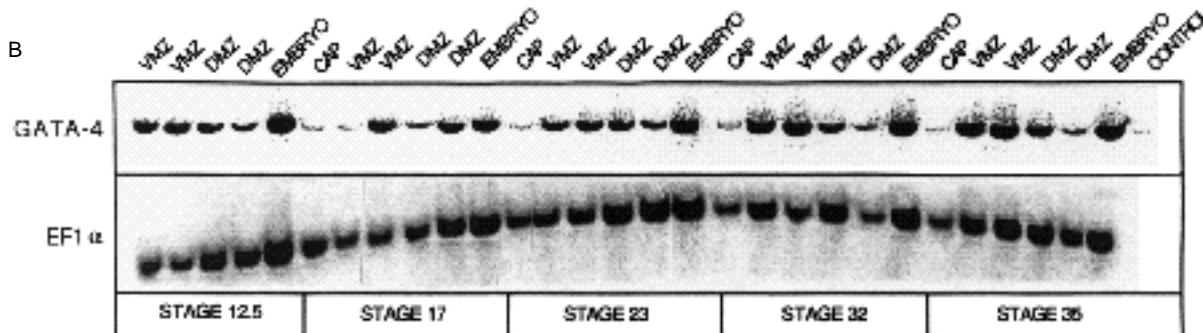
Regulation of GATA-4 during embryogenesis

The GATA-4 message is not present in oocytes or blastula stage embryos but is activated during gastrulation at about stage 11 (Fig. 5). This result has been confirmed by RT/PCR

Fig. 8. Quantitative RT/PCR analysis of GATA-4 RNA in embryo explants. (A) The GATA-4 gene is activated in the presumptive mesoderm and vegetal explants. Embryos were dissected at stage 8 and explants were incubated in $0.1 \times$ MBS until 30 hours



postfertilization. RNA was then prepared and analyzed as described in Materials and Methods. Multiplex PCR employed primers specific for either GATA-4 cDNA or ornithine decarboxylase (ODC) cDNA. The latter transcripts were co-analyzed as an internal control. A, animal caps; V, vegetal explants (three independent experiments are shown); M, presumptive mesoderm; E, whole embryo; NT, no RT included control. (B) The GATA-4 gene is activated in both the ventral marginal zone (VMZ) and the dorsal marginal zone (DMZ). Marginal zones were dissected at stage 12.25 and incubated in buffer alone until control embryos reached the indicated stages. RNA was analyzed as above. Two independent experiments are shown for each VMZ and DMZ explant. In the control lane reaction, RT was omitted.



(not shown); the initial expression coincides with the timing of activation for the other GATA factor genes (Zon et al., 1991). A transient drop in RNA levels after gastrulation is followed by a further rise in late neurula stages. The 1.8 kb RNA species is then maintained throughout tadpole stages and into adulthood. Note that zygotic activation of the *Ef-1* gene (Krieg et al., 1989) occurs somewhat earlier, at the mid-blastula transition.

We have performed whole-mount embryo in situ hybridization experiments to determine if the early activation of GATA-4 is localized during embryogenesis. Staged embryos were hybridized with antisense probes for xGATA-4a (Fig. 6A-H). GATA-4 is expressed at stage 18 of development in ventral cardiac mesoderm (Fig. 6A,B, see arrow). The analysis demonstrates that GATA-4 appears to be expressed in the primordial vascular 'tube' and therefore is an early marker of cardiogenesis. By stage 26 (Fig. 6C,D), the cardiac tube migrates and folds toward the anterior ventral region of the embryo. By stage 34 (Fig. 6E,F), GATA-4 transcription is largely confined to the heart and to the great vessels. By stage 40 (Fig. 6G,H), GATA-4 transcription has now increased extensively in the heart and is also detected in distinct organs. Essentially identical results were obtained if the xGATA-4b antisense RNA was used as a probe (not shown). Control experiments using sense probe demonstrated the specificity of the signal (not shown).

Analysis of sagittal sections from these stained embryos reveal that cardiac expression of GATA-4 RNA is restricted to endocardial cells rather than the myocardium. This is clearly seen in the example shown in Fig. 7 (using stage 38 embryos), in which the endocardium has produced sufficient cardiac jelly to be structurally distinct from the myocardium. As mentioned above, we have attempted in situ analysis of adult myocardial cross sections; we did not detect consistent signal above background. Adult endocardial cells are rare in

these sections and it might therefore be difficult to detect GATA-4 expression. Based on these results, the GATA-4 gene represents a very early molecular marker for endocardial-specific gene expression during the formation of the heart.

Transcription of GATA-4 in embryonic explants

Having found that GATA-4 transcription correlates with cardiovascular and gut development, we sought to determine if this transcription factor is induced in cultures of multipotential cells derived from animal caps. Typically, cultured animal caps will form a ball of ciliated epidermis. Uninduced animal cap cells, presumptive mesoderm, or vegetal regions were cultured in buffer for up to 30 hours. RNA was prepared at varying time intervals and was subjected to RT-PCR analysis for GATA-4 mRNA transcription (Fig. 8A). GATA-4 RNA was detected at a very low level in animal pole explants. As expected for a gene expressed in cardiac tissue, the GATA-4 gene is expressed in cells derived from presumptive mesoderm. The highest levels of GATA-4 can be detected in the vegetal pole explants from stage 8 embryos after culture for 48 hours. This correlates with the transcription of GATA-4 in the gut at a comparable stage of development. However, by analyzing the timing of transcription in embryonic regions isolated prior to gastrulation we find that the GATA-4 gene is first activated in both presumptive mesoderm and vegetal presumptive endoderm at the same time, around stage 11.

Addition of mesodermal inducing agents to animal pole explants leads to the maintenance of MyoD transcription and formation of muscle (Harvey, 1990). Neither activin nor bFGF induces or maintains GATA-4 transcription, despite characteristic elongation of the animal cap explants (data not shown). The cells fated to form cardiac mesoderm are derived from cells within the dorsal marginal zone (DMZ).

Both ventral marginal zone (VMZ) and DMZ explants express GATA-4 at a constant relatively low level throughout early development; the gene is expressed at a slightly higher level in the VMZ than the DMZ by stage 32 (Fig. 8B).

DISCUSSION

A new class of vertebrate transcriptional regulators has recently been identified and designated the family of GATA-binding factors, as each protein is extremely conserved in a central DNA-binding domain, which specifically recognizes a core WGATAR or closely related sequence (for reviews, see Evans et al., 1990; Orkin, 1990). In non-vertebrates, GATA factors have also been identified, either by homology to GATA-1 (Speith et al., 1991) or by genetic techniques (Cunningham and Cooper, 1991; Fu and Marzluft, 1990; Kudla et al., 1990). GATA-1 is restricted to erythroid, megakaryocytic and mast cell lineages (Evans and Felsenfeld, 1989; Martin et al., 1990; Sposi et al., 1992). There is abundant evidence to implicate this protein as a critical determinant of erythroid gene expression. GATA-2 has been shown to be expressed in vascular endothelial cells and has been implicated in regulating endothelial cell gene expression (Wilson et al., 1990; Lee et al., 1991; Dorfman et al., 1992). In addition, GATA-2 is highly expressed in mast cells and megakaryocytes; erythroid cells have low levels of GATA-2. The GATA-3 protein is most abundantly expressed in cells of T-lymphocyte lineage (Ko et al., 1991; Marine and Winoto, 1991), and is also expressed at a low level in mast cells.

We have isolated cDNA clones and determined patterns of transcription for a novel member of the GATA family of DNA-binding transcription factors. This new factor is designated GATA-4 and it contains the conserved central duplicated finger region which specifically recognizes and binds to the GATA core consensus *cis*-element. As the GATA-4 protein is not similar to other GATA factors outside of this region and is expressed in a distinct subset of cell types, we predict it functions as a positive acting transcription factor, but that it activates a unique set of target genes in the distinct set of cell types where it is expressed. Although we do not yet know the natural targets for GATA-4 action, we have shown that the expressed protein is capable of binding specifically to a GATA *cis*-element. In the adult frog, the GATA-4 gene is active in heart, endoderm-derived tissues and gonads.

Each of the known GATA factors is first activated in *Xenopus* embryos around stage 11 of development (Zon et al., 1991). At this time, GATA-2 is expressed at a high level in the entire animal pole; by the end of gastrulation transcription is completely localized to the ventral region (C. K. and L. I. Z., unpublished data) which includes the presumptive blood islands. However, some of this mesoderm will ultimately contribute to vascular development. Therefore, GATA-2 may be a very early marker for vasculogenesis. Later GATA-2 transcription in blood islands is much lower than GATA-1. Endothelial cells also will form from a subset of GATA-2-expressing mesodermal cells. It appears then that GATA-2 and GATA-4 may both be important in the developing cardiovascular system.

The fact that hematopoietic cells express GATA-1, 2 and 3, and vascular cells express GATA-2, 3 and 4, suggests a common mechanism for cell-specific gene regulation by this family of transcription factors. Multipotential cells express overlapping patterns of these factors and terminal differentiation is associated with a maintenance of expression of a restricted subset of the family. For instance, GATA-1 expression is maintained throughout hematopoietic commitment to the erythroid lineage. Similarly, GATA-4 transcription is maintained in the endocardium. Although the expression of GATA-binding proteins has only been studied in two mesodermal compartments, hematopoietic and endothelial cells, it is possible that each mesodermal program will express members of the family. Based on the limited differences in DNA-binding characteristics of GATA-1, 2, 3 and 4, the co-expression of these factors supports a model in which the relative level of distinct GATA-binding proteins is required for cell-specific gene regulation. Such a regulatory network would in many ways be strikingly similar to the occurrence of overlapping domains of family-related transcription factors during *Drosophila* development, which ultimately determines specific cell fates.

By analogy to GATA-1, we might predict that genes of the cardiac terminal differentiation pathway are good candidates for potential targets of GATA-4 action. Certainly GATA-4 could be activating genes important for endothelial function: providing a non-thrombogenic surface that can act as a permeability barrier, synthesizing specific vasoactive materials, growth regulatory factors and connective tissue macromolecules, and modifying plasma lipoproteins. However, the endocardium is also critical for the normal morphogenesis of the heart from a simple tube to a chambered structure connected to the rest of the circulatory system. Given the early expression of GATA-4 in the endocardium prior to and during this transformation, it is interesting to consider a role for GATA-4 in regulating genes involved in cardiac morphogenesis and endocardial cushion tissue function (French-Constant and Hynes, 1988; Icardo and Manasek, 1984). Considering that GATA-4 is the only cell-restricted transcription factor known to be expressed in the endocardium, these processes provide us with potential target genes, which can now be investigated directly.

Based on the expression patterns of certain cell adhesion molecules, it seems that the endocardial and myocardial components organize during morphogenesis along different pathways (Crossin and Hoffman, 1991; Manasek, 1981). Early endocardial cells, but not myocardial cells, express cytotoxin, which interacts with a specific proteoglycan apparently involved in providing migration clues. Vascular cells express a defined program of genes including Von Willebrand's Factor, P-Selectin, pre-proendothelin, V-CAM and the *flt* proto-oncogene. This expression pattern does not distinguish large vessel from small vessel programs. In addition, it has not previously been appreciated that endocardial gene expression is distinct from other vascular programs. The focal expression of GATA-4 in endocardium represents the first demonstration of a heterogeneity for vascular endothelial cells.

Interestingly, the GATA-4 gene is also transcribed in endoderm-derived tissues, particularly the stomach and throughout the small intestine. The *in situ* analysis of adult

tissue sections indicates that transcription is largely confined to the epithelium, suggesting that a unique set of genes might serve as targets for GATA-4 action during terminal differentiation of these cells. However, it is also intriguing to consider whether common molecules (potential target genes) might be co-regulated by GATA-4 in endocardium and endothelium. Both undergo extensive morphogenetic processes and interact with extracellular matrix and lumen. The whole-mount in situ hybridization analysis detects transcription in the endodermal tissues at relatively later stages of development. However, explants transcribe GATA-4 in vegetal cells of presumptive endoderm at approximately the same time as seen in marginal zone containing presumptive mesoderm. The whole-mount assay appears less sensitive at analyzing early gene transcription in presumptive endoderm due to the yolky composition of these cells (A. Hemmati-Brivanlou, personal communication). Alternatively, early transcription in vegetal explants may be artifactually induced by our culture system (or the lack of putative negative inducers from overlying marginal zone).

It is clear that the GATA-4 gene is not restricted in expression in the same manner as GATA-1. The original GATA-4 cDNA was isolated from tadpole liver RNA. It is therefore possible that this GATA factor may play a role in regulating transcription in other cell types, although the pattern of expression in embryos appears consistent with the hepatic vascular tree. Although the GATA-4 message is clearly concentrated within the developing heart of early embryos (Fig. 6), it is very likely (given the results of the explant assays and the pattern of expression in later development) that the gene is expressed during early embryogenesis at low levels in cells that do not contribute to the cardiovascular system. Therefore, although it seems likely that GATA-4 is important in heart development, it has not yet been determined whether the gene provides a bona fide assay for heart induction. We are currently further analyzing the localized expression of GATA-4 within explants, in the presence or absence of putative inducing molecules and embryonic regions, in order to determine the specificity of GATA-4 as a cardiovascular induction marker.

From an embryological standpoint, it may seem surprising that GATA-4 is expressed in cells that give rise to either mesoderm or endoderm tissues. We have confirmed that this pattern of GATA-4 expression (including transcription in intestinal epithelium) is not unique to amphibians, but is conserved in the chicken (J. Burch and T.E., unpublished results). Furthermore, similar results have been recently obtained with regard to the mouse homologue (D. Wilson, personal communication). A precedent for such gene activation is provided by the *mix-1* gene, originally isolated as an immediate response gene of mesoderm-inducing factors (Rosa, 1989). The *mix-1* mRNA is first transcribed in the marginal zone in addition to extensive portions of presumptive endoderm. In this regard, a *Drosophila* GATA-binding protein has recently been isolated that also localizes to both endodermal and mesodermal regions (T. Abel, A. Michelson and T. Maniatis, personal communication). It is possible that GATA-4 is activated by similar inductive mechanisms in both cell types, or that the expression in distinct regions occurs by secondary inductions. Our results do not support a simple immediate response to known

growth factor inducers such as activin or bFGF. These results are consistent with a previous report in which activin (PIF) failed to induce heart differentiation (Sokol et al., 1990).

However, a number of peptide growth factors have been implicated in the regulation of cardiovascular development and induction. The vascular endothelial growth factor (Ferrara et al., 1992) has mitogenic properties for vascular endothelial cells (and hematopoietic cells) and interacts with the *flt* proto-oncogene, which is expressed at high levels in the endothelium lining the atrioventricular and aortic valves (De Vries et al., 1992; Shibuya et al., 1990). Considerable evidence also exists for the roles of FGF, EGF, TGF- β , TGF- α , PDGF and PD-ECGF in this process (Muslin and Williams, 1991; Folkman and Shing, 1992). Clearly, the GATA-4 gene provides a new and very early marker for cardiac gene expression. It should now be possible to determine its usefulness in understanding the inductive interactions leading to heart formation.

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