Expression of zebrafish goosecoid and no tail gene products in wild-type and mutant no tail embryos

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SUMMARY

goosecoid is an immediate early gene expressed at the dorsal blastoporal lip of the Xenopus gastrula. Microinjection experiments have suggested a direct role for goosecoid in organizing the dorsoventral axis of the frog embryo. Here we characterize the zebrafish homologue of goosecoid (gsc) and compare its expression to that of Brachyury or no tail (ntl), another immediate early gene required in developing mesoderm. We show that gsc exhibits two independent phases of expression: an early one in cells anterior to the presumptive notochord, but not in cells of the notochord itself, and a later one in neural crest derivatives in the larval head. Zygotic gsc transcripts are detected soon after the midblastula transition, and at the blastula stage form a gradient with a maximum at the dorsal side. Use of gsc as a dorsal marker allowed us to demonstrate that ntl expression is initially activated at the dorsal side of the blastula. At this early stage, gsc and ntl show overlapping domains of expression and are co-expressed in cells at the dorsal midline of the early gastrula. However, gsc- and ntl-expressing cells become separated in the course of gastrulation, with gsc being expressed in the axial hypoblast (prechordal plate) anterior to the ntl-expressing presumptive notochordal cells. Studies with mutant embryos suggest that gsc is independent of ntl function in vivo.

Key words: Brachyury, gastrulation, goosecoid, mesoderm, no tail, zebrafish

INTRODUCTION

One of the common features of vertebrate gastrulation is the establishment of a body plan whose pattern is laid down concomitantly with the formation of the mesodermal layer (Slack, 1991). Members of the five different vertebrate classes use different gastrulation mechanisms, but all result in embryos with a remarkably similar body plan at mid-gastrulation (von Baer, 1828). For example, formation of the mesoderm in amphibian embryos invaginates through a structure called the blastopore (Keller, 1991). Several genes have been suggested to play a role in gastrulation and in the establishment of the vertebrate body axis. Among these, goosecoid and Brachyury have been demonstrated in functional studies to be particularly important.

The goosecoid homeobox-containing gene was first identified in Xenopus (Blumberg et al., 1991), in which it is expressed in the dorsal lip region of the blastopore of the early Xenopus gastrula (Cho et al., 1991). Microinjection of goosecoid mRNA into ventral blastomeres, where goosecoid is normally not expressed, leads to the formation of a secondary axis (Cho et al., 1991). The progeny of the injected cells have been shown to recruit those of uninjected cells into the secondary axis, mimicking Spemann’s organizer phenomenon (Niehrs et al., 1993). In mice, goosecoid is expressed at the anterior end of the primitive streak very early during gastrulation for a short period of time (Blum et al., 1992), reappearing at mid-gastrulation in the neural crest of the head region (Gaunt et al., 1993). In chick, goosecoid is first detected in the posterior marginal zone (Koller’s sickle) of the blastodisc, from where cells migrate to form the anterior end of the primitive streak (Hensen’s node) and subsequently the prechordal plate (Izpisua-Belmonte et al., 1993).

The Brachyury gene was identified by its short tail heterozygous mutant phenotype in the mouse (Chesley, 1935; Gluecksohn-Schoenheimer, 1944). Homozygous mutant embryos fail to develop a notochord and lack posterior structures. The Brachyury gene had been originally cloned from mouse (Herrmann et al., 1990), and homologous genes have been identified in Xenopus (Smith et al., 1991), and zebrafish (Schulte-Merker et al., 1992). In zebrafish, the gene no tail (ntl) has been identified by two mutant alleles with a phenotype very
similar to that of Brachyury mutations in the mouse (Halpern et al., 1993). Mutant embryos lack a differentiated notochord as well as the caudal half of their bodies. Molecular genetic evidence demonstrates that ntl is indeed the zebrafish homologue of the mouse Brachyury gene (Schulte-Merker et al., 1994). Following recent naming conventions, we will call the zebrafish homologue of the Brachyury gene ntl throughout this paper. We will refer to the frog and to the mouse homologues as Xbra and Brachyury, respectively.

In embryos of different vertebrate classes, Brachyury, Xbra and ntl are expressed in cells throughout the nascent mesoderm (Wilkinson et al., 1990; Smith et al., 1991; Schulte-Merker et al., 1992). In the course of gastrulation, expression becomes restricted to cells of the notochord and tailbud, while expression levels in the rest of the mesoderm decrease markedly. The ntl gene encodes a nuclear protein (Schulte-Merker et al., 1992) and, in Xenopus, has been shown to be able to act as a genetic switch inducing muscle differentiation (Cunliffe and Smith, 1992).

Both goosecoid and Xbra are remarkable in that they are ‘immediate early genes’ inducible by peptide growth factors even in the absence of protein synthesis in Xenopus (Smith et al., 1991; Cho et al., 1991). The nature of the in vivo inducing factors is unclear, however, members of the activin and the fibroblast growth factor (FGF) family are strong candidates for dorsal and ventral inducers, respectively (Amaya et al., 1991; Hemmati-Brivanlou and Melton, 1992; reviewed by Moon and Christian, 1992 and by Sive, 1993). While goosecoid can be induced by activin, but not by FGF (Cho et al., 1991), the Xbra gene can be induced by both factors (Smith et al., 1991), consistent with the expression pattern of goosecoid being confined to the dorsal side of the embryo and Xbra being expressed at both dorsal and ventrolateral positions.

Virtually nothing is known about mesoderm induction in the teleost embryo. The endoderm and mesoderm in cyprinid fishes such as the zebrafish (Brachydanio rerio) arises from cells that are located at the margin of the early gastrula (Kimmel et al., 1990), and which involute under the margin during the process of gastrulation (Wood and Timmermanns, 1988; Warga and Kimmel, 1990). These cells, collectively called the hypoblast, move towards the animal pole of the gastrula. The molecular nature and the origin of a putative mesoderm-inducing signal is unknown, but studies performed by Long (1983) suggest that a ‘dorsalizing’ signal might emanate from the yolk cell.

In this paper, we compare the expression of the zebrafish goosecoid gene (gsc) to that of ntl. Because in zebrafish the embryo is transparent and expression of gsc lasts until early somitogenesis, we have been able to perform a more extensive study of its mode of expression than previously possible in frog or mouse embryos. gsc is expressed initially as a gradient in the zebrafish blastula, and then as a patch on the dorsal side. After involution of dorsal mesoderm, gsc-expressing cells are found anterior to the notochord in the prechordal plate (which presumably includes pharyngeal endoderm and hatching gland as well as head mesoderm). We also report an independent phase of gsc expression in neural crest derivatives in the head region of the 2- to 5-day-old embryo. Furthermore, using gsc expression as a marker for the dorsal side of the embryo, we have investigated the onset of ntl expression prior to gastrulation. The ntl gene is expressed first at the dorsal side of the zebrafish embryo, colocalizing with gsc expression, before it is turned on in the rest of the future germ ring (marginal zone). The expression of gsc is normal in mutant ntl embryos, showing that ntl does not interact with gsc in vivo.

MATERIALS AND METHODS

Fish strains

All experiments, unless noted otherwise, were carried out with embryos from wild-type fish that have been kept in the Tübingen laboratory by inbreeding for several generations. The mutant ntl strains, ntl b190 and ntl b192, were kindly provided by M. E. Halpern and C. B. Kimmel (Eugene, Oregon).

Isolation of gsc cDNAs

A zebrafish gastrula cDNA library, constructed in AZAP (Stratagene), was screened with a random primed 1.1 kb Xenopus gsc cDNA probe (Cho et al., 1991) under middle stringent conditions. From 50,000 plaques screened, 4 plaques appeared positive on replica filters. The inserts of these 4 phage clones were converted into their plasmid forms by in vivo excision according to the manufacturer’s instructions. For all experiments described in this report the longest cDNA, pBS-gsc3, was used. This cDNA is 1234 nucleotides long and contains a single long open reading frame of 240 amino acids, which has a predicted molecular mass of 27×103 and a calculated isoelectric point of 8.74. Sequencing was performed using an ALF sequencer (Pharmacia). Sequencing reactions were carried out following the manufacturer’s protocol. The 5’ and the 3′ ends of all four cDNAs were sequenced. Then the complete sequence from two of the four cDNAs was determined. In each case, both strands of the respective cDNA were sequenced.

Northern analysis

Total RNA for northern analysis was prepared as previously described (Schulte-Merker et al., 1992). 10 μg of RNA was loaded per lane. Gel analysis and blotting was performed exactly as described previously (Schulte-Merker et al., 1992), except that Hybond N+ membrane was used for blotting. A random-primed EcoRI insert from pBS-gsc was used for probing.

In situ hybridizations

In situ hybridizations were carried out as described (Schulte-Merker et al., 1992) with some modifications. After hybridization and removal of the probe, washes were carried out as follows: 2× 30 minutes 55°C 50% formamide/2× SSCT, 1× 15 minutes 55°C 2× SSCT, and 2× 30 minutes 55°C 0.2× SSCT. Afterwards, samples were transferred to microtiter dishes and incubated in blocking solution for at least 30 minutes prior to the addition of anti-digoxigenin antibody. After stopping the colour reaction, embryos were washed in PBS and fixed for at least 1 hour in 4% paraformaldehyde in PBS. The fixation stabilizes the reaction product, which is not completely insoluble in anhydrous solutions. Clearing was performed in benzylbenzoate:benzylalcohol (2:1, v/v), or in permount (Polysciences).

Antibody purification and immunohistochemistry

The antiserum had been raised against, and purified with, the full-length Ntl protein (Schulte-Merker et al., 1992), making use of the pET3A expression vector by Studier and Moffat (1986). Since pre-gastrulation embryos still exhibited considerable background staining even when using affinity-purified serum, the antibody used in this study was purified against recombinant Ntl protein from a new source. Briefly, the coding region of the Ntl cDNA was amplified using primers containing BamHI and BgII recognition sites. The reaction product was cloned into the vector pQE 9 (Qiagen) which had been digested with the same two enzymes. The resulting vector pQE 9-Ntl
was transformed into E. coli M 15 cells. The protein was then expressed and purified according to the manufacturers instructions (Quiagen). The purified Ntl protein was used for affinity purification of the antiserum (Driever and Nüsslein-Volhard, 1988). Antibody stainings were performed exactly as previously reported (Schulte-Mmerker et al., 1992).

RESULTS

Zebrafish goosecoid
We isolated zebrafish gsc from a gastrula cDNA library (see Methods). The sequence has been submitted to the EMBL database, and is identical to the sequence reported by Stachel et al. (1993). Fig. 1 shows a comparison of the predicted sequences of gsc proteins from zebrafish, chick, mouse and frog. There is extensive conservation. The homeobox is entirely conserved, except for a single amino acid change in Xenopus. Three other regions of high conservation are also found, two flanking the homeobox and the other at the amino terminus (Fig. 1). These conservations should help in the cloning of other gsc homologues.

Temporal expression of the gsc gene
The time course of gsc expression is shown in Fig. 2. The size of the gsc transcript is about 1.5 kb, in good agreement with the length of the cDNA, assuming a poly(A) tail of a few hundred nucleotides. Stachel et al. (1993) reported a doublet of 1.3 and 1.4 kb, as well as additional, much weaker bands. Northern analysis reveals that gsc message can be detected weakly in RNA from adult females and ovaries, but not in RNA from adult males (not shown). These maternal transcripts are only detectable after long exposures. Xenopus also has maternal transcripts (H. Steinbeisser and E. D. R., unpublished observations). In zebrafish the firstzygotic gsc transcripts can be detected between 3 and 4 hours of development, i.e. at blastula stages prior to the onset of epiboly. Maximal amounts of transcripts are present during gastrulation, between 50% and 100% epiboly stages. During early somitogenesis, the level of gsc transcript decreases markedly. No message can be detected between 3 and 4 hours of development (end of somitogenesis). However, starting from 36 hours there exists a second, inde-

Spatial distribution of gsc expression in the gastrulating embryo
Whole-mount in situ hybridizations were performed in order to reveal the distribution of gsc transcripts in the embryo. At 3.5 hours of development, gsc transcripts are detected in the zebrafish blastula (Fig. 3A,B). There is a gradient of intensity of expression with the maximum at one side of the embryo. It is only in this region of the embryo that the signal persists, as an increasingly sharper patch, throughout the stages prior to the onset of involution and during early epiboly stages (Fig. 3D, 40% epiboly). With the onset of involution and the concomitant formation of the embryonic shield, the localization of these cells is at the dorsal side of the early gastrula (Fig. 3E), and the gsc-expressing cells are among the first to involute under the margin. Due to dorsal convergence at this stage, there is an increase in the number of gsc-expressing cells at the dorsal midline, at the expense of the signal at the periphery of the gsc-expressing domain (Fig. 3E). The signal at this stage can only be detected in the hypoblast, and the epiblast (arrowhead in Fig. 3C) is devoid of staining (compare also Fig. 5C). As gastrulation proceeds, gsc-expressing cells migrate towards the animal pole of the embryo (Fig. 3F; 75% epiboly). Examination of the anterior tip of the hypoblast at this stage reveals that in front of the cells expressing gsc there are about two or three rows of cells which show no detectable signal (not shown).

Fig. 3G (lateral view) and 3H (animal pole view) show different views of the same tailbud stage embryo (10 h). The cells expressing gsc are arranged as a patch of cells at the anterior of the hypoblast (which at this stage already forms the prechordal plate, from which head mesoderm and endoderm presumably derive), with the remaining cells trailing back along the anterior part of the dorsal midline. The most anterior cells, which form the leading edge of the prechordal plate are positive for gsc.

By the two somite stage only a weak signal can be detected in the dorsal midline (Fig. 3I; compare with Fig. 3G). It is not clear from this data whether the loss of gsc expression in the posterior of the prechordal plate is due to a rapid degradation of the mRNA or to cells moving to the anterior edge. During early somitogenesis the gsc-expressing cells at the anterior edge of the hypoblast seem to segregate into two symmetric

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Fig. 1. The sequences of the goosecoid homeodomain proteins of mouse, fish, chicken and Xenopus are highly conserved. The deduced protein sequences differ in numbers of total amino acids, but show regions of considerable conservation. The shaded box indicates the homeodomain; the lysine residue indicated with a star is involved in DNA-binding specificity.
and adult females after longer exposures. Weak gsc expression decreases during early somitogenesis. An independent phase of transcription commences at 3-4 hours, peaks at 50-100% epiboly, and decreases during development. An independent phase of transcription can be observed starting from 36 hours of development. Weak gsc expression can be seen in RNA from ovary and adult females after longer exposures.

Fig. 2. Early and late phases of zygotic gsc expression are revealed by Northern analysis. The respective stages are indicated at the top. The lower panel displays 28S rRNA as a loading control. Zygotic expression commences at 3-4 hours, peaks at 50-100% epiboly, and decreases during early somitogenesis. An independent phase of gsc transcription can be observed starting from 36 hours of development. Weak gsc expression can be seen in RNA from ovary and adult females after longer exposures.

populations (not shown, but see Stachel et al., 1993). This could either be the first sign of the beginning differentiation of these cells, or an indication of further migratory activity of gsc-expressing cells, with cells moving away from the dorsal midline into a lateral position. During later somitogenesis, gsc transcripts are gradually lost, so it is not possible to determine the ultimate fate of these cells.

A second phase of gsc expression in the head region

While our results about the early expression of gsc are consistent with the observations reported by Stachel et al. (1993), we have detected a second phase of gsc expression in the head of the larva. Neural crest derivatives in the mandibular arch (especially Meckel’s cartilage) and parts of the hyoid arch express high levels of gsc RNA (Fig. 4A,B). In addition, a set of cells shows strong labelling in the anterior part of the head, particularly after 3 days of development (Fig. 4C,D); it is not clear at present whether these cells are part of the forebrain or whether they are the cells of the neural crest surrounding the olfactory epithelium in mouse embryos (Gaunt et al., 1993). These late patches of gsc expression in the larval head should provide useful reference points for the phenotypic analysis of zebrafish mutants, particularly those in which the neural crest is affected. We conclude that gsc in the zebrafish has two independent phases of expression, in agreement to what has been reported for the mouse embryo (Gaunt et al., 1993).

Staining for both gsc and ntl in individual embryos

The expression of goosecoid and Brachyury has been analyzed in Xenopus, chick and mouse (see Introduction), and now in zebrafish. Their expression domains should define homologous anatomical regions despite the apparent differences in their mechanisms of gastrulation, and for this reason a precise comparison would be desirable. Because an anti-Ntl (Brachyury) antibody exists (Schulte-Merker et al., 1992), it is possible to perform double labelling experiments in which gsc mRNA is developed with alkaline phosphatase and Ntl protein is developed with a peroxidase reaction.

Expression of Ntl protein in the blastula

We have reported previously (Schulte-Merker et al., 1992) that the distribution of Ntl protein is asymmetrical prior to the onset of involution (sphere stage, 4.0 h). However, we were unable to say whether the staining was dorsal or ventral due to the lack of morphological markers in the blastula. Determining where ntl is initially expressed was of interest because in Xenopus it is under the control of both the activin and FGF systems (Smith et al., 1991). We therefore performed double labelling experiments using gsc as a dorsal marker.

Ntl protein is present at one margin of the embryo in a small number of cells (Fig. 5A). The total number of cells at this stage is approximately 4000. The size of the Ntl-expressing domain can vary significantly, however it normally does not exceed 30% of the egg’s circumference. Double labelings demonstrate that Ntl protein and gsc RNA colocalize (Fig. 5B). Thus, ntl is first expressed at the dorsal side of the blastula, suggesting that both genes respond to the same signal.

After having established the position of ntl expression to be at the dorsal side of the embryo, we asked whether ntl expression in the future germ ring develops in a graded fashion with the high point at the dorsal side (as has been reported in the case of Xbra RNA by Ruiz i Altaba and Jessell, 1992), or whether the presumptive endoderm and mesoderm cells of the non-dorsal part of the germ ring start expressing Ntl protein more or less simultaneously. In the majority of

Fig. 3. Expression of zebrafish gsc during gastrulation. Dorsal is to the right except in B, D, E and H, which are animal pole views. Lateral view (A) and animal pole view (B) of a 3.5-hour-old blastula embryo; note the graded distribution of gsc transcripts. In an embryonic shield stage embryo (C), gsc is exclusively expressed in the first involuting cells at the dorsal side. Dorsal convergence at this stage leads to an accumulation of gsc-expressing cells in the hypoblast of the dorsal midline (E), leading to a thinning-out of gsc expression to be at the dorsal side of the hypoblast. (G,H) Lateral and animal pole views of the same embryo (tailbud stage, 10 h). There is strong staining in cells of the prechordal plate, with an arrowhead-shaped widening at the anterior most end of the hypoblast. One hour later (I), in a 2-somite embryo, staining in the dorsal midline has decreased in intensity, but there are still cells expressing high levels of gsc at the anterior tip of the hypoblast.
Expression of *goosecoid* in zebrafish embryos

(A) Image A

(B) Image B

(C) Image C

(D) Image D

(E) Image E

(F) Image F

(G) Image G

(H) Image H

(I) Image I
cases examined, no gradient of Ntl protein could be observed (data not shown). Furthermore, some cells at more ventral positions often showed higher amounts of the antigen than cells at more dorsal positions. Even though stainings of embryos at this stage are somewhat variable, the results therefore do not support a model of graded activation of Ntl protein in the germ ring.

The intensity of staining at this very early stage is not high enough to permit analysis of the expression of both genes in sections. Therefore it was not possible to answer unambiguously the question of whether gsc and ntl are expressed in identical cells right from the beginning of their expression. Based on sections of slightly older embryos, where hypoblast cells express both gsc and ntl (Fig. 5C), and examination of specimens such as that in Fig. 5B, it seems likely, however, that the same cells express gsc and ntl very early on.

The gsc and ntl expression domains separate during gastrulation
During the course of gastrulation, the positions of the expression domains of gsc and ntl change relative to each other. While an overlap of gsc RNA and Ntl protein expression can be observed in sections (Fig. 5C), and in whole-mounts of 60% epiboly embryos (Fig. 6A,B), there is virtually no overlap in slightly older embryos (Fig. 6C,D; 90% epiboly). As Ntl protein and gsc are expressed in the same mesodermal cells at the start of involution but not at its end (Fig. 7A), it follows that during the course of involution the cells of the prechordal plate turn off expression of ntl. The notochord itself expresses ntl but not gsc (Fig. 7A).

The no tail mutation has no effect on gsc expression
At least during early gastrulation, gsc and ntl are expressed in the same cells (Fig. 5C), while the two domains of expression separate later. Therefore it seems plausible that ntl might repress gsc expression in the presumptive notochord, or that gsc might repress expression of ntl in hypoblast cells anterior to the presumptive notochord. To test the former hypothesis, we asked whether the pattern of gsc expression is perturbed in mutant embryos. Analysis of mutant embryos during all stages of development shows that this is not the case (Fig. 7B shows one example at 95% epiboly), and that neither the amount of gsc message, nor its spatial distribution (in particular the posterior border of gsc expression), differed from wild-type embryos. We conclude that ntl does not repress gsc in vivo.
DISCUSSION

**Zygotic gsc expression starts before the onset of gastrulation**

The significance of the low levels of maternal gsc transcript is not clear at present. Zygotic gsc expression starts right after midblastula transition, which in fish takes place at 3 hours of development (Kane et al., 1992). This early zygotic transcription is consistent with experiments showing that in *Xenopus goosecoid* is a member of the class of 'immediate early genes', whose expression can be induced even in the presence of cycloheximide (Cho et al., 1991). Therefore, expression of gsc in the embryo is most likely regulated by maternal transcription factors.

At the blastula stage there is a clear gradient of gsc transcripts along the dorsal-ventral axis (Fig. 3A, B). This gradient persists only for a short period of time, after which expression of gsc is restricted exclusively to the dorsal-most cells of the future germ ring. This early gradient presumably reflects the distribution of the dorsal signal in the late blastula.

Maternal transcripts for zebrafish gsc exist, but they are below the limits of detection by in situ hybridization (this study; Stachel et al., 1993). The situation is reminiscent of what has been described in the case of MyoD in *Xenopus* (Rupp and Weintraub, 1990), where ubiquitously detectable transcripts at midblastula transition become restricted to muscle precursor cells in the marginal zone of the embryo, in a process apparently dependent on mesoderm induction. As maternal transcripts also have been detected in the case of *Xbra* (Smith et al., 1991) and *Xenopus goosecoid* (H. Steinbeisser and E. D. R., unpublished observations), ubiquitous expression with subsequent restriction to specific subpopulations of future mesodermal cells might be a general principle for genes responding to mesoderm induction.

**gsc-expressing cells involute very early during gastrulation**

In *Xenopus* embryos goosecoid expression is strongest at the dorsal lip of the early blastopore (Cho et al., 1991), in gastrulating mouse embryos in an equivalent region of the primitive streak (Blum et al., 1992), and in chick embryos in the early Hensen's node (Izpisua-Belmonte et al., 1993). In zebrafish embryos, we have shown that the cells expressing gsc are involuted very early during gastrulation. In *Xenopus* embryos goosecoid expression is strongest at the dorsal lip of the early blastopore (Cho et al., 1991), in gastrulating mouse embryos in an equivalent region of the primitive streak (Blum et al., 1992), and in chick embryos in the early Hensen's node (Izpisua-Belmonte et al., 1993). In zebrafish embryos, we have shown that the cells expressing gsc are involuted very early during gastrulation.

![Fig. 5. Ntl protein expression starts at the dorsal side of the late blastula. (A) Blastula (4.0 h, animal pole view) stained with an antiserum raised against the Ntl protein shows asymmetrical distribution at the margin. (B) Double staining of gsc RNA (blue) and Ntl protein (brown) demonstrates that Ntl protein is expressed first at the dorsal side of the zebrafish blastula (animal pole view). (C) Histological section through the dorsal margin of an embryonic shield stage embryo after double staining as in (B) shows that some cells, but not all, express both gsc RNA (blue) in the cytoplasm and Ntl protein (yellow) in the nucleus. The arrowhead marks the epiblast.](image-url)
among the first ones to involute under the margin at the dorsal side of the early gastrula. During gastrulation, they move ahead of the cells that will form the notochord, and the domains of \textit{gsc} and \textit{ntl} expression, which initially overlap, separate from each other. The posterior boundary of \textit{gsc} expression clearly does not reach beyond the anterior end of the notochord. As evident from double stainings with the antibody against the Ntl protein, there is, at most, an overlap of a few cells between the domains of cells expressing \textit{gsc} and \textit{ntl}.

Even though expression of \textit{gsc} lasts until early somitogenesis in zebrafish, it is not sufficiently long to determine the ultimate fate of the \textit{gsc}-positive hypoblast cells. Based on the fate map (Kimmel et al., 1990) of hypoblast cells in the area of the embryonic shield, and on the observation that \textit{gsc}-expressing cells are anterior to the presumptive notochord, it seems likely that these cells will give rise either to hatching gland (which does not have an obvious homologous structure in other vertebrates), to pharyngeal endoderm, to head mesoderm, or to a combination thereof.

In \textit{Xenopus}, \textit{goosecoid} expression can be followed from the dorsal lip to the prechordal plate of the late neurula (H. Steinbeisser and E. D. R., unpublished data). In mice, the anterior tip of the primitive streak, which expresses \textit{goosecoid} (Blum et al., 1993) will later form the head process (from which the anterior endoderm, head mesoderm and notochord are derived; Snell and Stevens, 1966). In chick, \textit{goosecoid} mRNA expression lasts well into somitogenesis, and high expression is found in pharyngeal endoderm and head mesoderm (Izpisua-Belmonte et al., 1993). In summary, although the ultimate fate of \textit{gsc}-expressing cells in the vertebrate embryo is not yet certain, anterior endoderm and head mesoderm are good candidates. It is clear, however, that in zebrafish the anterior edge of the hypoblast (or prechordal plate) expresses \textit{gsc}.

\textbf{Mesoderm induction in zebrafish}

Morphologically, the dorsal side of the zebrafish blastula is indistinguishable from its ventral or lateral sides at 4 hours of development. Using \textit{gsc} expression as a marker for the dorsal side of the blastula, we have shown in this study that \textit{ntl}, the zebrafish homologue of the mouse \textit{Brachyury} (Herrmann et al., 1990) and of the \textit{Xenopus Xbra} (Smith et al., 1991) genes, is first expressed at the dorsal side of the pregastrula embryo. The co-expression of both \textit{gsc} and \textit{ntl} in the same cells demonstrates that dorsal blastomeres at this early point are specified and molecularly different from other, non-dorsal blastomeres. Using the amphibian embryo as a model (Nieuwkoop, 1969a,b), it seems possible that in zebrafish also different inducing activities might lead to the specification of dorsal versus non-dorsal mesoderm (and perhaps endoderm) very early in development. In \textit{Xenopus}, cells have the ability to induce or to be induced at the 32-cell stage (Jones and Woodland, 1987). In teleosts, mesoderm induction is not well understood, and it is not clear when inducing signals exert their effect on presumptive mesodermal cells, and whether this

\textbf{Fig. 6.} The \textit{gsc} and \textit{ntl}-expressing regions resolve into two distinct cell populations in the course of gastrulation. In situ hybridizations revealing \textit{gsc} message (blue), combined with antibody stainings showing the distribution of Ntl protein (brown). Two different magnifications of a 60\% epiboly (A,B) and a 90\% epiboly embryo (C,D) are shown, each one in a dorsal view. The pictures demonstrate overlap of \textit{gsc} and \textit{ntl} expression early during gastrulation, while 2 hours later the domains of expression have separated. The whole-mount preparations were dissected and flattened for photography.
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process occurs as early as in Xenopus. Given the fact that until the 16-cell stage all cells of the zebrafish embryo are part of a syncytium (Kimmel and Law, 1985), the initial events of mesoderm induction might be somewhat different from Xenopus.

We find that the ntl gene has a biphasic mode of expression during pregastrula stages, first in the dorsal side and second in a ring comprising all cells of the future hypoblast. It is interesting to compare this observation with the results obtained by Green and colleagues (1992). They have shown that Xenopus animal cap cells can respond to exposure to activin by expressing Xbra, and in doing so recognize two distinct concentration ranges of activin. There are two windows of Xbra response to activin, separated by a window where cells do not respond to activin by expressing Xbra, but rather express other markers (muscle actin and XlHbox6). The higher activin dose also induces goosecoid, which is in agreement with our observation that both gsc and ntl are expressed in dorsal cells of the zebrafish blastula. The lower activin dose could, theoretically, be responsible for the non-dorsal expression domain of ntl. However, a gradual spreading of an initially dorsal activin signal along the entire germ ring seems unlikely, as the results from Green et al. (1992) would lead one to predict a gap of ntl expression between the cells of the presumptive notochord and the non-dorsal presumptive mesoderm. Such a gap has not been observed; however, it is possible that this apparent contradiction is due to the fact that Green et al. (1992) were assaying for Xbra expression at a much later time (stage 17.5, i.e. very late during gastrulation).

We favour the idea that a signal different from activin (possibly a member of the FGF family) induces the non-dorsal aspect of ntl expression. Independent of the nature of the dorsal and ventral signals, ntl, unlike other genes of the presumptive mesoderm, reflects the presence of two signals by its biphasic mode of expression.

**gsc expression is independent of ntl function**

Both gsc and ntl are likely to be transcription factors. In the case of gsc, DNA binding to target sites similar to those of Drosophila bicoid has been demonstrated (Blumberg et al., 1991). In the case of the Brachyury gene homologues, nuclear localization of the Ntl protein (Schulte-Merker et al., 1992), DNA binding of the protein product of the optomotor-blind gene, a relative of Brachyury in Drosophila (Plaggefelder et al., 1992), and the ability of ectopically expressed Xbra to induce expression of mesodermal markers (Cunliffe and Smith, 1992), are all supportive of ntl being a transcription factor. We have therefore asked whether gsc and ntl interact. As both genes have been shown to be ‘immediate early’ genes (Smith et al., 1991; Cho et al., 1991), it seems unlikely that either gene is involved in the initiation of transcription of the other gene. However, given that some cells, early during gastrulation, express both genes (Fig. 5C), it could have been possible, for example, for ntl to control the posterior limit of gsc expression. The normal expression of gsc in homozygous ntl mutant embryos shows that this is not the case, and that ntl does not control gsc expression in the embryo. It is possible, however, that gsc represses ntl expression in hypoblast cells anterior to the presumptive notochord. This could not be tested with the reagents available.

**goosecoid and Brachyury in the vertebrate gastrula**

As mentioned initially, morphologically the gastrulae of zebrafish, Xenopus, chick and mouse are very different. However, using just two markers, goosecoid and Brachyury, it is possible to define regions of homology. Thus the embryonic shield (fish), dorsal lip (frog), early Hensen’s node (chick) and...
tip of the primitive streak (mouse) are considered homologous, for they express high levels of goosecoid mRNA and initiate cell movements that will lead to the development of the mesendoderm of the head region. Similarly, the germ ring (fish), marginal zone (frog) and primitive streak (chick and mouse) are homologous, because they express Brachury and direct the formation of the rest of the mesoderm. The same cells that express gsc in the early embryo also initiate the expression of Brachury on the dorsal side, at least in zebrafish. This is intriguing, for goosecoid expression ends up in the most anterior tissue, the tailbud, at least in zebrafish (Schulte-Merker et al., 1992), Xenopus (Gont et al., personal communication) and mouse (Herrmann, 1991) embryos.

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