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# Over-expression of GATA-6 in *Xenopus* embryos blocks differentiation of heart precursors

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***Xenopus*** **GATA-6 transcripts are first detected at the beginning of gastrulation in the mesoderm, and subsequent domains of expression include the field of cells shown to have heart-forming potential. In this region, GATA-6 expression continues only in those cells that go on to form the heart; however, a decrease occurs prior to terminal differentiation. Artificial elevation of GATA-6, but not GATA-1, prevents expression of both cardiac actin and heart-specific myosin light chain. This effect is heart-specific because cardiac actin expression is unaffected in somites. Expression of the earlier marker XNkx-2.5 was unaffected and morphological development of the heart was initiated independently of the establishment of the contractile machinery. We conclude that a reduction in the level of GATA-6 is important for the progression of the cardiomyogenic differentiation programme and that GATA-6 may act to maintain heart cells in the precursor state. At later stages, when the elevated GATA-6 levels had decayed, differentiation ensued but the number of cells contributing to the myocardium had increased, suggesting either that the blocked cells had proliferated or that additional cells had been recruited.**

*Keywords*: cardiac development/differentiation/GATA transcription factors/RNA injection/*Xenopus*

## Introduction

The consensus DNA sequence (A/T)GATA(A/G), known as the GATA motif, has been found in the promoters and enhancers of a large number of genes expressed in a wide range of tissues, where it is thought to function as either a positive or a negative regulatory element. So far, six related zinc finger-containing proteins have been described which recognize and bind this *cis* element (Yamamoto *et al.*, 1990; Laverriere *et al.*, 1994). They fall into two subgroups containing GATA-1, -2 and -3 in one and GATA-4, -5 and -6 in the other. The subgroups are defined by both sequence homology and expression pattern, with GATA-1, -2 and -3 predominating in blood and ectodermal derivatives and GATA-4, -5 and -6 featuring in heart and endodermal derivatives.

In the developing mouse, GATA-4 is expressed in the heart, intestinal epithelium, primitive endoderm and gonads (Arceci *et al.*, 1993) and in homozygous deficient embryoid bodies differentiation of the visceral endoderm is disrupted (Soudais *et al.*, 1995). Putative GATA-binding *cis* elements have been identified in the promotors of a number of genes specifically expressed in cardiac tissue, including the genes encoding the heart-specific isoforms of myosin light and heavy chains, cardiac actin, troponin C and atrial natriuretic factor (Grepin *et al.*, 1994; Ip *et al.*, 1994; Molkentin *et al.*, 1994) and in some of these promoter elements, specific interaction with GATA-4 has been demonstrated (Grepin *et al.*, 1994; Ip *et al.*, 1994; Molkentin *et al.*, 1994). Roles for GATA-4 and GATA-5 in heart development are suggested by their expression patterns in *Xenopus*, mouse and chicken, with transcripts in the anterior mesoderm that gives rise to cardiac progenitor cells and, in later embryonic stages, in both endocardium and myocardium (Kelley *et al.*, 1993; Heikinheimo *et al.*, 1994; Laverriere *et al.*, 1994). Furthermore, inhibition of GATA-4 expression prevents differentiation of a pluripotent cell line to beating cardiac muscle (Grepin *et al.*, 1995).

We have investigated the role of GATA-6 in the development of the heart in *Xenopus laevis*. We show that GATA-6 transcripts are present in the presumptive heart mesoderm from the earliest time of heart specification, with expression declining prior to terminal differentiation, as defined by the expression of cardiac actin and heart-specific myosin light chain. By artificially elevating the levels of GATA-6 in developing embryos, terminal differentiation of the heart was blocked but when exogenous GATA-6 levels dropped, the differentiation programme was completed. We therefore suggest that reduction in the GATA-6 level is a critical step in differentiation of heart precursors.

## Results

### *Isolation and characterization of X.laevis GATA-6 cDNA*

GATA-6 cDNA was isolated by screening an adult *Xenopus* liver cDNA library (Xu and Tata, 1992) with the *Xenopus* GATA-2 zinc finger region at moderate stringency. Regions of homology throughout the complete predicted amino acid sequence and particularly within the zinc finger region indicate that the GATA factor cDNA isolated is most closely related to GATA-6. Within the zinc finger region the peptide sequence is 97% identical to the recently published *Xenopus* GATA-6 (Jiang and Evans, 1996; Figure 1) and 95/96% identical to chicken and rat GATA-6 (Tamura *et al.*, 1993; Laverriere *et al.*, 1994). In contrast,

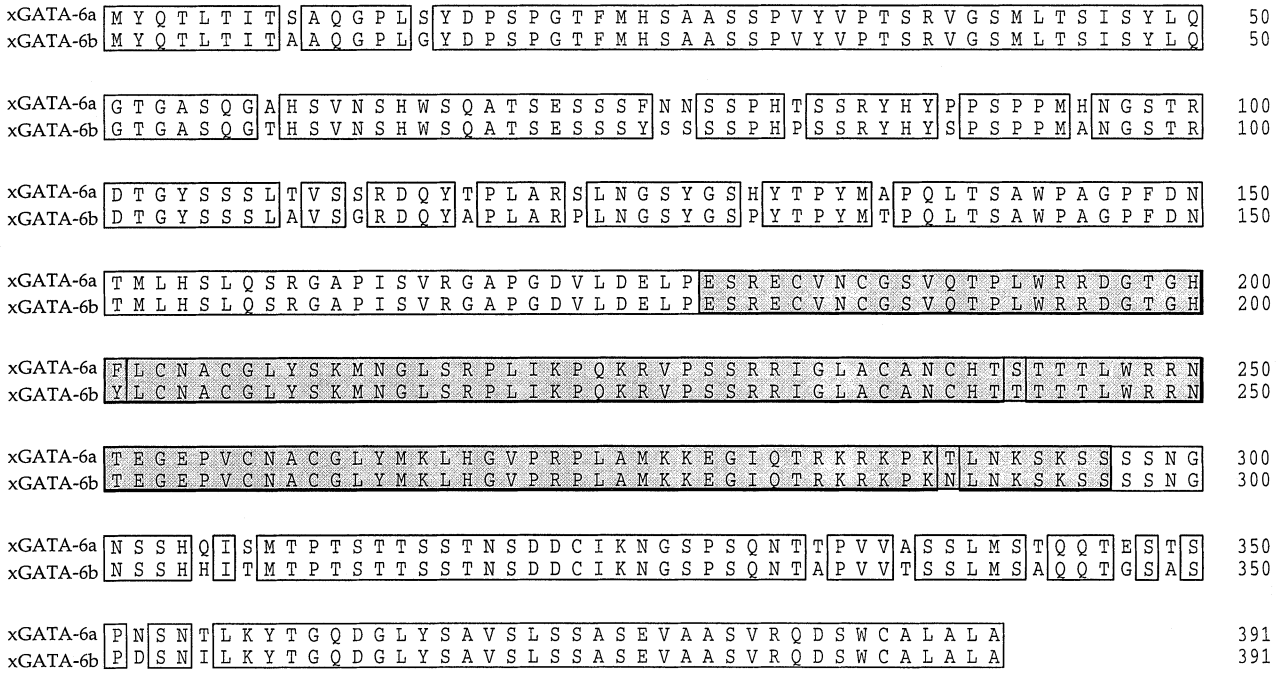


**Fig. 1.** Comparison of xGATA6 predicted amino acid sequences for the two non-allelic forms. The peptide sequences for xGATA-6a (Jiang and Evans, 1996) and xGATA-6b (this study) were compared using the Pileup and Prettybox functions of the GCG package (University of Wisconsin). Identity of 93% was observed over the entire sequence and 97% within the zinc finger domain (shaded). Sequence data from this article have been deposited with the GenEMBL Data Library under accession No. Y08865.

only 91% identity is observed for *Xenopus* GATA-4, 87% for GATA-5 and 72, 75 and 74% for GATA-1, -2 and -3 respectively. In addition, seven residues are unique to the GATA-6 group and six more, which are only conservative changes compared with the chicken and rat proteins, are unique to the cDNA described here and the *Xenopus* GATA-6 sequence of Jiang and Evans (1996). Comparison of the complete amino acid sequence with the published xGATA-6 revealed 93% identity (Figure 1), which is more divergent than expected between polymorphic variants but within the range observed for non-allelic loci of the pseudotetraploid *Xenopus* genome (Kobel and Du Pasquier, 1986). Within the GATA family, 89% identity has been observed between the two non-allelic copies of GATA-1 and 96% between the two copies of GATA-5. These considerations, taken together with sequence analysis of the untranslated regions of the cDNAs, have led us to the conclusion that the xGATA-6 sequences described by Jiang and Evans (1996) and herein represent the two non-allelic forms of xGATA-6 and we have therefore designated them a and b respectively.

### *DNA binding and transactivation by xGATA-6*

RNA transcribed from xGATA-6b cDNA was translated *in vitro*, revealing a single major protein band of ~48 kDa (Figure 2A). Confirmation that xGATA-6 is a GATA-binding protein was obtained by incubating the translation mix with labelled oligonucleotides containing known GATA-binding sites and mutant sequences. The oligonucleotide αG2, a well-characterized GATA binding site containing the motif GATAAG found in the mouse α1 globin gene promoter, was retarded by xGATA-6 in a gel shift assay (Figure 2B, lane 1). Binding to αG2 was competed by a 50-fold excess of unlabelled αG2 oligonucleotide (lane 2) and by an oligonucleotide containing the GATA element from the promotor of the chicken β hatching globin gene, βHB (lane 6). No competition was evident with oligonucleotides containing point mutations within the consensus binding region (lanes 3–5), with an oligonucleotide, GATC, that binds GATA-2 and -3 but not GATA-1 (Ko and Engel, 1993; G.Partington and R.Patient, unpublished results) (lane 7) or with an oligonucleotide containing the octamer consensus (lane 8). By comparing lane 1 (programmed) with lane 9 (unprogrammed) it is evident that GATA-6 gives rise to a number of retarded complexes, one major and several minor bands, all of which were competed by unlabelled αG2 (lane 2). Since the *in vitro* translated protein migrated predominantly as a single band on SDS–PAGE (Figure 2A), it does not appear that different protein products are the reason for the multiple bands. The highest mobility band may represent a proteolytic cleavage product of the major band and by analogy to GATA-1 the doublet of low mobility is likely to reflect aggregation of specifically bound protein complexes (Visvader *et al.*, 1995). We conclude that xGATA-6 binds to known consensus GATA-binding sites.

The ability of GATA-6 to transactivate gene expression was demonstrated using a luciferase reporter construct, containing multiple GATA-binding sites in the promotor, co-injected with GATA-6 mRNA and a *Hin*dIII-linearized TKβ-gal construct (as injection control) into the animal pole of single cell *Xenopus* embryos. Transactivation, related to the amount of co-injected xGATA-6 mRNA, was detected in the injected region (animal caps) which had been dissected at the mid blastula stage (stage 8), when gene expression begins (MBT), and cultured overnight (Figure 2C). Aliquots of 20 pg GATA-6 mRNA and 20 pg GATA-1 mRNA produced equivalent levels of transactivation (~4- and 3-fold respectively) compared with the reporter construct alone, while 60 pg GATA-6

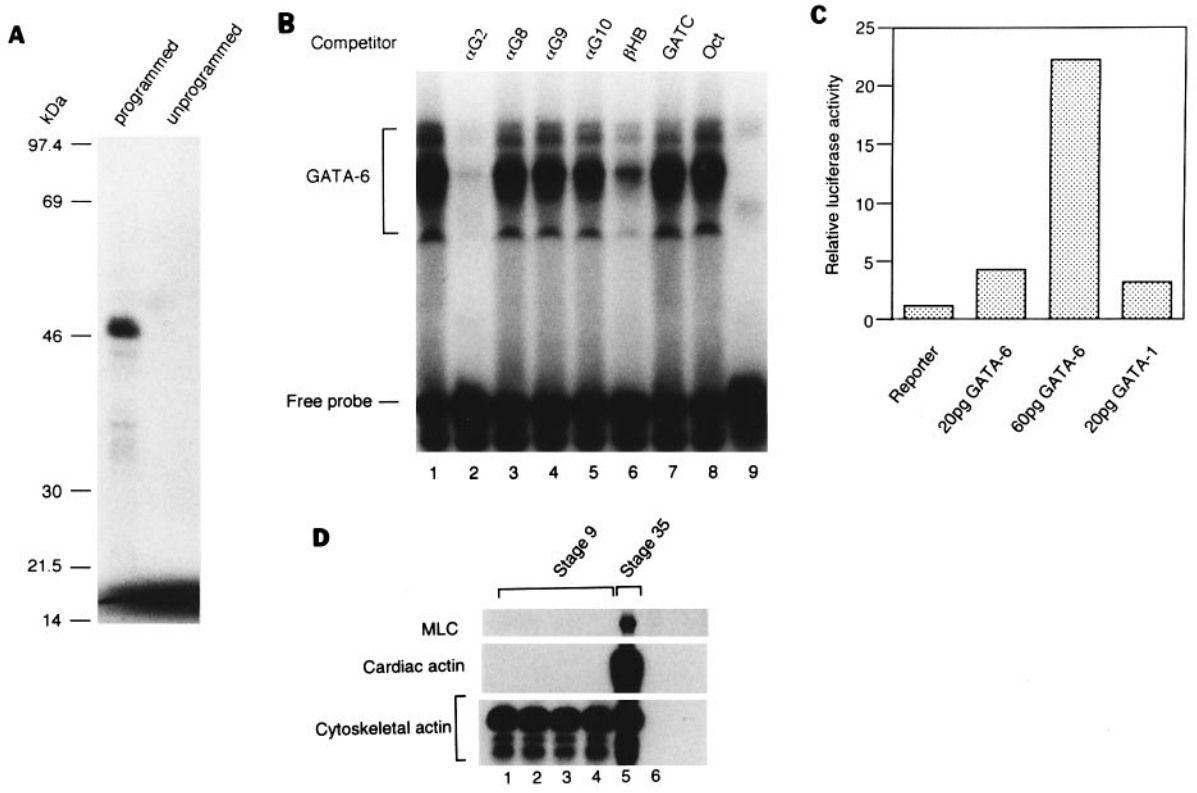


**Fig. 2.** Translation of GATA-6 mRNA *in vitro* produces a protein product of 48 kDa which binds specifically to the WGATAR consensus sequence, transactivates a co-injected reporter construct but does not prematurly activate endogenous potential target genes. (**A**) [$^{35}$S]Methionine-labelled translation products from a reticulocyte lysate programmed with GATA-6 mRNA and from an unprogrammed lysate were analysed by SDS–PAGE. Molecular sizes are indicated on the left. (**B**) Translated products (lanes 1–8) and unprogrammed lysate (lane 9) were incubated directly with a $^{32}$P-labelled oligonucleotide, αG2, containing a WGATAR sequence and analysed on a 4% non-denaturing polyacrylamide gel. Unlabelled oligonucleotides were added to the reaction in 50-fold excess as indicated: lane 2, αG2; lanes 3–5, oligomers containing a mutated consensus binding region; lane 6, βHB, a WGATAR-containing oligonucleotide from the promotor of the chicken β-hatching gene; lane 7, an alternative consensus site, GATC, that binds chicken GATA-2 and -3 (Ko and Engel, 1993); lane 8, the octamer motif; lanes 1 and 9, no unlabelled oligonucleotides. (**C**) Relative luciferase activity in neurula stage animal caps. Aliquots of 30 pg linearized luciferase reporter construct, which contains multiple GATA-binding sites in the promoter (Igarashi *et al*., 1994) and 20 pg *Hin*dIII-linearized TKβ-gal construct (as injection control) were injected into the animal pole of stage 1 embryos alone (lane 1) or together with 20 or 60 pg GATA-6 mRNA (lanes 2 and 3 respectively) or 20 pg GATA-1 mRNA (lane 4). At stage 8, animal caps were dissected and cultured overnight at 19°C to the equivalent of neurula stages judged from sibling embryos. Luciferase activity was normalized to β-galactosidase activity. (**D**) Total RNA extracts were analysed by RNase protection for cardiac actin, XMLC2 and control cytoskeletal actin mRNAs. RNA extracts were prepared from stage 9 embryos (pools of 20) and 10 embryo equivalents were analysed from uninjected embryos (lane 1) and embryos injected with 20 or 60pg GATA-6 mRNA (lanes 2 and 3 respectively) or 20 pg GATA-1 mRNA (lane 4). Two embryo equivalents of RNA from uninjected stage 35 embryos were run as a control (lane 5); the tRNA control is in lane 6.

mRNA produced a 19-fold increase. No transactivation of a reporter lacking the GATA sites was seen (data not shown). Intermediate levels of transactivation have been seen using 50 pg of each mRNA (T.Sykes and R.Patient, unpublished results). In contrast, RNase protection analysis of mRNA extracted from blastulae (stage 9 embryos, post MBT, from the same batch of embryos as in Figure 2C) injected at the single cell stage with 20 or 60 pg GATA-6 mRNA showed no precocious transactivation of the endogenous cardiac-specific genes XMLC2 and cardiac actin (Figure 2D, lanes 2 and 3), which disagrees with a recent report (Jiang and Evans, 1996; see Discussion). Thus, xGATA-6 can activate transcription through GATA sites but is unable to elicit premature activation of endogenous potential target genes (see also below).

### *GATA-6 mRNA is widely distributed in adult tissues but predominates in the heart*

The distribution of GATA-6 transcripts in a variety of adult tissues was examined by RNase protection (Figure 3). Protected bands corresponding to GATA-6 were apparent in all tissues studied except brain (not shown) and blood. When these bands were scanned and normalized to the EF-1α loading control, the strongest signal was present in heart but strong signals were also evident in ovary, skin, gut (duodenum), muscle (skeletal), testis and liver. Only weak signals were seen in kidney, lung, pancreas and spleen. Two major RNA species were detected and their relative intensities varied between tissues. For example, the larger species predominates in heart (lanes 2 and 14) whilst the shorter protected product is stronger in skeletal muscle (lane 3). The significance and cause of these differences are currently unclear. Sequence analysis of the region protected by the 178 nt probe ruled out the possibility that the two RNA species reflect differential expression of the two non-allelic copies of the gene, as reported for GATA-1 (Zhang and Evans, 1994). Similarly, the position of intron/exon boundaries in the genomic organization of GATA-6 (S.Nijjar, A.Bomford and C.Gove, unpublished results) make it unlikely that

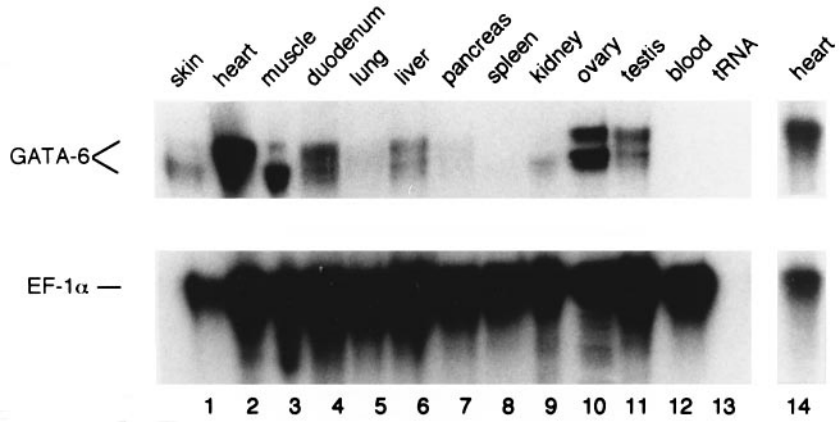


**Fig. 3.** Expression of GATA-6 in adult tissues. Total RNA was extracted from adult *Xenopus* tissues and analysed by RNase protection for the presence of GATA-6 (178 bp probe) and control EF-1α mRNAs. Lane 14 is a lower exposure of the heart sample in lane 2 which shows that the larger protected species predominates (see text).

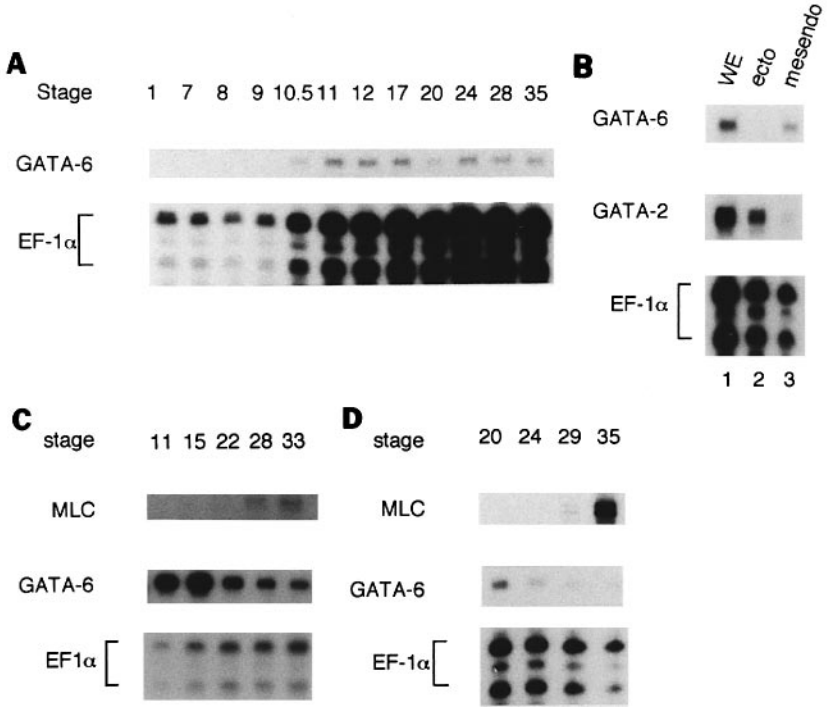


**Fig. 4.** GATA-6 is expressed from early gastrulation (stage 10.5) in *Xenopus* embryos, but not in ectoderm. Expression in the developing presumptive heart field *in vitro* and *in vivo* declines prior to the appearance of the terminal differentiation marker myosin light chain (XMLC2). Total RNA extracts were analysed by RNase protection for GATA-2, GATA-6 (using the 155 bp probe), XMLC2 and control EF-1α mRNAs. (**A**) RNA extracts were prepared from staged embryos (pools of 20) at the time points indicated and four embryo equivalents were analysed. (**B**) After removal of vitelline membranes at stage 8, embryos were incubated overnight in 1× MBS to induce exogastrulation. At the equivalent of stage 13, embryos were dissected into ectoderm and mesendoderm fragments. RNA was prepared from 10–12 pooled embryos or fragments. Lane 1, whole embryos; lane 2, ectoderm; lane 3, mesendoderm. (**C**) DLMZ (presumptive heart tissue) explants were dissected from stage 10.5 embryos and cultured to the equivalent of stages 11, 15, 22, 28 and 33 as judged from control sibling embryos. RNA was prepared from 10 pooled explants at each stage and four embryo equivalents were analysed. (**D**) Ventral portions of presumptive heart fields were dissected from embryos at stages 20, 24 and 29 and beating hearts at stage 35. RNA was extracted from pools of 10 fragments at each stage.

splice variants are responsible for this observation, although it is difficult to completely exclude splicing to a cryptic site within the coding sequence. Whatever their origins, the two RNA species give rise to a single protected band with a probe from the 5′ non-coding region (see for example Figure 4). In conclusion, consistent with its position in the -4, -5, -6 branch of the family, xGATA-6 is strongly expressed in heart and endodermal derivatives. However, we also detect significant expression in additional tissues, including skin, skeletal muscle and the germline.

### *Temporal and spatial expression of GATA-6 during early development*

Temporal expression of GATA-6 was determined by RNase protection analysis using RNA extracted from embryos at various stages of development (Figure 4A). Transcripts were first detected in early gastrulae (stage 10.5 of

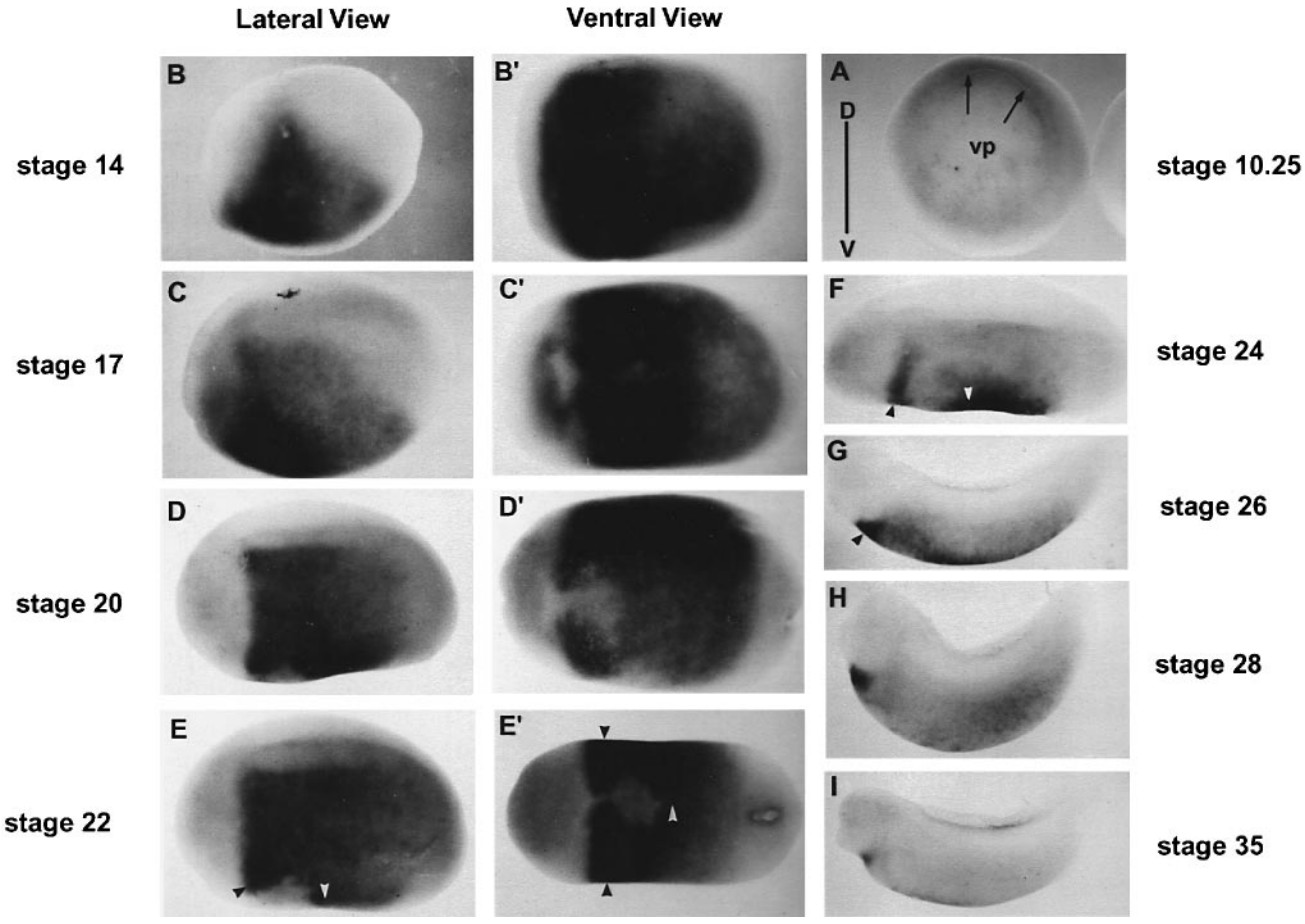


**Fig. 5.** GATA-6 expression is observed in the marginal zone of gastrulae, is widely distrubuted in the ventro-lateral region in early neurula embryos and is progressively restricted to regions corresponding to the heart and blood islands in mid to late tailbud stages. Embryos were probed for GATA-6 by whole mount *in situ* hybridization at (**A**) stage 10.25, (**B** and **B′**) stage 14, (**C** and **C′**) stage 17, (**D** and **D′**) stage 20, (**E** and **E′**) stage 22, (**F**) stage 24, (**G**) stage 26, (**H**) stage 28 and (**I**) stage 35. Anterior is to the left in all views except (A) and dorsal is to the top in the lateral views. Weak staining is first detected as a ring around the vegetal pole (vp) which is more intense in the region of the dorsal lip (black arrows in A). Following gastrulation extensive ventro-lateral staining is evident in early and mid neurula stage embryos (B, B′, C and C′), by early to mid tailbud stages strong staining is evident in presumptive heart (black arrows) and blood island (white arrows) regions (D, D′, E, E′ and F). In mid to late tailbud embryos staining in the heart field and in the blood islands is progressively restricted until by stage 35 a strong signal is evident only in the heart itself (G, H and I).

development) at the same time as the increase in the signal for EF-1α was observed (Figure 4A). Peak expression occurred at neurula stages 11–17, with progressive reduction from the mid tailbud stage (stage 24) onwards, but transcripts were still clearly present at stage 40 (swimming tadpole), the latest stage examined (data not shown). Normalization of the signals to the EF-1α control signal shows that the apparent reduction in expression at stage 20 was due to uneven RNA loading (Figure 4A). To investigate which germ layers express GATA-6, ectoderm and mesendoderm fragments from exogastrulated embryos at the equivalent of stage 13 of development were analysed by RNase protection analysis (Figure 4B). GATA-6 transcripts were evident in whole embryos (lane 1) and in the mesendoderm fragments (lane 3), but not in ectoderm (lane 2). Expression of GATA-6 in whole embryos and mesendoderm was similar when normalized to the EF1α signal. In contrast, GATA-2 was most strongly expressed in the ectoderm fragments (lane 2), with low levels of expression detectable in the mesendoderm (lane 3), as previously noted by Walmsley *et al.* (1994).

Whole mount *in situ* hybridization of *Xenopus* embryos between stages 10.25 and 35 (Figure 5) shows that GATA-6 expression can first be detected weakly as a ring around the vegetal pole which is more intense in the region of the dorsal lip (arrowed in Figure 5A). After gastrulation (stages 14 and 17), the signal is strong throughout the ventro-lateral region (Figure 5B/B′ and C/C′), which includes precursors of the heart and blood (Kau and Turpen, 1983; Sater and Jacobson, 1990). Transverse sections of stage 14 embryos confirm that expression is in the mesodermal layer (Figure 6I) and the anterior limit of GATA-6 staining at this stage is close to the anterior boundary of the ventral mesoderm (Figure 5B′) (Hausen and Riebesell, 1991). During neurula stages, elongation of the embryo occurs in an anterior direction as the neural tube grows and extends and, at the same time, prospective heart mesoderm begins to move laterally from its dorsal position towards the ventral midline. By late neurula the two heart anlagen fuse immediately posterior to the cement gland and anterior to the ventral blood island, leaving a hole between the two (Mangia *et al.*, 1970; Hausen and Riebesell, 1991). Thus by stage 22 the anterior boundary of the ventral mesoderm observed at stage 14 resides at

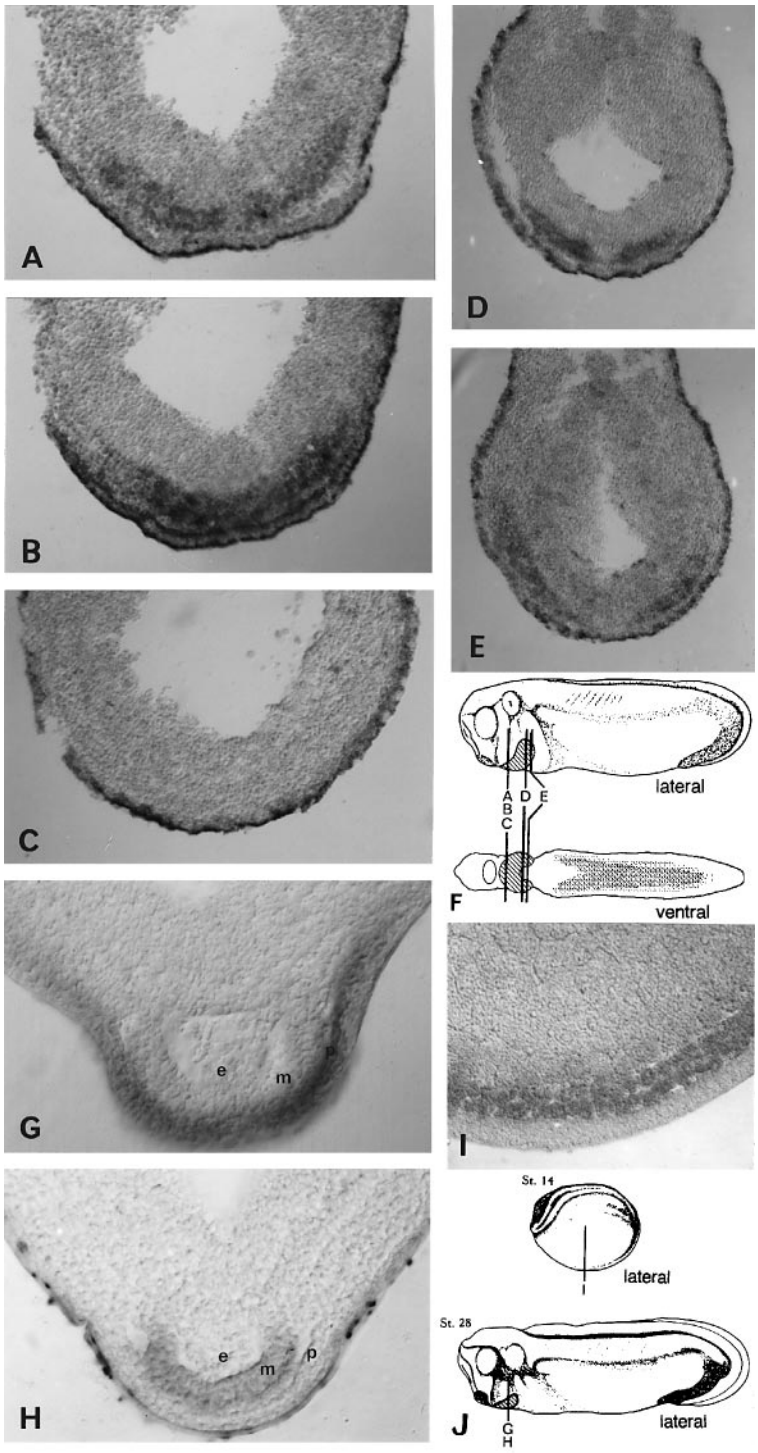


**Fig. 6.** GATA-6 is expressed in the developing heart field in the antero-ventral mesoderm of mid tailbud *Xenopus* embryos where it co-localizes with cardiac actin, a marker of terminal cardiac differentiation. Later, as the heart tube closes, expression of GATA-6 is in the myocardium and pericardium but not the endocardium. Serial transverse sections of embryos at developmental stage 26 were hybridized *in situ* to digoxigenin-labelled RNA probes. (**A**) GATA-6 staining revealed a continuous arc of expression in the ventral mesoderm; (**B**) in the adjacent section probed with cardiac actin, staining was seen in the corresponding area of ventral mesoderm and unexpectedly in sensorial ectoderm; (**C**) the specificity of these signals is shown by the lack of staining in the adjacent section probed with the digoxigenin-labelled sense strand of GATA-6. Sections taken more posteriorly show that the ventral mesodermal expression domain of GATA-6 is increasingly separated into two symmetrical patches (**D** and **E**). The dark colour of the outer ectodermal cells (A–E) is due to pigmentation, as these are unbleached sections of non-albino embryos. (**F**) Viewed from the ventral aspect the staining pattern describes a V shape with the arms pointing backwards (hatched). The blood islands (stippled) and the cement gland (ellipse) are also marked. Transverse sections through whole mount embryos [position of sections indicated in (**J**)] hybridized *in situ* show: (**I**) GATA-6 to be expressed in the ventral mesoderm of early neurula embryos (stage 14); (**G**) at the closing heart tube stage (stage 28) GATA-6 is expressed in the myocardium (frequently stains blue with BM purple) and pericardium but not in the endocardium; (**H**) at the same stage (stage 28) transcripts for the terminal differentiation marker, XMLC2, are present in the myocardium.

the point indicated by the white arrow in Figure 5E/E′ and the migrating heart mesoderm has reached the point indicated by the black arrows in Figure 5E/E′. All this mesoderm is GATA-6 positive. However, viewed from the ventral aspect there is a clear gap in the GATA-6 staining of the heart mesoderm spanning the midline (Figure 5D′ and E′).

In *Xenopus* at stage 20 of development, a field of mesodermal cells, present as a broad ventro-lateral band posterior to the cement gland, has the ability to form beating heart tissue, while by stage 28 the cells with heart-forming potential have become restricted to the most ventral part of this region (Sater and Jacobson, 1990). By whole mount *in situ* hybridization at stage 20, the GATA-6 expression domain includes the region capable of forming heart tissue (Figure 5D). Subsequently, the signal for GATA-6 is steadily restricted, so that by stage 28, expression in this region is limited to the most ventral mesoderm forming the heart (black arrows in Figure 5E–H). Further evidence that this expression culminates in heart mesoderm was provided by hybridizing serial sections of a mid tailbud embryo (stage 26) to GATA-6 and cardiac actin probes (Figure 6A and B respectively). Both probes labelled the mesodermal layer to a similar extent, suggesting that many of the cells in this region express both mRNAs. No staining was observed when the opposite strand of the GATA-6 probe was used (Figure 6C). We therefore conclude that the predominant site of GATA-6 expression at the time of heart tube formation is in cells with heart-forming potential.

Sections taken more posteriorly show that the ventral mesodermal expression domain of GATA-6, which is in a continuous arc anteriorly (Figure 6A), is increasingly separated into two symmetrical patches (Figure 6D and E). Thus, viewed from the ventral aspect, the expression domain describes a V shape with the arms oriented posteriorly (hatched region in Figure 6F), this boundary defining the anterior edges of the hole in the mesoderm described earlier (Figure 5E′).

Members of the GATA-4, -5, -6 subfamily of transcription factors show early developmental expression in myocardium (GATA-4 and -6) (Heikinheimo *et al.*, 1994; Jiang and Evans, 1996) and endocardium (GATA-5 and -6) (Kelley *et al.*, 1993; Jiang and Evans, 1996). We examined the distribution of xGATA-6 transcripts within the developing heart tube in transverse sections through mid tailbud embryos (stage 28) which had been hybridized *in situ.* We found that, at this stage, when expression of the heart-specific isoform of myosin light chain (XMLC2) is found in the myocardium (Figure 6H), GATA-6 is expressed in myocardium and pericardium but not in the endocardial tube (Figure 6G). The absence of GATA-6 expression in the endocardial precursors is also evident at stage 20 as the gap in GATA-6 staining , described earlier, in the ventral midline anterior to the hole in the mesoderm (Figure 5D′). Fusion of the two presumptive heart anlagen begins at stage 16 and is completed by stage 19 to produce a single, continuous anlage with the endocardial element in the ventral midline (Nieuwkoop and Faber, 1967). The narrowing of the gap in GATA-6 staining at stage 22 (Figure 5E′) and closure by stage 26 (Figure 6A) reflects the continued wrapping of the myocardial and pericardial elements around the rudiment of the endocardium (Balinsky, 1970).

### *GATA-6 expression falls as heart precursors differentiate*

Comparison of the intensity of staining for GATA-6 in the developing heart from neurula to late tailbud suggests that expression is reduced as differentiation proceeds (Figure 5C–I). To quantitate expression during this period dorso-lateral marginal zone (DLMZ) explants (presumptive heart tissue; Sater and Jacobson, 1990) were dissected from stage 10.5 embryos (early gastrulae) and cultured until sibling embryos reached stages 11, 15, 22, 28 and 33. GATA-6 expression, as determined by RNase protection, was highest at stages 11 and 15, thereafter declining progressively to stage 33 (Figure 4C), in a similar way to that observed in whole embryos (Figure 4A). In contrast, XMLC2 expression was first apparent at stage 28 and increased by stage 33 (Figure 4C), in agreement with observations *in vivo* (Chambers *et al.*, 1994). DLMZ explants are capable of differentiating into tissues other than heart and a possible explanation for the reduction in GATA-6 signal is the progressive dilution when normalized to the EF-1α signal originating from other types of tissue. As a second more rigorous test and to avoid this dilution effect, similarly sized explants of developing heart tissue (corresponding to the discrete cardiac region seen in Figure 5H) were dissected from the antero-ventral region of embryos at stages 20, 24 and 29 and, finally, at stage 35 the beating heart itself was removed (Figure 4D). When four separate experiments were quantitated by scanning densitometry, relative to the EF-1α loading control, a consistent reduction in GATA-6 expression level was revealed. Although the major decrease occurred between stages 20 and 24, this was followed by a more gradual decline to stage 35, in agreement with the observations in *in situ* probed embryos (Figure 5). The decrease in the EF-1α signal probably reflects reduced efficiency of RNA extraction as organogenesis proceeds. Expression of the terminal heart differentiation marker XMLC2 was first detected at stage 29, with a strong signal present in the beating heart at stage 35 (Figure 4D). Taken together, these findings suggest that a reduction in the level of GATA-6 expression occurs prior to the appearance of terminal differentiation markers. These observations in *Xenopus* are in agreement with data from the chick (Laverriere *et al.*, 1994).

### *Over-expression of GATA-6 blocks differentiation of the heart*

To test whether the decline in levels of GATA-6 expression are essential for the completion of the cardiomyogenic programme we artificially maintained GATA-6 expression in developing embryos by mRNA injection. Cardiac actin (Mohun *et al.*, 1988) and XMLC2 (Chambers *et al.*, 1994), detected by whole mount *in situ* hybridization followed by sectioning, were used as markers for terminal differentiation of cardiomyocytes. Embryos at the two cell stage were injected with GATA-6 mRNA into the mesoderm-forming equatorial region of a single blastomere, leaving the uninjected side of the embryo as a control. The injected side was identified in developing embryos by staining for β-galactosidase produced from co-injected mRNA. At

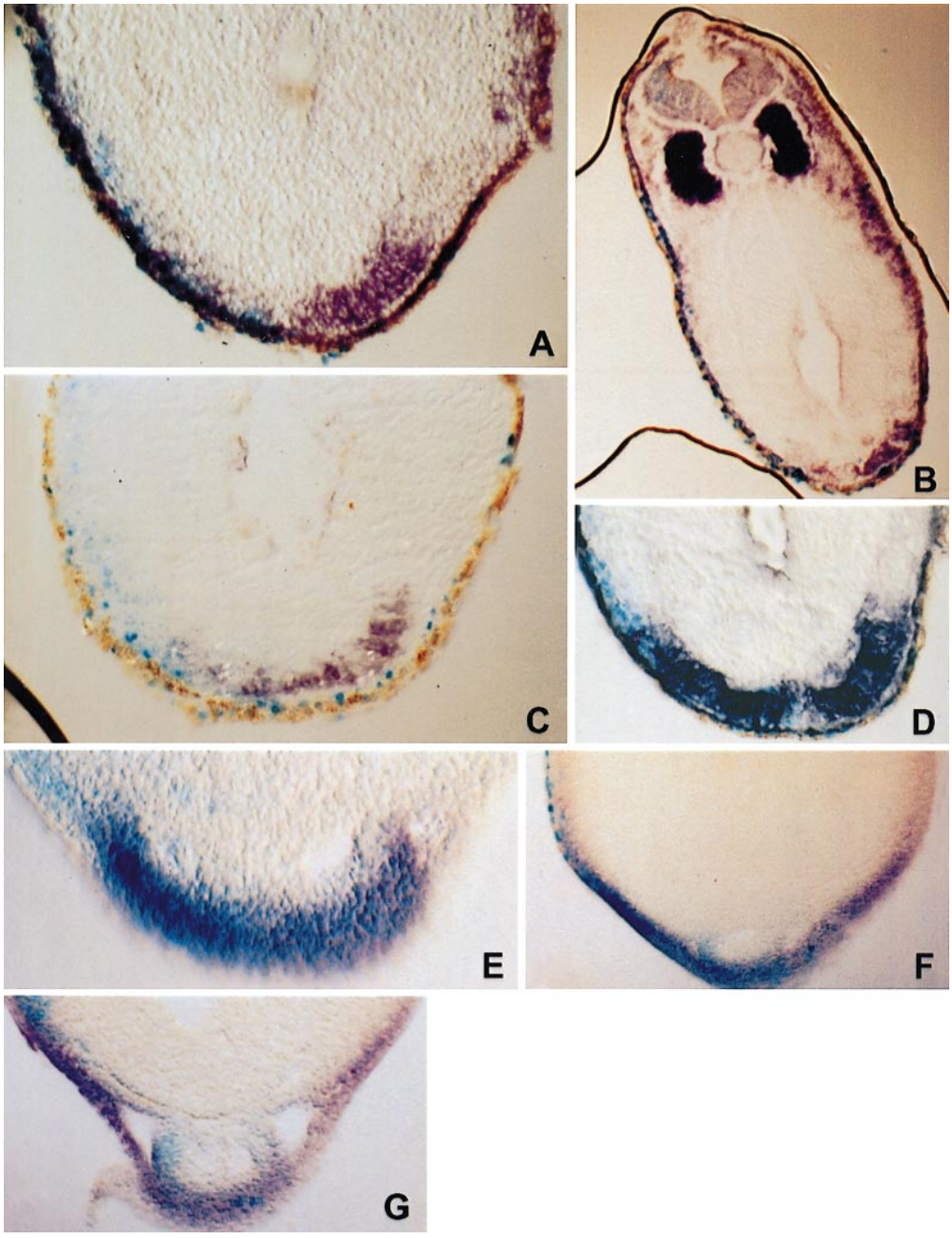


**Fig. 7.** Perturbation of the developing heart field in the ventral mesoderm in tailbud embryos by over-expression of GATA-6. *Xenopus* embryos were injected in a single blastomere at the two cell stage with 50 pg GATA-6 mRNA or with mutant GATA-1 or GATA-1 mRNA as controls along with 50 pg β-galactosidase mRNA as a lineage tracer. Embryos were harvested and fixed at developmental stages 27, 28 and 29 (tailbud) and the lineage tracer developed to give a turquoise nuclear stain. Embryos were analysed by whole mount *in situ* hybridization using probes for the terminal cardiac differentiation markers cardiac actin and XMLC2 and sectioned transversely. (**A**) Section of a tailbud embryo (stage 27) probed with cardiac actin (purple stain) revealing markedly reduced staining in the ventral mesoderm on the side injected with GATA-6 mRNA (indicated by the turquoise nuclear staining) as compared with the uninjected side. (**B**) The specifity of this reduction in expression of cardiac actin in the ventral mesoderm is evident by comparing this signal with the unperturbed signal in the somites. (**C**) A second cardiac differentiation marker, XMLC2, which is normally expressed concurrently with cardiac actin in the ventral mesoderm, showed a parallel reduction in signal on the GATA-6 mRNA-injected side. These effects were not produced by injection of mutated GATA-1 mRNA (**D**) or GATA-1 mRNA (**E**), where the intensity of staining was similar on both sides of the section. In (**F**) stage 28 and (**G**) stage 29 embryos, the signal for XNkx-2.5, which is expressed in presumptive heart cells from early gastrulation, was not perturbed by GATA-6 over-expression.

stage 27/28, transverse sections through the developing heart region showed a marked reduction of the field of expression of both cardiac actin and XMLC2 on the injected (turquoise nuclear stained) side of the embryo (Figure 7A and C). Because of incomplete cleavage during early cell division in *Xenopus*, occasional turquoise cells are seen on the uninjected side of the embryo (e.g. in the sensorial ectoderm in Figure 7C). In a very few embryos where the β-galactosidase had spread throughout the complete heart field, obliteration of the XMLC or cardiac actin signal occurred on both sides (data not shown). This effect was specific to GATA-6-injected embryos and was

**Table I.** GATA-6 down-regulates cardiac actin and myosin light chain 2 expression in heart precursors without altering XNkx-2.5

| mRNA injected | Embryos injected | Embryos surviving to stage 28 | Unilateral β-gal in heart region | Embryos with decreased signal | Percentage affected |
|---|---|---|---|---|---|
| (**A**) Myosin light chain (XMLC2) or cardiac actin (CA) | | | | | |
| GATA-6 | c.600 | c.450 | 24 | 20 | 83 |
| GATA-1 | c.300 | c.250 | 18 | 2 | 11 |
| mGATA-1 | c.600 | c.500 | 8 | 0 | 0 |
| **(B)** XNkx-2.5 | | | | | |
| GATA-6 | c.300 | c.120 | 21 | 0 | 0 |
| GATA-1 | c.100 | c.70 | 12 | 0 | 0 |

Embryos were injected into one of two cells with the synthetic RNA shown (50 pg) together with RNA encoding β-galactosidase (50 pg). When embryos had developed to stage 28 they were fixed, stained for β-galactosidase and analysed by whole mount *in situ* hybridization for the RNA shown. Sectioned embryos were then scored for the presence of β-galactosidase unilaterally in the heart region and asymmetry of the mRNA expression under test (A and B).

not seen in control embryos injected with mRNA for either mGATA-1 (encoding a severely truncated GATA-1 protein) (Figure 7D) or GATA-1 itself (Figure 7E). The specificity of this effect for heart mesoderm can be seen by comparing the disturbed actin signal in the heart precursor cells with the undisturbed staining in the somites (Figure 7B). A major problem with these over-expression experiments is the difficulty in achieving precise targeting of injected mRNA to the region of interest. Thus, of ~450 GATA-6 injected embryos that appeared normal as intact embryos at stage 28, only 24 had unilateral β-galactosidase staining in the presumptive heart region when sectioned. Importantly, 83% of these correctly targeted embryos showed a reduction in XMLC2/cardiac actin staining on the injected side (Table IA). The majority of the remaining GATA-6-injected embryos showed unilateral β-galactosidase staining in a variety of areas outside the presumptive heart region (including adjacent tissues) but no reduction in XMLC2/cardiac actin staining was observed on the injected side of these embryos. In contrast, only 11% of GATA-1-injected and none of the mGATA-1-injected embryos showed a reduction on the injected side (Table IA). We conclude that over-expression of GATA-6 in the heart-forming cells of the antero-ventral mesoderm prevents their differentiation to the stage at which they express myosin light chain and cardiac actin.

This reduction in the numbers of differentiating heart cells could result from loss, respecification or a block to their differentiation. Two approaches were used to investigate this question. First, whole mount *in situ* hybridization followed by sectioning was carried out on embryos injected with GATA-6 or GATA-1 mRNA, as described above, with a probe for the homeodomain-containing gene XNkx-2.5, which is expressed in the mesoderm, including presumptive heart cells, from early gastrulation (Tonissen *et al.*, 1994). No perturbation of this marker (purple staining in Figure 7F and G) was seen in any of the preparations in which there was unilateral staining for β-galactosidase in the antero-ventral mesoderm (Table IB). Second, the blocked cells were able to initiate the wrapping of the endocardium apparently normally (Figure 7F and G). These observations suggest that the heart precursors were not destroyed or respecified by GATA-6 over-expression but rather that their terminal differentiation was blocked.

### *Recovery from ectopic GATA-6 expression results in increased myocardial muscle thickness*

Despite the earlier block to differentiation, injected embryos allowed to develop to the beating heart stage had macroscopically normal, beating hearts. Whole mount *in situ* hybridization with XMLC2 at stage 35 confirmed that well-defined hearts were formed, both in embryos injected at the one cell stage (data not shown) and unilaterally at the two cell stage (Figure 8B compared with uninjected controls in Figure 8C). Whilst these hearts could derive from the remaining unaffected cells, the increase in myocardial tissue (see below), together with the continued expression of XNkx-2.5 in the injected cells, suggests an alternative explanation, namely that the block to differentiation is reversible. A likely cause of such reversibility would be turnover of the injected RNA. To monitor this, RNase protections were carried out using the β-globin 5′- and 3′-untranslated sequences present in the injected GATA-6 RNA. The β-globin RNA containing these sequences is not itself expressed in the embryo until metamorphosis (Banville and Williams, 1985). Protected bands corresponding to the 5′- and 3′-untranslated regions of the injected GATA-6 RNA were detected up to and including stages 24 and 28, with a much reduced signal by stage 35 (Figure 8A). Similar half-lives of injected RNAs have been estimated by others (Harland and Misher, 1988; Turner and Weintraub, 1994). Thus, the majority of injected RNA present at stage 28 when the block to terminal differentiation is occurring has turned over by stage 35 when an apparently normal heart is seen. It therefore seems that the block to differentiation is reversible and that the cells prevented from differentiating by elevated GATA-6 expression can still differentiate when the injected RNA decays. The transient nature of this effect coupled with the need for accurate targeting probably explains the failure by other workers to observe any effect (Jiang and Evans, 1996).

Closer inspection of the heart in recovered embryos, by vibratome sectioning, revealed an increase in the intensity of staining for XMLC2 on the GATA-6-injected side in both the atria (Figure 8D) and ventricles (Figure 8E), in contrast to the symmetrical signal in the uninjected controls (Figure 8F and G). Increased XMLC2 expression in injected embryos was confirmed by RNase protection analysis (data not shown). To determine if this reflected

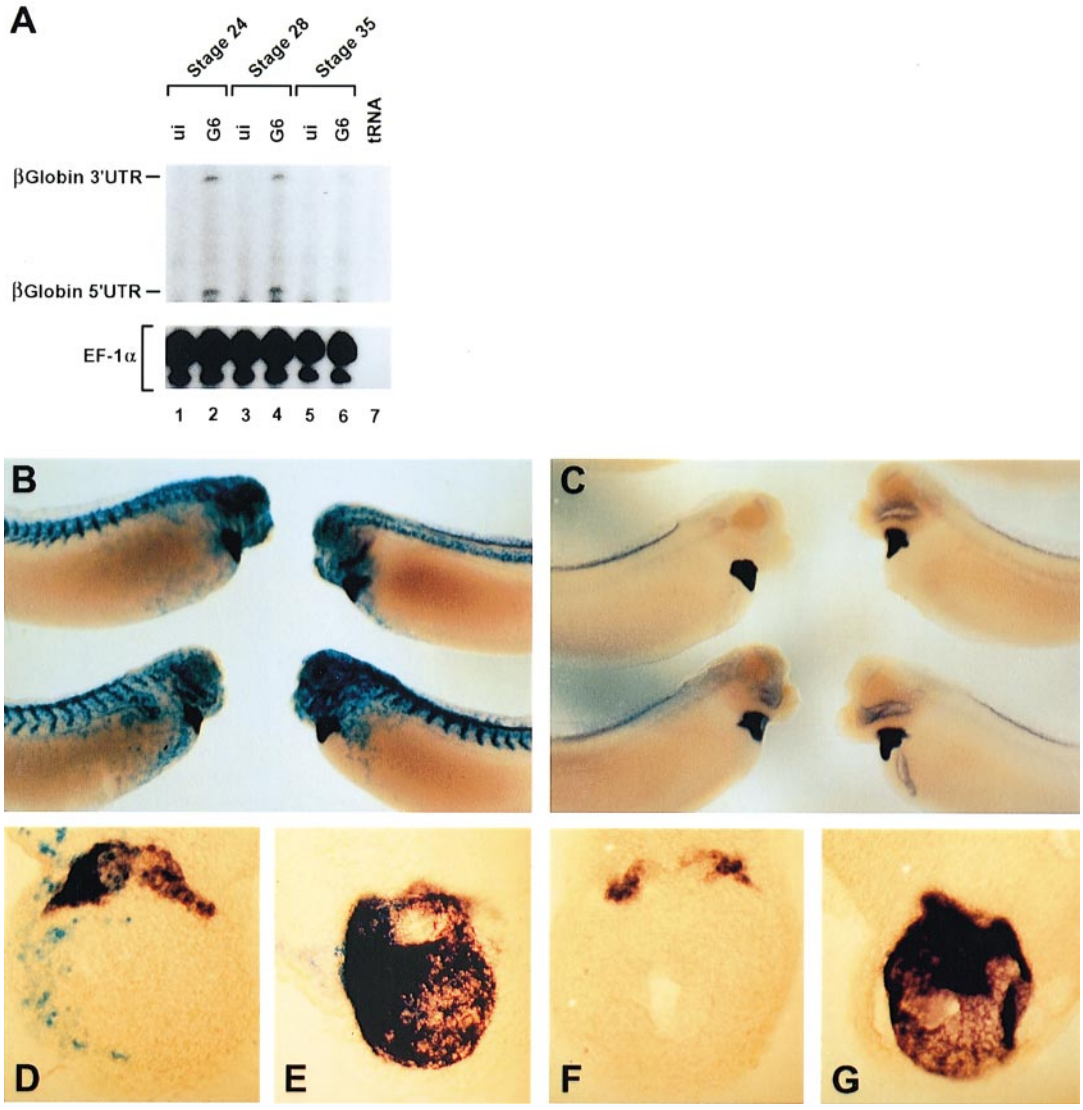


**Fig. 8.** Injected *in vitro* synthesized mRNA persists to stage 28 and then declines in *Xenopus* embryos. XMLC2 expression is increased in the beating heart (stage 35) by GATA-6 over-expression. (**A**) Total RNA extracts (five embryo equivalents) were analysed by RNase protection for β-globin 3′- and 5′-untranslated regions (UTR) and control EF-1α mRNAs. Embryos injected with 120 pg GATA-6 mRNA at stage 1 and uninjected control embryos were analysed at the time points indicated. (**B**) Lateral views from the injected side of cleared stage 35 embryos injected with 50 pg GATA-6 mRNA into a single blastomere at the two cell stage and probed *in situ* with XMLC2 show apparently normal heart morphology when compared with (**C**) uninjected embryos. (**D**) Transverse section through the atria and (**E**) ventricle of one of the embryos in (B) showing increased staining for XMLC on the injected side in both parts of the heart compared with the symmetrical staining in the uninjected embryo (**F** and **G**).

increased tissue, the myocardial thickness was measured in photographs of transverse sections through the heart region. Care was taken to ensure that sections were precisely transverse. There was a 14.8% ($P < 0.001$) increase in myocardial thickness on the injected as compared with the control uninjected side of stage 35 embryos (Table II). The injected side was not consistently left or right and uninjected embryos revealed no left/right differences in thickness. In contrast to the situation in stage 35 injected embryos, at stage 28/29 no difference in myocardial thickness was seen between the injected and uninjected sides (Table II). It therefore seems that the hearts formed in injected embryos contain increased myocardial tissue, as revealed by increases in thickness and XMLC2 expression.

## Discussion

We report here the cloning of *Xenopus* GATA-6 cDNA, a member of the family of zinc finger transcription factors that recognize the consensus sequence WGATAR. The emerging pattern of expression profiles for this family is that GATA-1, -2 and -3 are expressed in blood, with GATA-2 and -3 additionally in ectodermal derivatives, whereas GATA-4, -5 and -6 (Laverriere *et al.*, 1994; Jiang and Evans, 1996) are predominantly expressed in heart and endodermal derivatives. We show here that xGATA-6 follows this trend, although we also detect expression in skeletal muscle, skin and gonads. The RNase protection probe used here is specific for xGATA-6b while it is not clear to what extent the RT–PCR primers used by Jiang

**Table II.** Recovery from ectopic GATA-6 expression results in increased myocardial muscle thickness

| | Percentage difference in myocardial thickness | |
|---|---|---|
| | GATA-6 injected embryos (Injected versus uninjected side) | Uninjected embryos (Left versus right side) |
| Stage 28/29 | –2.1 ± 2.1[a] $n = 6$ | +1.2 ± 1.7[a] $n = 10$ |
| Stage 35 | +14.8 ± 3.3[b] $n = 6$ | +2.5 mean of 2 |

Embryos were injected into one of two cells with *in vitro* transcribed GATA-6 mRNA (50 pg) together with RNA encoding β-galactosidase (50 pg). When embryos had developed to stages 28/29 and 35 they were fixed, stained for β-galactosidase and analysed by whole mount *in situ* hybridization for XMLC2 or cardiac actin expression and sectioned transversly. Results are expressed as the mean ± SEM; *P* values were calculated using Student's *t*-test.
[a] $P > 0.25$
[b] $P < 0.001$

and Evans (1996) would cross react with xGATA-6b. Thus the different approaches used could explain the observed expression differences in lung and skeletal muscle. Although we detect no GATA-6 in adult blood, high levels of expression are seen in ventral blood island mesoderm from early neurula through to late tailbud, suggesting that GATA-6 may play an early role in embryonic blood formation. Thus GATA-6 appears to be unusual for this branch of the family in being expressed in mesoderm giving rise to both embryonic blood and heart. The role of GATA-6 in blood mesoderm is currently under investigation.

Expression of GATA-6 begins at the onset of gastrulation, concomitant with specification of mesodermal cell types, including the heart (Sater and Jacobson, 1989). As gastrulation proceeds the signal fades dorsally but is maintained broadly in the dorso-lateral, ventro-lateral and ventral mesoderm. The domain of expression is subsequently restricted to the morphogenetic fields of the heart and blood (Sater and Jacobson, 1990; Walmsley *et al.*, 1994; Bertwistle *et al.*, 1996). Terminal differentiation of both tissues is associated with a quantitative reduction in GATA-6 expression. Thus, GATA-6 is switched on early in cells with heart- and blood-forming potential and subsequently switched off in those cells which do not contribute to these tissues but is also down-regulated in cells which do. These observations indicate that GATA-6 may play important roles in heart and blood specification and also in controlling their differentiation.

The role played by GATA-6 in heart formation was investigated by over-expressing the protein in developing embryos. We found that differentiation of the myocardium to the myosin light chain- and cardiac actin-expressing stage was blocked, an effect specific both to the heart versus the somites and to GATA-6 versus GATA-1. Specificity within the GATA family has also been seen in experimental manipulation of erythroid differentiation (Briegel *et al.*, 1993), but these data and our own contrast with recent reports suggesting interchangeability of family members (Blobel *et al.*, 1995; Visvader *et al.*, 1995). The unchanged XNkx-2.5 expression pattern shows that the heart progenitors were not respecified or destroyed by GATA-6 overexpression. These experiments also indicate that the morphological development of the heart can at least initiate independently of the establishment of the contractile machinery.

As the levels of injected GATA-6 RNA decreased, cardiac differentiation escaped from the block, giving rise to normal cardiac structure but increased myosin light chain expression and myocardial thickness, implying an increase in cell number. Whilst normal hearts could derive from the remaining unaffected cells, the increase in the number of cardiomyocytes suggests an alternative explanation. In experiments involving the over-expression of another member of the GATA family, GATA-2, erythroid differentiation was blocked leading to continued proliferation of cells (Briegel *et al.*, 1993). Like GATA-6 in the embryonic heart lineage (Laverriere *et al.*, 1994; this study), GATA-2 expression drops as haematopoietic progenitors differentiate (Briegel *et al.*, 1993; Leonard *et al.*, 1993; Nagai *et al.*, 1994). In the case of embryos injected with GATA-6 mRNA, it would appear that terminal cardiomyocyte differentiation is prevented until the exogenous mRNA and protein turn over. The subsequent increase in myosin light chain expression may reflect the higher proliferative capacity of cardiac muscle precursors compared with their differentiated derivatives. Holding the cells in the precursor state for a longer period would likely increase their number. An alternative explanation could involve cell recruitment. If GATA-6 plays a role in specification of heart mesoderm, as suggested by its expression pattern, part of which reflects the formation and restriction of the heart field during development, maintenance of expression in the more lateral cells of the heart field could facilitate their recruitment into the pool of cells for later terminal differentiation.

What is the mechanism by which GATA-6 specifies heart mesoderm and prevents its premature differentiation? GATA-binding sites have been identified in the control regions of a number of cardiac-specific genes and GATA-4 has been shown to bind to some of these and to transactivate transcription (Arceci *et al.*, 1993; Grepin *et al.*, 1994; Ip *et al.*, 1994; Molkentin *et al.*, 1994). An essential role for GATA-4 in the expression of cardiac-specific terminal differentiation markers has been demonstrated using antisense transcripts in cultured cells (Grepin *et al.*, 1995). More recently, mouse GATA-6 has been demonstrated to bind *in vitro* a specific element within a putative natural target, the heart-specific cTnC promoter enhancer element, and be capable of transactivating a co-transfected reporter construct containing this element in a non-cardiac cell line, CT3 (Morrisey *et al.*, 1996). We show here that

GATA-6 can bind canonical GATA-binding sites and transactivate reporter constructs containing multimerized copies of them when injected into *Xenopus* embryos. However, in contrast to a recent report (Jiang and Evans, 1996), we were unable to transactivate the endogenous cardiac actin gene in *Xenopus* blastulae and neither was the endogenous XMLC2 gene transactivated. We note that in the reported experiments, transactivation by GATA-4 and -5 was significantly stronger and less variable than by GATA-6. However, our results are in agreement with those of Morrisey *et al.* (1996), where GATA-4 and -6 over-expression failed to transactivate endogenous cardiac-specific genes in the non-cardiac CT3 cell line. Thus it appears that both the temporal and spatial context of the binding element may be important for transactivation. The effects of GATA-6 over-expression which we report in the present study are the only ones carried out in the correct developmental context, including the background of GATA-4 and -5 expression levels and the spatial location. These observations are consistent with the view that GATA-6 expression is necessary but not sufficient to drive the complete cardiogenic programme. It is therefore possible that the targets for GATA-6 may not be the cardiac genes associated with terminal differentiation but genes associated with the precursor state. Terminal differentiation is more likely to be driven by GATA-4 or -5, since their concentrations are maintained as the embryonic heart differentiates, in contrast to GATA-6 (Laverriere *et al.*, 1994; this study). It will be important to compare the effects of correctly targeted GATA-4 and -5 over-expression with those reported here for GATA-6. We conclude that the important role played by GATA-6 in heart cell precursors is mediated at least in part by target genes yet to be identified.

## Materials and methods

### *Isolation and characterization of cDNA clones*

An adult *Xenopus* liver cDNA library (Xu and Tata, 1992) in λZAPII (Stratagene) was screened at moderate stringency (3× SET, 65°C) using a 350 bp fragment from the zinc finger region of *Xenopus* GATA-2 (Walmsley *et al.*, 1994). Inserts from a number of plaque-pure, positive clones were rescued into Bluescript phagemids and characterized initially by restriction analysis and sequencing using the *fmol* DNA Sequencing System (Promega). This allowed the identification of seven overlapping clones containing a 5′-region of variable length. Two of the clones encoded a complete open reading frame of 391 amino acids. The presumptive initiation codon appears to be the true translation start site because an in-frame stop codon is located 210 nt upstream and the ATG is flanked by nucleotides appropriate for translation initiation (Kozak, 1981; Cavener and Ray, 1991). A segment of 2.1 kb of the largest insert (3.3 kb), which contained the complete open reading frame, was sequenced on both strands. Confirmatory sequence of the full-lengh cDNA was obtained from the overlapping clones. Sequence identity demonstrated that only one of the two non-allelic copies of the gene (Kobel and Du Pasquier, 1986) had been isolated.

### *RNA isolation and RNase protection analysis*

Total RNA was isolated from staged embryos as described in Walmsley *et al.* (1994) and from dissected adult tissues by homogenization in guanidinium isothiocyanate followed by acidic phenol/chloroform extraction (Chomczynski and Sacchi, 1987). Two RNase protection probes for GATA-6 were used, one of which protected 178 residues spanning nt 1143–1321 in the 3′ coding region, outside the zinc finger domain, and was prepared by subcloning an 804 bp fragment (nt 517–1321) into pGEM-3Z. After linearizing with *Hin*fI, antisense RNA was transcribed with SP6 polymerase. The second probe, which protected 155 residues, was prepared by subcloning nt 1–155 of the 5′-untranslated region of the largest cDNA into pGEM-3Z. Antisense RNA was transcribed with T7 polymerase after linearizing with *Bam*HI. The EF-1α probe (see Sargent and Bennett, 1990) was synthesized using SP6 polymerase from the *Hin*fI-linearized plasmid pSP72 as described by Krieg and Melton (1987). Hybrids were digested in 40 μg/ml RNase A and 2 μg/ml RNase T1 for 1 h at 37°C, followed by digestion with proteinase K (50 μg/ml) in the presence of 0.5% SDS. The XMLC2 probe was prepared as described (Chambers *et al.*, 1994) and digested with 2 μg/ml RNase T1 for 45 min. The β-globin untranslated region probe was synthesized using T7 polymerase and *Hin*dIII-linearized plasmid pβ-UT2 (a kind gift of A.Rodaway), which contains the β-globin 5′- and 3′-untranslated regions separated by a polylinker sequence. Hybrids were digested in 2 μg/ml RNase T1 for 1 h at 37°C. Samples were phenol extracted and precipitated with ethanol or propan-2-ol with tRNA as carrier. RNA samples were resuspended, denatured and resolved on 6% denaturing polyacrylamide gels. After autoradiography, the intensities of protected species and their respective EF-1α signals were quantitated by scanning densitometry of multiple exposures in the linear range.

### *In vitro synthesis of Xenopus GATA-6 and DNA binding studies*

PCR amplification of the open reading frame of xGATA-6 was carried out with primers (incorporating *Bam*HI compatible ends) spanning the initiation and stop codons. The product was sequenced after digestion with *Bam*HI and subcloning into the *Bgl*II site of pSP64T (Krieg and Melton, 1984). For *in vitro* transcription of xGATA-6 RNA the plasmid was linearized with *Eco*RI. Transcription was carried out using SP6 polymerase as described previously (Krieg and Melton, 1987) with the addition of GpppG (0.5 mM) to cap the RNA, which was translated in nuclease-treated rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions in the presence of [$^{35}$S]methionine. The protein product was resolved by SDS–PAGE in 12% gels (Laemmli, 1970) and visualized by fluorography. Binding of xGATA-6 to GATA *cis* elements was determined by gel shift assays as described by Perkins *et al.* (1989). Synthetic, double-stranded oligonucleotides used to determine binding activity were αG2 (GATCCGGGCAACTGATAAGGATTCCCA), which contains the core WGATAR motif from the promotor of the mouse α1 globin gene, and mutants of the region underlined in αG2. These were αG8 (GAcAAG), αG9 (GATAta) and αG10 (GATAca) (Plumb *et al.*, 1989). Additionally βHB, a WGATAR-containing oligonucleotide, GATCCGAAGGAAAGATAGCAAATTTTA, from the promotor of the chicken β-hatching globin gene (Plumb *et al.*, 1989), a site-selected synthetic element which contains the sequence GATC that binds chicken GATA-2 and -3 (Ko and Engel, 1993) and the octamer consensus from the mouse immunoglobulin heavy chain gene promoter were used.

### *Preparation and characterization of GATA-1 and mGATA-1 expression plasmids*

Full-length GATA-1 cDNA (Zon *et al.*, 1991) in pGEM-7 was partially digested with *Nco*I and *Hin*dIII, the 1.2 kb fragment flanking the open reading frame was isolated and the overhanging ends filled in using Klenow fragment. This was then ligated into pSP64T which had previously been cut with *Bgl*II and filled in. *In vitro* transcription and translation produced a polypeptide of the expected size and injection of synthetic RNA into embryos produces an αG2 (GATA)-binding activity with the same mobility as the major *Xenopus* blood GATA in a gel shift assay (M.Guille and R.Patient, unpublished data). mGATA-1 was produced by partial digestion of the GATA-1 expression construct with *Bam*HI, filling in with Klenow fragment and isolating clones which had lost the *Bam*HI site at nt 336 of the cDNA. This produced a highly truncated peptide which did not bind DNA.

### *Embryos and RNA injections*

*Xenopus* embryos were obtained by artificial fertilization as described by Smith and Slack (1983). They were dejellied in 2% cysteine, pH 7.8, and incubated in 1× MBS until stage 6, when they were transferred to 0.1× MBS (Gurdon and Wickens, 1983). The embryos were staged according to Nieuwkoop and Faber (1967). Capped RNA (50–500 pg), prepared as described above and quantitated by UV absorption, was injected either at the one or the two cell stage. For two cell injections β-galactosidase RNA was included as a tracer (see Griffin *et al.*, 1995). The toxic level for each RNA preparation varied between experiments but where mortality exceeded 10% the embryos were discarded. To induce exogastrulation embryos were devittelined at stage 8 and incubated overnight in 1× MBS (Kintner and Melton, 1987). At the equivalent of

stage 13 (judged from sibling embryos) the embryos were dissected into ectoderm and mesendoderm fragments. RNA was prepared from 10–12 pooled fragments. Explants of the DLMZ (presumptive heart tissue; Sater and Jacobson, 1990) were dissected from stage 10.5 embryos and cultured to stages equivalent to 11, 15, 22, 28 and 33, as assessed by comparison with sibling embryos. At each stage RNA was extracted from 10 pooled explants. Similarly sized explants of developing heart tissue (corresponding to the discrete cardiac region seen in Figure 5H) and representing the mid ventral part of the presumptive heart field described by Sater and Jacobson (1990) were dissected from intact embryos at stages 20, 24 and 29 and the beating heart itself at stage 35 and RNA was extracted from 10 pooled heart fields/hearts at each stage.

### *In situ hybridization*

xGATA-6 antisense and sense digoxigenin-labelled RNA probes were transcribed from the complete largest cDNA (3299 bp) and hydrolysed to ~700 residues. Specificity of the signals was verified with probes transcribed from a 578 bp *Xba*I–*Eco*RI fragment from the 3′-untranslated region of the cDNA. The cardiac actin and XMLC2 probes were prepared as described by Mohun *et al.* (1988) and Chambers *et al.* (1994) respectively.

Whole mount *in situ* hybridization was performed on staged embryos essentially as described by Harland (1991), with the modifications described in Bertwistle *et al.* (1996). Colour development of β-galactosidase activity was carried out as described in Griffin *et al.* (1995) before bleaching.

Transverse thin sections of wax-embedded *Xenopus* embryos at stage 27 were mounted on slides coated with 2% 3-aminopropyl triethoxysilane as described by O'Keefe *et al.* (1991). *In situ* hybridization of sections was carried out using a modification of the whole mount protocol as described by Walmsley *et al.* (1994).

### *Sectioning of whole mount embryos*

Following whole mount *in situ* hybridization, thin sections of wax-embedded embryos were cut with a microtome. Alternatively embryos were embedded in a gelatin/albumen mix (phosphate-buffered saline containing 0.5% gelatin, 11% egg albumen and 20% sucrose) which was set by the addition of a 1/10 vol. of 25% glutaraldehyde according to the protocol of J.D.W.Clark (personal communication) and 50 μm sections cut with a vibrotome.

### *GATA transactivation assay*

Aliquots of 30 pg of *Sal*I-linearized luciferase reporter construct pRBGP3-MapX3, which contains multiple GATA binding sites in the promoter (Igarashi *et al.*, 1994; a kind gift of M.Yamamoto) and 20 pg of a *Hin*dIII-linearized TKβ-gal construct (used to control for injection variation; see Brewer *et al.*, 1995) were injected into the animal pole of stage 1 embryos either alone or along with GATA-6 or GATA-1 *in vitro* synthesized RNA. At stage 8, animal caps were dissected and cultured overnight at 19°C to the equivalent of neurula stages judged from sibling embryos. Assay of luciferase and β-galactosidase proteins in animal caps was carried out by measurement of chemiluminescence in a luminometer according to the instructions of the kit manufacturers (Luciferase assay system, Promega and Galacto-light TM, Tropix).

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