Induction of the mesendoderm in the zebrafish germ ring by yolk cell-derived TGF-β family signals and discrimination of mesoderm and endoderm by FGF

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This paper is dedicated to the memory of Nigel Holder who died tragically as this work was being written up for publication

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SUMMARY

The endoderm forms the gut and associated organs, and develops from a layer of cells which emerges during gastrula stages in the vertebrate embryo. In comparison to mesoderm and ectoderm, little is known about the signals which induce the endoderm. The origin of the endoderm is intimately linked with that of mesoderm, both by their position in the embryo, and by the molecules that can induce them. We characterised a gene, zebrafish gata5, which is expressed in the endoderm from blastula stages and show that its transcription is induced by signals originating from the yolk cell. These signals also induce the mesoderm-expressed transcription factor no tail (ntl), whose initial expression coincides with gata5 in the cells closest to the blastoderm margin, then spreads to encompass the germ ring. We have characterised the induction of these genes and show that ectopic expression of activin induces gata5 and ntl in a pattern which mimics the endogenous expression, while expression of a dominant negative activin receptor abolishes ntl and gata5 expression.

Injection of RNA encoding a constitutively active activin receptor leads to ectopic expression of gata5 and ntl. gata5 is activated cell-autonomously, whereas ntl is induced in cells distant from those which have received the RNA, showing that although expression of both genes is induced by a TGF-β signal, expression of ntl then spreads by a relay mechanism. Expression of a fibroblast growth factor (eFGF) or a dominant negatively acting FGF receptor shows that ntl but not gata5 is regulated by FGF signalling, implying that this may be the relay signal leading to the spread of ntl expression. In embryos lacking both squint and cyclops, members of the nodal group of TGF-β related molecules, gata5 expression in the blastoderm is abolished, making these factors primary candidates for the endogenous TGF-β signal inducing gata5.

Key words: GATA-5, GATA transcription factors, Heart, Gut development, TGF-β, squint, cyclops, nodal, activin, no tail

INTRODUCTION

The endoderm is the germ layer which forms the gut lining and contributes to organs forming as outgrowths of the gut, including the lungs, pancreas and liver. Of the three germ layers that emerge during gastrulation, the induction and patterning of the mesoderm and ectoderm have been much studied. In contrast, little is known about these processes in the endoderm, largely due to a lack of markers of cell differentiation during the early stages of endoderm formation. According to fate maps of Xenopus and zebrafish, cells of the future endoderm are located at the vegetal and lateral margin of the embryo prior to gastrulation. In Xenopus these are the large yolk cells of the vegetal hemisphere and the superficial layer of the involuting marginal zone (Keller, 1975, 1976; Dale and Slack, 1987; Moody, 1987; Minsuk and Keller, 1997). In zebrafish the endoderm is derived from cells closest to the blastoderm margin, adjacent to the vegetal yolk cell (Kimmel et al., 1990; Warga and Nusslein-Volhard, 1999).

Amongst vertebrates, endoderm specification has been best studied in Xenopus. In early studies, whole vegetal pole pieces were cultured (Holtfreter, 1938; Okada, 1960; Takata, 1960) or single blastomeres transplanted (Heasman et al., 1984; Wylie et al., 1987). These showed that endodermal commitment occurs gradually from blastula to early gastrula stages, implying that genes necessary for this commitment have been induced at late blastula stages and that further cell interactions are not necessary to maintain expression. More recent studies addressed the signals involved in mesendoderm specification. These imply that a TGF-β signal can induce endoderm (Gamer and Wright, 1995; Henry et al., 1996, Jones et al., 1993). Activin or processed Vg-1 are capable of inducing the markers...
In *Xenopus* it is unclear whether activin is the endogenous mesoderm inducer (Dyson and Gurdon, 1997; Schulte-Merker et al., 1994), however in teleosts, there is evidence, from injection of RNA encoding interfering variants of activin, that it is involved in mesoderm specification (Wittbrodt and Rosa, 1994). If activin is also involved in mesoderm induction in zebrafish, it is likely that it acts in concert with other members of the TGF-β family, since loss of function of two members of the nodal-related subfamily cyclops (cyc) and squint (sqt) leads to loss of endoderm (Feldman et al., 1998).

The role of FGFR in endoderm induction is unclear. Studies using animal caps and/or vegetal explants by Jones et al. (1993) and Henry et al. (1996) showed that FGFR can induce some endoderm–expressed genes, whereas Gardner and Wright (1995) demonstrated that FGFR inhibits expression of *Hxbox8*, while it is not involved in induction of endoderm (Sasai et al., 1996). Low levels of FGFR can induce mesoderm-specific gene expression in vegetal blastomeres implying that FGFR is not normally active in these cells (Cornell and Kimmelman, 1995). It appears likely that a TGF-β family member is required for the induction of both endoderm and mesoderm while FGFR signalling plays a role in the maintenance of the mesoderm in *Xenopus* (Isaacs et al., 1994, Schulte-Merker and Smith, 1995) and zebrafish (Griffin et al., 1995). This conclusion is supported by the observation that the endoderm marker, Xsox17α, is induced by activin but not FGFR (Hudson et al., 1997).

In order to analyse endoderm induction in zebrafish we have cloned *gata5* cDNA. We show that *gata5* is expressed in cells with an endodermal fate from the earliest stages of the induction process. In the blastula *gata5* is coexpressed with the mesodermally expressed gene *no tail* (*ntl*), the zebrafish brachury homologue (Schulte-Merker et al., 1994). However by the start of gastrulation, *ntl* expression has spread to encompass the germ-ring, while *gata5* remains confined to the marginal blastomeres. Transplants show that *gata5*, as well as *ntl* (Mizuno et al., 1996), is induced by a signal from the yolk cell. It is likely that this signal involves TGF-β family members, since a dominant negative activin receptor blocks expression of both genes in the germ ring, and injection of activin RNA results in

**Fig. 1.** (A) cDNA sequence and the ATG beginning the long open reading frame is in bold, as is an upstream, in-frame termination codon. Within the translation, the conserved cysteine residues of the ‘zinc fingers’ are in bold and underlined. The sequence 3’ to base 1912 is not shown since the partial cDNAs which run beyond this diverge in sequence. (B) Alignment of the predicted sequence of *gata5* protein with GATA5 from *Xenopus* (Kelley et al., 1993), chicken (*c*) (Laverriere et al., 1994) and mouse (*m*) (Morrissey et al., 1997). Residues identical in two or more sequences are boxed in black, and those where conservative substitutions are present in two or more sequences are boxed in grey. Alignment was performed using the programme Pileup (GCG) and shaded using Boxshade.
ectopic induction of these genes in a distribution which mimics their endogenous arrangement. Injection of constitutively active activin receptor RNA induces both genes. At higher levels of injected RNA, gata5 is expressed cell autonomously, while ntl is expressed in cells distant to those receiving the RNA, implying a relay signal. The relay is likely to be FGF since this signal is necessary for ntl expression in all but the most marginal blastomeres, while gata5 is unresponsive to perturbation of FGF signalling. Fish doubly mutant for squint and cyclops do not express gata5 in the marginal blastomeres, implying that the TGF-β signals include these nodal-related factors.

MATERIALS AND METHODS

Cloning of zebrafish gata5

A partial cDNA for gata5 was cloned from a zebrafish neurula library (gift of D. J. Grunwald) by hybridisation with a fragment encoding the conserved DNA binding domain of Xenopus GATA-2A as described by Neave et al. (1995). This cDNA was used to probe, at high stringency, a random primed zebrafish 3- to 16-hour library (gift from J. Campos-Ortega) and a further 6 overlapping partial cDNAs were obtained. Since none of the partial cDNAs extended to the translation start site, a ‘Marathon’ cDNA synthesis kit (Clontech) was used to obtain three separate 5' RACE clones. The cDNA clones from the libraries and the 5’RACE clones were sequenced by a combination of manual (Sequenase, Amersham), and automated fluorescent cycle sequencing (ABI). The sequence of zebrafish gata5 has the accession number AJ242515.

Cloning of PCR fragments encoding zebrafish activins A and B

PCR fragments encoding zebrafish activins βA and βB were obtained using primers identical to those described by Thomsen et al. (1990). Amplification was performed on 0.5 μg of boiled zebrafish genomic DNA and products of 365 bp were ligated into pSP73 and sequenced.

Fig. 2. Expression of zebrafish gata5 detected by in situ hybridisation. Stages according to Kimmel et al. (1995). In A and B the animal pole is up and in C-F anterior is to the left and dorsal is up. (A) 40% epiboly, gata5 expression in the germ ring. (B) 80% epiboly. gata5-expressing cells have involuted and dispersed towards the animal pole. (C) 2 somites. gata5-expressing cells are in the lateral mesoderm anteriorly, and in presumptive endoderm cells converging towards the midline. (D) 11 somites. The anterior extent of the expressing cells is beneath the midbrain. (E) 22 somites. The heart is expressing gata5 (arrowheads). Caudally, expressing cells are present around the yolk cell extension (arrows). (F) 24 hours. The arrowhead marks the heart and the arrows expression around the yolk cell extension. (G) 24 hour, frontal view. gata5 expression in the heart on the left hand side of the embryo (arrowhead). More caudally, expression is across the midline in a thin layer of presumptive endoderm, and in the anterior lateral mesoderm (lm). (H) 30 hours (thick section). Cells expressing gata5 are close to the yolk cell (arrows). neural keel (nk); notochord (n); somites (s). At 4 days (10 μm sections) gata5 expression is maintained in the heart (arrow in I) and the gut (J). Labels in J are n, notochord; m, muscle; y, yolk. Scale bars, 250 μm (A-G); 50 μm (H); 25 μm (IJ).
The sequences of the activin βA and βB clones have accession
tables AJ238980 and AJ238981 respectively.

Whole-mount in situ hybridisation

Antisense probes for xata5 (digoxigenin labelled) and ntl (fluorescein labelled) were synthesised and single-colour and two-colour whole-mount in situ hybridisations were performed essentially as previously described (Gering et al., 1998). The signal for xata5 was developed using BM-Purple, and that for ntl using Fast Red (both Boehringer Mannheim). Single-colour in situ were sectioned after mounting in JB4 methacrylate resin (Agar Scientific), and counterstained using Nuclear Fast Red (Vector). Two-colour in situ were mounted in agarose, frozen and sectioned using a cryostat. To more clearly distinguish overlapping gene expression, we developed a technique that allowed the separate detection of two genes on the same section. Briefly, after detection using Fast Red (Gering et al., 1998), the embryos were photographed before developing with BM-purple. Finally, the Fast Red stain was removed by washing (4×5 minutes) in methanol, allowing the BM-Purple signal to be seen alone.

Immunohistochemistry

Rabbit polyclonal antiserum directed against zebrafish Fkd2 was a gift from Rachel Warga (Warga and Nusslein-Volhard, 1999). Embryos stained by whole-mount in situ hybridisation for xata5 message were re-fixed in 4% paraformaldehyde then washed into MABTw (0.1 M maleic acid, 0.15 M NaCl, pH7.5 using NaOH, 0.1% Tween 20). After blocking (2 hours RT, 2% Boehringer Block in MABTw), embryos were incubated (overnight, 4°C) with anti-Fkd2 antiserum (1:200 in 2% Boehringer Block in MABTw). After washing, detection was carried out with the ABC system using DAB substrate (Vector Laboratories). Embryos were sectioned (14 µm) using a cryostat.

Preparation of synthetic RNA and RNA injections

RNA for injection was synthesised from templates linearised with the appropriate restriction enzyme, using the MEGAScript kit (Ambion Inc.) according to the manufacturer’s instructions, except that 1.25 µl of 40 mM Cap analogue [mG(5’)ppp(5’)]G and 1 µl of 75 mM GTP were used in place of 2 µl of 75 mM GTP. Embryos were injected with 200-400 pl of RNA solution in deionised water into the yolk-free animal pole cytoplasm at the 2-4 cell or 16-32 cell stages (see below).

The template for synthesis of Xenopus eFGF (Isaacs et al., 1992) was as described by Griffin et al. (1995): pSP64-T-ZZ (zebrafish activin βB) was a gift from Jochen Wittbrodt (Wittbrodt and Rosa, 1994). Dominant negative FGF receptor, XFD was a gift from Enrique Amaya (Amaya et al., 1992). Dominant negative Xenopus activin receptor dnXAR is a gift of Dan Mahony and is essentially the same as ΔXR1 (Hemmatti-Brivanlou and Melton, 1992) except that it is cloned in pBscRN3 (Lemaire et al., 1995). Constitutively active murine Type 1 activin receptors, ALK-2α and ALK-4α (ten Dijke et al., 1993), contain a single amino acid substitution in the juxtamembrane activation domain (Weiser, et al., 1995), and can be used to assess function of signalling through the pathways normally activated by ligand binding (Armes and Smith, 1997).

Yolk cell transplantation

The method used was that described by Mizuno et al. (1996). Briefly, the blastoderm was removed from a yolk cell at the high to sphere stages (staged according to Kimmel et al., 1995). The donor yolk cell was then transplanted onto the animal pole region of a recipient embryo of approximately the same stage. Grafted embryos were allowed to develop until 50-60% epiboly before fixation and in situ hybridisation using a xata5 probe.

RESULTS

Features of the zebrafish xata5 cDNA and protein

We screened a zebrafish neurula cDNA library using a fragment of the Xenopus GATA-2A cDNA (Zon et al., 1991) encoding the conserved zinc-finger DNA-binding domain. One clone appeared to be a partial cDNA encoding zebrafish xata5. We used this to rescreen a random-primed cDNA library, obtaining six overlapping clones. Since the initiating methionine codon was absent, we used 5’ RACE to extend the cDNA sequence. The largest contig obtained was 2.6 kb, however since the two cDNAs containing the 3’ end diverge in sequence beyond base 1912, we only show sequence 5’ to this in Fig. 1A. The first ATG, which is in-frame with a termination codon 60 bp upstream, begins an open reading frame encoding 383 amino acids. The predicted Mr 41.5×103 protein was shown by database searching to be a member of the 4/5/6 subfamily of GATA factors, and by pairwise comparison with these proteins that it was considerably more similar to GATA-5s from other species. We therefore propose that we have cloned zebrafish xata5. Use of a zebrafish ‘GATA-5’ gene to mark the developing pancreas has been described (Pack et al., 1996), however it is now clear that this was in fact xata6 (J. Reiter and D. Y. Stainier, personal communication).

Fig. 1B shows a translation alignment between xata5 and GATA-5 cDNAs cloned from other species. The most highly conserved region is the DNA-binding domain, containing two zinc fingers, characteristic of vertebrate GATA factors. The sequence C-terminal to this region is considerably less conserved than that N-terminal, which has recently been shown to contain transcriptional activation domains conserved amongst members of the GATA-4,5/6 subfamily (Morrisey et al., 1997). The amino-acid residues identified as being vital in the function of these domains in mGA TA-4 are either conserved or, in the case of Y21 (F26 in mGA TA-4) and D117 (E149 in mGA TA-4), conservatively substituted.

xata5 expression in the embryo occurs in gut and heart cells

xata5 transcripts are first detected in the blastula, at 30% epiboly, in cells around the blastoderm margin. This expression is initially symmetrical and at 40% epiboly is in a 3-4 cell deep rim around the blastoderm close to the yolk cell (Fig. 2A). At the onset of gastrulation (50% epiboly) xata5 is expressed in cells that are amongst the first to involute. By mid-gastrula stages (80% epiboly; Fig. 2B), expression can be seen in the hypoblast, more strongly on the ventral side of the embryo, probably representing precursors of the later expression in the anterior lateral mesoderm. By the end of gastrulation (2 somites; Fig. 2C), the xata5 expression has begun to move towards the dorsal midline. Expression is stronger in the anterior half of the embryo. At 11 somites (Fig. 2D), this anterior expression is in the anterior mesodermal lateral to the mid- and hindbrain, including the cells that will fuse to form the heart ventral to the midbrain. In addition to this mesodermal expression, a thin layer of xata5-positive cells begins to form between the embryo and the yolk cell, running from the midbrain posteriorly to the tail-bud, likely representing the endoderm. At 22 somites (Fig. 2E), xata5 expression can be seen in the fusing heart-tubes (arrowheads), and in a layer of cells between the embryo and the yolk cell which now extends to surround the posterior end of the yolk-plug extension (arrows). This expression pattern is maintained at 24 hours post fertilisation (hpf). At this stage (Fig. 2F,G) the heart is beginning to circulate the blood and has moved to lie...
on the left side of the embryo (Fig. 2G), while gata5 expression is still also present in the anterior lateral mesoderm (lm). Expression of gata5 in the endoderm between the embryo proper and the yolk cell can be seen in Fig. 2G, but is more obvious in a transverse section (Fig. 2H) where a monolayer of cells lies across the width of the embryo (arrows). gata5 expression is maintained in the hatched larva (4 days) in both the heart (Fig. 2I arrow), and in the epithelium of the gut (Fig. 2J).

The gata5-expressing cells in the blastoderm margin are the first cells to involute during gastrulation and lineage labelling studies indicate that these cells give rise to the endoderm (Kimmel et al., 1990; Warga and Nusslein-Volhard, 1999). Two additional findings indicate that the gata5-expressing cells in gastrulae include endoderm precursors. Firstly, examination of the cells expressing gata5 at 80% epiboly shows them to be relatively large, flat cells located close to the yolk cell surface (Fig. 3E, white arrow), which is characteristic of gut precursors (Warga and Nusslein-Volhard, 1999). Secondly, in dorsal and lateral regions of the germ ring, gata5 coexpresses with the endoderm-expressed protein forkhead-2 (fkd2; Warga and Nusslein-Volhard, 1999) (Fig. 3A,B,D). Ventrally (Fig. 3C), a proportion of gata5-expressing cells, those closest to the yolk cell surface, coexpress fkd2 (arrow), whereas other, more superficial gata5-expressing cells, possibly fated to contribute to the anterior lateral mesoderm and heart, do not.

gata5-positive cells coexpress no tail at the onset of gastrulation
In zebrafish fate maps, endoderm precursors lie at the margin of the blastoderm. This region also gives rise to some mesodermal derivatives, while the germ-ring more distant from the margin gives rise to mesoderm alone (Kimmel, et al., 1990; Warga and Nusslein-Volhard, 1999). To define these mesendodermal cells in terms of gene expression we compared the expression of gata5 with the mesodermal regulatory gene no tail (ntl; Schulte-Merker et al., 1994). As determined by whole-mount double in situ hybridisation at 50% epiboly, gata5 is expressed in a marginal subset of ntl-expressing cells (Fig. 4A). To more precisely map the spatial relationship of these genes, we developed a technique for sequential visualisation of the ntl and gata5 signals on the same section (see Materials and methods). gata5 is expressed approximately 3-4 cell diameters from the blastoderm margin, whereas ntl expression extends 8-10 cell diameters from the margin (Fig. 4C,E). Thus, by the beginning of gastrulation (50% epiboly), the fate of the cells in the germ-ring, is mirrored by the genes they express. Earlier in development (at 30% epiboly), gata5 and ntl are coexpressed to a depth of 2-3 blastomeres from the blastoderm margin, adjacent to the yolk cell (Fig. 4B,D). gata5, but not ntl, is also expressed in the yolk syncitial layer (YSL) underlying the blastoderm margin. Thus at blastula stages there may be no distinction between cells fated to form endoderm or mesoderm.

gata5 expression in the blastoderm is induced by signals from the yolk cell
Since ntl is induced by signals from the yolk cell (Mizuno et al., 1996), and the expression of gata5 overlies with ntl in the margin of the blastoderm, we investigated whether gata5 is also induced by signals from the same source. We performed 33 successful yolk cell transplantations at high to sphere stages. Embryos were fixed between 50 and 60% epiboly. In 31 of the transplanted embryos ectopic gata5 expression was detected (Fig. 5A). In sections of these embryos the normal gata5 domain lies adjacent to the host yolk cell (hye) and a second localised band of expressing cells abuts the grafted, biotin-labelled yolk cell (dye, Fig. 5B). This domain is similar in position and dimensions to the normal gata5 expression domain. This result shows that, as for ntl, the yolk cell is the origin of inducing signals for gata5 in the blastoderm.

Ectopic activin induces gata5 and ntl in the blastula in distinct patterns
TGF-β signals have been implicated in endoderm induction and are therefore candidates for the yolk cell signal (Gamer and Wright, 1995; Henry et al., 1996; Jones et al., 1993). To assess the role of the TGF-β family of signalling proteins in induction of mesendodermal gene expression in zebrafish, we used activin and a dominant negative activin receptor. To generate a local, ectopic source of activin, we injected activin RNA (10 μg/ml) apically into 16-32 cell embryos (Fig. 6A-D). RNA (100 μg/ml) coding for a nuclear form of β-galactosidase (nls-β-gal) was coinjected as a lineage label. Embryos were fixed at 50% epiboly and the location of the injected RNA detected by staining for nuclear β-gal activity (turquoise spots). The cytoplasmic in situ signal for ntl (red) was developed and photographed followed by staining for cytoplasmic gata5 (blue-purple), ntl is ectopically induced in activin-injected embryos in a region that overlaps the staining for β-gal and extends 4-8 cell diameters beyond the injected cells (Fig. 6A,C). Sometimes ntl expression is absent from some of the β-gal stained cells, probably as a result of suppression of ntl expression by high levels of activin signalling, as is seen with Xbra in Xenopus (Green et al., 1992). Ectopic gata5 expression is induced in the injected cells and spreads beyond them by, at most, 2-3 cell diameters (Fig. 6B,D). This results in a ‘bull’s eye’ appearance, with the central gata5- and ntl-expressing cells surrounded by a halo of cells expressing ntl but not gata5. This mimics the expression of these genes in the germ ring, where the cells closest to the source of the endogenous signal (the yolk cell) express both gata5 and ntl, while more distant cells express only ntl.

To investigate further the possibility that endogenous induction of ntl and gata5 involved a TGF-β family member we injected embryos with RNA encoding a dominant negative activin receptor, dnXAR, which blocks signalling by a number of TGF-β family members including activin and Vg-1 (Schulte-Merker et al., 1994). Expression of dnXAR results in inhibition of the germ-ring expression of both gata5 and ntl coinciding with the expression of the coinjected β-gal tracer (Fig. 6E-G).

Differential expression of gata5 and ntl in response to constitutively active activin receptor; evidence for a relay signal for ntl
There are two possible interpretations of the pattern of ntl and gata5 expression induced by ectopic activin expression: (1) the cells expressing activin act as the high point of an activin diffusion gradient, and that ntl has a lower concentration threshold for induction by activin, and so is induced further from the activin source than gata5, or (2) that the activin signal...
is able to cause cells to produce a second ‘relay’ signal which is able to induce ntl but not gata5, so resulting in the spread of ntl expression beyond the cells that directly receive the activin signal. To distinguish between these hypotheses, we injected RNA encoding constitutively active forms of the type I activin receptor, ALK-4 (ALK-4*) and the type I BMP2/7 receptor ALK-2 (ALK-2*) (Jones et al., 1996, Weiser et al., 1995). Such receptors activate the intracellular signals normally driven by their ligands, but work in a cell-autonomous manner.

RNA for ALK-4* was injected at three different concentrations, 1, 10 and 100 \( \mu \text{g/ml} \), and different patterns of gene expression were observed at 50% epiboly. At the lowest concentration (Fig. 7A), gene expression was little affected except where the injected RNA (localised by \( \beta \)-gal staining) overlapped the germ-ring, resulting in slight spreading of ntl and gata5 expression away from the margin. At 10 \( \mu \text{g/ml} \) (Fig. 7B), ectopic expression of both genes was clearly induced. This often resembled the expression induced by injection of activin RNA, with the patch of injected cells expressing ntl and gata5 fringed by cells expressing ntl. These ntl-expressing cells generally did not contain \( \beta \)-gal staining. This is more clearly seen at the highest concentration of injected ALK-4*, where ntl expression is suppressed in a proportion of the injected cells.

Fig. 3. gata5 is expressed in endodermal cells in the gastrula embryo. (A-C) Cryostat sections of a 60% epiboly embryo double stained for gata5 message (in situ hybridisation; blue cytoplasmic staining) and fkd2 protein (antibody; brown nuclear staining). (A) Dorsal view. Coexpression of gata5 and fkd2 in the mesendoderm of the prechordal plate. (B) Lateral view. Coexpression of gata5 and fkd2 in endodermal precursors adjacent to the yolk cell, in the innermost layer of the hypoblast (arrow). fkd2 also expressed in the YSL (arrowhead) which weakly expresses gata5. (C) Ventral view. fkd2 is expressed predominantly in the YSL (arrowhead) with only the occasional hypoblast cell, coexpressing gata5 (arrow). gata5 is expressed in several layers of hypoblast cells without coexpression of fkd2. These may include precursors of the anterior lateral mesoderm. (D) Diagram showing the plane of sections (blue lines) and position in sections (red lines) in parts A-C. (E) High power lateral view of 80% epiboly embryo whole-mount focused immediately above the yolk cell. gata5 is expressed in cells (arrowed) having the characteristic position (adjacent to the yolk cell) and shape (large, flat cells) of endodermal precursors (Warga and Nusslein-Volhard, 1999).

Fig. 4. gata5 and ntl coexpress in the blastoderm margin. (A) Two-colour whole-mount in situ of a 50% epiboly embryo showing gata5 (blue) expression in the margin of the blastoderm, and wider expression of ntl (red). Scale bar,150 \( \mu \text{m} \). (B-E) sequential in situ detection on the same sections of gata5 (blue; B and C) and ntl (red fluorescence; D and E), at 30% epiboly (B,D) and 50% epiboly (C,E). (B) The initial expression of gata5 in the blastoderm extends 2-3 cell diameters from the blastoderm margin. Expression is also detected in the YSL (white arrow). (C) At 50% epiboly, gata5 expresses for approximately the same distance from the blastoderm margin, which now represents 3-4 cell diameters. (D) Initially ntl expresses in the same blastomeres as gata5, but not in the YSL. (E) 50% epiboly, ntl expression has spread to encompass blastomeres within 8-10 cell diameters of the margin. Thus, by the beginning of gastrulation, gata5-expressing cells are a marginal subset of those expressing ntl.
while neighbouring patches of uninjected cells strongly express \textit{ntl} (Fig. 7C,D white arrows and arrowhead, respectively). In contrast, \textit{gata5} is expressed cell-autonomously only in the cells that have received the ALK-4* message.

These data imply that cells responding to a TGF-\(\beta\) signal are able to produce a secondary relay signal, and that it is this signal which induces \textit{ntl} expression in cells distant from the source of the TGF-\(\beta\) signal. In contrast, the induction of \textit{gata5} is restricted to cells expressing the constitutively active receptor, indicating that the spread of expression seen with activin RNA injections is due to activin diffusion.

Injection of RNA encoding ALK-2* (BMP-2/7 receptor) at a concentration of 100 \(\mu \text{g/ml}\) did not perturb expression of \textit{gata5} or \textit{ntl} at the onset of gastrulation (Fig. 6E). The resulting embryos were, however, severely abnormal showing that the receptor was active.

\textbf{Unlike \textit{ntl}, \textit{gata5} expression in the blastoderm is not regulated by FGF}

Expression of the \textit{Xenopus} homologue of \textit{ntl}, Xbra, is maintained by a positive feed back loop involving FGF during blastula and gastrula stages (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). A similar mechanism acts on zebrafish \textit{ntl} (Griffin et al., 1995). We therefore examined whether \textit{gata5} is regulated by FGF by injecting RNA encoding either eFGF or the dominant negative FGF receptor, XFD (Amaya et al., 1992).

Unlike \textit{ntl} (Fig. 8C), \textit{gata5} is not ectopically expressed in response to injection of eFGF RNA (Fig. 8D), while expression of XFD does not downregulate \textit{gata5} expression in the germ ring (Fig. 8F). XFD does cause localised loss of \textit{ntl} expression in the germ ring (Fig. 8E) in cells containing this dominant negative FGFR RNA. However in the presence of XFD, a depth of 1-2 cells at the blastoderm margin continue to express \textit{ntl} prior to involution (Fig. 8E arrow) implying that an endogenous non-FGF signal induces the most marginal \textit{ntl} expression at this stage, but that FGF is required for the spread of \textit{ntl} expression. The FGF independence of \textit{gata5} expression is further confirmed by the fact that it is unaltered in \textit{no tail} mutant embryos in which functional \textit{ntl} protein is absent (data not shown).

\textbf{Nodal-related TGF-\(\beta\) family signals are required for expression of \textit{gata5} in the blastoderm margin}

Since ectopic activin is able to induce expression of \textit{gata5} and \textit{ntl}, we were interested to determine whether activin could be the endogenous yolk cell-derived signal inducing these genes. We isolated partial cDNAs to activin \(\beta\)A and \(\beta\)B, and RNase protection assays indicated that activin \(\beta\)A was not detectable until 5 hpf (data not shown), too late to be the endogenous inducing signal for \textit{gata5} expression which is detectable by 3 hpf. In contrast, \(\beta\)B RNA is present at low levels maternally, and increases soon after MBT, a result we have confirmed by semi-quantitative RT-PCR (data not shown), however we have...
not been able to reproducibly detect localised activin βB expression in the zebrafish blastula by in situ hybridisation or immunohistochemistry (Bartlett, 1995), while RT-PCR on dissected blastulae does not indicate that activin RNA is concentrated in the yolk cell relative to the blastomeres (data not shown). There is therefore currently insufficient evidence to establish a role for activin in endoderm induction in zebrafish.

Recently, it has been shown that the zebrafish mutants cyclops (cyc) and squint (sqt) result from mutations in the nodal-related TGF-β family members znr1 and znr2 respectively (Sampath et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998; Erter et al., 1998). While the loss of either of these genes predominantly affects the development of the prechordal plate mesendoderm and the ventral midline of the nervous system, embryos lacking the function of both these genes have essentially no mesoderm or endoderm (Feldman et al., 1998). We therefore examined the effect of these mutations on the induction of gata5 expression. In crosses between carriers of the cyc mutation, we could detect no effect on gata5 expression between 30% epiboly and shield stages (data not shown), with normal expression in the germ ring and YSL (see Fig. 9A,D open and black arrowheads respectively). In sqt crosses however, approximately one quarter of the embryos showed reduced gata5 expression: at 30% epiboly, expression in the blastoderm margin was weaker and sometimes patchy, while at 50% epiboly and shield stages, gaps in the ring of expression were clear (data not shown, but see Fig. 9B,E with black arrows indicating weak expression and white arrowheads delineating gaps). In a cross between sqt; cyc double heterozygotes (a gift from Will Talbot), embryos showing the phenotype for absence of sqt were seen as expected (Fig. 9B,E). In embryos lacking both sqt and cyc, expression of gata5 was completely absent from the blastoderm (Fig. 9C,F), with only some expression in the remaining YSL (Fig. 9C, black arrowhead). This shows that full induction of gata5 in the blastoderm margin is dependent on the overlapping activities of sqt and cyc.

**DISCUSSION**

**gata5 is expressed in the endoderm and its precursors**

To study the induction of endoderm in zebrafish, we have cloned zebrafish gata5, and conclude, for the following reasons, that it is expressed in endoderm and its precursors from 30% epiboly. (1) gata5 is expressed in the margin of the blastula, which contains the cells fated to be endoderm (Kimmel et al., 1990; Warga and Nusslein-Volhard, 1999). (2) During gastrulation gata5 is coexpressed with the endoderm-expressed nuclear protein, fkd2 (Fig. 3A-D). (3) During gastrulation gata5 is expressed in cells with the characteristics of endoderm (large, flat cells adjacent to the yolk cell; Fig. 3E). (4) Later, gata5 expression is found where endoderm forms in the embryo (Fig. 2D-H). (5) Eventually, gata5-expressing cells are located in the differentiating gut (Fig. 2I), consistent with the expression pattern of GATA-4/5/6 genes in other species (Kelley et al., 1993; Laverriere et al., 1994).

The expression pattern of gata5 and ntl in the blastoderm margin reflects cell fate

Fate maps show that the mesoderm and endoderm of zebrafish derive from cells near the blastoderm margin (Kimmel et al., 1990). Even in the late blastula, cells can give rise to clones which contribute to both germ layers (Warga and Nusslein-Volhard, 1999). Within the blastoderm margin the cell fates are to some extent segregated, in that cells within 4 cell diameters of the margin can give rise to endoderm and/or mesoderm, while cells further than this only generate mesodermal clones (Warga and Nusslein-Volhard, 1999). The expression of gata5 and ntl at the beginning of gastrulation (Fig. 4) mirrors this fate restriction: cells destined to form mesoderm alone express ntl alone, while cells in the region that gives rise to both mesoderm and endoderm express both ntl and gata5. This is, however, a simplification, in that endodermal structures derive more often from the dorsal and lateral regions of the blastoderm margin, whereas gata5 expression is symmetrical. The expression domain of fkd2 includes endoderm precursors from gastrula stages, and this protein is coexpressed with gata5 in dorsal and lateral regions of the hypoblast, while ventrally only a few of the gata5-expressing cells surface coexpress fkd2 (Fig. 3). It may be that some of the ventral gata5-expressing cells contribute to the anterior lateral mesoderm and heart, which express gata5 at later stages. Our studies do not address how gata5+ endoderm and gata5+ heart cells are differentiated, however there is a ventral to dorsal gradient of nkd2.5 in the germ ring (Chen and Fishman, 1996) which is known in other systems to cooperate with GATA factors to activate heart gene expression (Durocher et al., 1997). Stronger gata5 expression becomes apparent on the ventral side of the embryo at mid-gastrula stages, and may indicate gata5 expression in additional cells which will give rise to anterior lateral plate mesoderm and the heart.

**gata5 and ntl are induced by a TGF-β-like signal derived from the yolk cell**

As has been previously demonstrated for ntl and gsc (Mizuno et al., 1996), transplantation experiments indicate that the signal to induce expression of gata5 derives from the yolk cell. Like ntl and gsc, gata5 induction occurs only at the margin of the blastoderm: cells contacting most of the transplanted yolk cell surface do not express gata5. It is likely that this is due to the endogenous signal emanating only from the yolk cell where it contacts the blastoderm margin, since the response to ectopically expressed activin demonstrates that blastomeres in other locations are competent to respond by expressing gata5 and ntl.

The signal emanating from the yolk cell is likely to be a member of the TGF-β family of growth factors. Expression of a dominant negative activin receptor (which is able to block signalling by activin, Vg1 and possibly other TGF-β family members (Schulte-Merker et al., 1994)) prevents expression of both ntl and gata5 in the margin of the blastoderm. Ectopic expression of the TGF-β family member, activin, is able to induce expression of these genes in a pattern that mimics their endogenous expression: gata5 and ntl are coexpressed close to the source of the signal, and ntl alone is expressed 3-6 cell diameters beyond the expression domain of gata5.

**Different responses to a relay signal account for the patterned expression of gata5 and ntl in the blastoderm margin**

The patterned expression of ntl and gata5 after ectopic
expression of activin raised the possibility that this represented a differential response of these genes to an activin gradient, however it was also possible that activin was acting via a second, relay signal. The relative importance of diffusion and relay mechanisms in mesoderm induction is an area of considerable debate. It has been shown that activin can act as a morphogen by diffusing within explants and differentially inducing gene expression in distant cells (Jones et al., 1996; McDowell et al., 1997). These studies also provided evidence that the effect of activin on distant cells did not depend on a secondary relay signal. However, it has also been shown that *Xenopus* animal caps exposed to activin are able to induce mesoderm in untreated caps with which they are combined (Cooke et al., 1987), and Reilly and Melton (1996) showed that cells induced to form mesoderm by coinjection of TGF-β1 and its receptor were able to induce muscle differentiation in uninjected animal cap cells. Both of these studies therefore imply the presence of a relay signal in mesoderm induction.

Our studies support this conclusion: expression of a constitutively active activin receptor (ALK-4*), which activates intracellular responses to activin signalling, showed that while *gata5* appears to respond cell-autonomously to such signalling, *ntl* is induced in distant, uninjected cells showing that it is induced by a relay signal.

This result and that of Reilly and Melton (1996) appear to contradict the findings of Jones et al. (1996) and McDowell et al. (1997). However, this might result from the different experimental techniques used. In Jones et al. and McDowell et al., the response to TGF-β and potential relay signalling was assayed in late blastula animal caps, whereas in Reilly and Melton and in our study, the cells that produce the relay signal were present in the responding tissue throughout the early development of the embryo. It is therefore possible that the relay signal requires longer contact between signalling and responding tissue than was allowed in the experiments of Jones et al. and McDowell et al.

The identity of the relay signal as FGF was implied by studies which showed positive feedback between FGF signals and *ntl/Xbra* expression (Griffin et al., 1995; Isaacs et al., 1994; Schulte-Merker et al., 1995). In this study we confirm the induction of *ntl* by FGF and show that the spread of *ntl* expression beyond the most marginal 1-2 tiers of blastomeres depends on FGF signalling (Fig. 8E). Previously we had assayed *ntl* expression in XFD-injected embryos at early gastrula stages (Griffin et al., 1995), at which stage the most marginal cells have involuted and downregulated *ntl*, and so had not detected this residual expression of *ntl*. This expression likely represents a direct response to the endogenous yolk cell-derived signal and may correspond to the transient expression of Xbra detected in *Xenopus* when FGF signalling is blocked (Schulte-Merker and Smith, 1995).

Unlike *ntl*, *gata5* in the blastula is not induced by FGF, nor does it require FGF signalling. We therefore propose that the patterned expression of *ntl* and *gata5* in the blastoderm margin results from differential responsiveness to TGF-β and FGF signalling. Thus, initial TGF-β signals derived from the yolk cell induce both genes in the most marginal tiers of blastomeres (cf. Fig. 4B,D). This also induces (either directly, or via *ntl*) expression of an FGF, which can induce *ntl* but not *gata5*. *ntl*, but not *gata5*, expression is then able to spread beyond those cells directly exposed to the yolk cell TGF-β, either by means of the diffusion of FGF which has recently been shown to occur (Christen and Slack, 1999), or by a relay working through positive feedback between *ntl* and FGF. Thus by means of its responsiveness to FGF, *ntl* can spread to occupy the whole depth of the germ ring, while *gata5* is confined to the marginal 3-4 tiers.

This is consistent with results in *Xenopus* where the endoderm-specific genes *Xlhbox8* and *Xsox17* are not induced by FGF (Hudson et al., 1997; Gamer and Wright, 1995) (but see Henry et al. (1996) who show that expression of Xlhbox8 in mid-tailbud stages can be blocked by injection of XFD). It is also consistent with the model put forward by Cornell et al. (1995) where FGF signalling is involved in inducing or maintaining mesoderm in the equatorial region of the *Xenopus* embryo, and is not active in the vegetal region. It is possible that lack of a requirement for FGF signalling for induction of gene expression is a characteristic of endoderm.

The inducing signal for *gata5* involves the nodal-related proteins *sqt* and *cyclops*

The mesendodermal-inducing TGF-β signal has been much investigated in *Xenopus*, however its identity is still unclear. Candidates proposed include activin (Asashima et al., 1990; Green and Smith, 1990), Vg1 (Dale et al., 1993) and nodal related proteins (Jones et al., 1995). This controversy is in part due to the unsuitability of *X. laevis* for genetic study. Here we have used mutants in nodal-related candidate signals which are available in zebrafish to define a role for these factors in the induction of germ ring expression of *gata5*. Feldman et al. (1998) showed that fish mutant for both *sqt* and *cyc* had essentially no mesoderm or endoderm. We therefore tested whether these factors were necessary for the induction of *gata5* in the blastoderm margin. In embryos lacking *cyc*, *gata5* expression appeared normal, whereas in those lacking *sqt*, the early expression was weak and patchy, and by the beginning of gastrulation gaps were present in the ring of *gata5* expression. Despite the apparent dispensability of *cyc*, a role for this molecule was shown by the phenotype of the double *sqt;cyc* mutants. In these embryos, all expression of *gata5* was lost from the blastoderm, with only some residual expression in the YSL remaining. These data show that a combination of *sqt* and *cyc* is necessary for the full induction of *gata5* in the zebrafish blastoderm (see also Warga and Nusslein-Völlhard, 1999).

*sqt* is expressed in the YSL from the earliest stages of zygotic transcription, while *cyc* does not seem to be expressed in the YSL. *sqt* is therefore likely to be yolk cell-derived signal detected in yolk cell transplants. *sqt* and *cyc* are later expressed in the margin of the blastoderm and it is probable that this expression is responsible for maintaining *gata5* expression. Interestingly, in *sqt;cyc* double mutants *ntl* expression is only lost dorsally. This may imply that another TGF-β signal is present which is able to induce expression of this mesodermally expressed gene, but our data clearly show that *gata5* cannot be fully induced without both *sqt* and *cyc*.

Is there a role for activin in mesendodermal induction?

That the absence of *sqt* and *cyc* prevents induction of *gata5* in the zebrafish does not preclude a role for activin in *gata5*
induction. Activin and the activated activin receptor ALK-4* are able to induce gata5, and activin bB message is present at the appropriate time, albeit apparently not localised to the yolk cell. Experiments in medaka using an interfering mutant of activin imply that activin signalling is necessary in mesoderm induction (Wittbrodt and Rosa, 1994). In Xenopus, expression of the extracellular domain of activin receptor IIB, which appears to block activin but not other TGF-βs tested, interfered with induction of Xbra (Dyson and Gurdon, 1997). Furthermore, activin is able to diffuse and activate genes at a distance from its source, whereas BMP-4 and the nodal-related Xnr-2 cannot (Jones et al., 1996; McDowell et al., 1997). There is, however, also evidence that activin is not the endogenous mesodermal inducing signal in Xenopus (Schulte-Merker et al., 1994). Mice disrupted at the activin βB locus are viable (Schrewe et al., 1994), however the role of cyc in inducing gata5 is only evident in the absence of sqt, so the role of activin βB in these mice may be similarly masked.

It is therefore still possible that activin could be playing a role in the induction of mesendoderm in zebrafish. Even if activin is not localised within the blastula, activin signalling may be required to potentiate signalling by nodal related local signals in a similar manner to the synergism of the BMP-related signalling molecules DPP and SCW in patterning Drosophila (Nguyen et al., 1998). A better understanding of the induction of the mesendoderm will require further investigation of the signalling pathways involved; for example it is not clear which receptors act to transduce the nodal-related signals nor how the downstream signalling events link with those triggered by other TGF-β family signals. The role of activin may be revealed by the isolation of fish mutated in these genes and the investigation of the interactions of these mutations with sqt and cyc.
Fig. 9. gata5 expression phenotypes obtained from a cross of fish doubly heterozygotic for squnt (sqt c35) and cyclops (cycm294). Expression was assayed at 30-40% epiboly (A-C, left embryos oblique animal pole view, right embryos lateral view) and shield stage (D-F, animal-lateral view). A and D show the normal expression pattern seen in most embryos. (A) At 30-40% epiboly, expression is in the margin of the blastoderm (open arrowhead) and the underlying YSL (black arrowhead). At shield stage (D) gata5 is expressed in the newly involuted hypoblast. One abnormal phenotype (B and E) was the same as that found in embryos from crosses of sqt single heterozygotes. At 30-40% epiboly (B), expression of gata5 in the blastoderm margin was generally weaker (black arrows) and in some embryos expression was absent from part of the blastoderm margin (white arrowheads). At shield stage (E), expression was absent from typically one quarter of the hypoblast (white arrowheads), and reduced in the remainder. In the second abnormal phenotype, representing embryos homozygous for both sqt c35 and cycm294, gata5 was completely absent from blastomeres at both stages (C and F). Some remaining expression could be seen in the YSL (C, black arrowhead).

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