INTRODUCTION

Induction and patterning of the three germ layers are two of the earliest and most crucial events of embryogenesis. Studies in *Xenopus* have demonstrated that a vegetally localized maternal T-box transcription factor, VegT, is required for the transcription of inducing factors that position the endoderm at the bottom of the embryo, and the mesoderm in the overlying cells (reviewed in Kimelman and Griffin, 1998). In *zebrafish*, the mechanism of mesendoderm induction is less clear. Transplantation experiments demonstrate that the extra-embryonic yolk cell, and an associated syncytial layer of cytoplasm and nuclei known as the yolk syncytial layer (YSL), is capable of ectopically inducing mesoderm and endoderm when transplanted to the animal pole of a host embryo (Mizuno, 1996; Ober and Schulte-Merker, 1999; Rodaway et al., 1999). This suggests that the mesendoderm is induced by YSL-derived signals.

Although it is clear that the YSL is sufficient to induce mesoderm and endoderm, whether it is required to do so was not known. Consistent with the ability of the YSL to induce dorsal mesoderm, the nodal-related factor Squint (Sqt; Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998) and the homeodomain protein Bozozok (Boz; Fekany et al., 1999; Koos and Ho, 1998, 1999; Yamanaka et al., 1998), both of which have been shown to be necessary and sufficient for dorsal mesoderm induction, are expressed in the dorsal YSL. However, both of these genes are also expressed in the marginal blastomeres (Erter et al., 1998; Feldman et al., 1998; Koos and Ho, 1998; Rebagliati et al., 1998), suggesting that the marginal blastomeres contain sufficient factors to induce mesodermal genes independently of the YSL.

We have developed a novel technique to study signaling from the YSL in which we specifically eliminate RNAs in the YSL, thus addressing the in vivo requirement of RNA-derived signals from this region in mesendoderm induction. We show that injection of RNase into the yolk cell after the 1K cell stage (3 hours) effectively eliminates YSL transcripts without affecting ubiquitously expressed genes in the blastoderm. We also present data that indicate the stability of existing proteins in the YSL is unaffected by RNase injection. Using this technique, we show that RNA in the YSL is required for the formation of ventrolateral mesendoderm and induction of the nodal-related genes in the ventrolateral marginal blastomeres, revealing the presence of an unidentified inducing signal released from the YSL. We also demonstrate that the dorsal mesoderm can be induced independently of signals from the YSL and present evidence that this is due to the stabilization of β-catenin in the dorsal marginal blastomeres. Our results demonstrate that germ layer formation and patterning in *zebrafish* uses a combination of YSL-dependent and independent inductive events.

Key words: Zebrafish, Squint, Yolk syncytial layer, Bozozok, Germ Layer

SUMMARY

Formation of the three germ layers requires a series of inductive events during early embryogenesis. Studies in *zebrafish* indicate that the source of these inductive signals may be the extra-embryonic yolk syncytial layer (YSL). The characterization of genes encoding the nodal-related factor, Squint, and homeodomain protein, Bozozok, both of which are expressed in the YSL, suggested that the YSL has a role in mesendoderm induction. However, these genes, and a second nodal-related factor, cyclops, are also expressed in the overlying marginal blastomeres, raising the possibility that the marginal blastomeres can induce mesendodermal genes independently of the YSL.

We have developed a novel technique to study signaling from the YSL in which we specifically eliminate RNAs in the YSL, thus addressing the in vivo requirement of RNA-derived signals from this region in mesendoderm induction. We show that injection of RNase into the yolk cell after the 1K cell stage (3 hours) effectively eliminates YSL transcripts without affecting ubiquitously expressed genes in the blastoderm. We also present data that indicate the stability of existing proteins in the YSL is unaffected by RNase injection. Using this technique, we show that RNA in the YSL is required for the formation of ventrolateral mesendoderm and induction of the nodal-related genes in the ventrolateral marginal blastomeres, revealing the presence of an unidentified inducing signal released from the YSL. We also demonstrate that the dorsal mesoderm can be induced independently of signals from the YSL and present evidence that this is due to the stabilization of β-catenin in the dorsal marginal blastomeres. Our results demonstrate that germ layer formation and patterning in *zebrafish* uses a combination of YSL-dependent and independent inductive events.

Key words: Zebrafish, Squint, Yolk syncytial layer, Bozozok, Germ Layer
not the expression of sqt and boz in the YSL is required for dorsal mesoderm and endoderm formation, it is necessary to specifically eliminate the expression of these genes in the YSL.

In order to determine whether or not the YSL is required for establishing the mesendoderm, we chose to eliminate all RNA transcripts from the YSL by injecting RNase into the yolk cell at the time that the YSL forms. Formation of the YSL at the 1K cell stage (3 hours) marks the time at which gap junctions between the yolk cell and the overlying marginal blastomeres are closed, resulting in the inability of large proteins to freely pass between the extra-embryonic and embryonic compartments (Kimmel and Law, 1985). Therefore, injection of RNase was expected to target transcripts in the YSL specifically, thus addressing whether or not the expression of sqt and boz in this region is required for dorsal mesoderm and endoderm formation. As no factors have yet been identified that are required for ventrolateral mesoderm induction, injection of RNase into the yolk cell should also indicate whether transcripts in the YSL are required for all mesoderm formation.

We show that injection of RNase specifically eliminated RNA in the YSL within 20 minutes, and we present evidence that protein stability is not affected. We demonstrate that the YSL is required for ventrolateral mesendoderm induction, and that RNA in the YSL is required between the 1K cell stage (3 hours) and sphere stage (4 hours) for ventralateral ntl expression. We also show that the expression of sqt and cye in the ventrolateral blastomeres depends on the YSL. In contrast, we find that dorsal mesoderm induction occurs independently of the YSL via stabilization of β-catenin in the dorsal blastomeres. These results demonstrate a required role for the YSL in establishing the germ layers, and indicate that an unidentified signal in the YSL is needed to induce ventrolateral mesendoderm.

**MATERIALS AND METHODS**

**Embryos**

Adult AB strain zebrafish and embryos were raised at 28.5°C as described (Westerfield, 1989). Embryonic stages were determined by observation (Westerfield, 1989). Embryos were dechorionated in 2 mg/ml Pronase (Roche Molecular Biochemicals) prior to injection (Westerfield, 1989).

**Dye, RNase and GFP injections**

All injections were made into the yolk cell of dechorionated 1K cell stage (3 hours) embryos, unless otherwise indicated, using a Picospritzer II (Parker Hannifin Corporation). Approximately 1 nl was injected per embryo. Lysine fixable biotinylated dextran or rhodamine-labeled biotinylated dextran (10kMW, Molecular Probes) was dissolved in water to a 20 mg/ml stock solution, and injected at 4 mg/ml, with or without RNase. Dextran were visualized using biotin-avidin peroxidase staining (Vector Laboratories). RNase, DNase free (0.5 mg/ml, Roche Molecular Biochemicals) was diluted 1/50 in water, for a final concentration of 10 µg/ml and injected with 4 mg/ml dextran. Recombinant green fluorescent protein (GFP; Roche Molecular Biochemicals) was diluted to 0.5 mg/ml in water, and injected into embryos with or without RNase and dextran.

**Inactivation of RNase with DEPC**

A 25 µl volume of 0.2 mg/ml RNase, with or without 280 µM diethyl pyrocarbonate (DEPC; Sigma) was incubated in closed eppendorf tubes for approximately 20 hours in a 37°C air incubator. The tubes were then transferred to a 37°C heat block and incubated for 10 minutes with the caps open to allow volatile DEPC to escape. The overnight incubated RNase, with or without DEPC, was diluted 1:2 in water with dextran such that the final concentration of RNase and dextran was 10 µg/ml and 4 mg/ml. This mixture was then injected into embryos. For in vitro analysis of RNase activity, the overnight incubated RNase with or without DEPC was diluted 1:2 in water, and 6 µl of this was added to 1 µg of capped RNA (synthesized using the Ambion Message Machine System) and incubated at room temperature for 1 hour.

**In situ hybridization**

Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense RNA and visualized using anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (Roche Molecular Biochemicals) as previously described (Griffin et al., 1995; Melby et al., 1997). Double staining was carried out by detecting the fluorescein-labeled probe first, using Fast Red (Sigma) as a substrate. The reaction was stopped by washing the embryo in PBST (PBS/0.1% Tween) several times, followed by two washes in 100 mM glycine, pH 2.5, for 10 minutes each. Embryos were rinsed several times in PBST, and blocked for at least 1 h in 2% goat serum and 2 mg/ml BSA in PBST. The second probe was visualized using anti-digoxigenin Fab fragments.

In situ probes used: boz (Koos and Ho, 1998; Yamanaka et al., 1998), ntl (Schulte-Merker et al., 1992), gsc (Stachel et al., 1993), gta5 (Rodaway et al., 1999), sqt (Erter et al., 1998; Rebagliati et al., 1998) and cye (Rebagliati et al., 1998; Sampath et al., 1998). Embryos were photographed in either Permout (Fisher) or 70% glycerol.

**Dorsalization with LiCl**

Embryos were treated in their chorions with 0.3M LiCl for 9 minutes at the 64-cell stage (2 hours) (Stachel et al., 1993). The embryos were then Pronase treated, washed and incubated at 28.5°C.

**RESULTS**

To address the requirement of the YSL for mesendoderm induction, we wanted to specifically affect the ability of the YSL to send signals to the blastoderm. Studies in Xenopus indicate that the mesoderm-inducing signals are zygotically expressed (reviewed in Kimelman and Griffin, 1998). As the YSL forms at the 1K cell stage (3 hours), soon after zygotic transcription is initiated at the midblastula transition (MBT; Kane and Kimmel, 1993), injection of RNase into the yolk cell at the 1K cell stage (3 hours) should eliminate all zygotic transcripts before they are translated. Proteins can not diffuse into the blastomeres after the YSL forms (Kimmel and Law, 1985) therefore RNase injected as a protein should result in the rapid degradation of RNAs specifically within the YSL. We chose to inject DNase-free RNase (Roche Molecular Biochemicals), which contains a mixture of different RNases, although we have also individually injected RNase A and RNase T1 and obtained similar results (data not shown). We found that the results obtained by injecting this RNase were specific to the activity of the RNases (see below) and determined that it is necessary to inject a carrier along with the RNase to obtain consistent results. As it was useful to detect the location of the injected material, the carriers we used were either rhodamine-labeled biotinylated dextran, or unlabeled biotinylated dextran. By injecting the RNase into the yolk cell,
we could specifically target RNAs in the YSL and ask whether or not the YSL is required for mesendoderm induction.

**Injected RNase is limited to the YSL**

Until the YSL is completely formed at the 1K cell stage (3 hours), marginal blastomeres retain cytoplasmic bridges with the yolk cell (Kimmel and Law, 1985). We wanted to ensure that material injected into the YSL at this stage would not be inherited by the marginal blastomeres, which are also a source of inducing signals, such as sqt and cyc (Erter et al., 1998; Feldman et al., 1998; Gritsman et al., 2000; Rebagliati et al., 1998). To determine the extent to which an injection of RNase into the yolk cell at the 1K cell stage (3 hours) would populate marginal blastomeres, we injected a similarly sized biotinylated dextran molecule, which was visualized by biotin-avidin peroxidase staining. We observed that the injected dextran was mostly limited to the YSL, although a few marginal cells and their descendents were labeled by 30% epiboly (4.7 hours; Fig. 1A). Since the labeled blastomeres represented a very small percentage of the marginal blastomeres, we concluded that even if the RNase were to enter a few marginal blastomeres, there were a large number of marginal cells remaining that could induce mesendodermal genes, if they had the capacity to do so.

Although the dextran injections revealed that injected RNase was likely to be restricted to the YSL, it was also important to establish that the expression of RNAs in the blastoderm that are not dependent on signals from the YSL are unaffected by the injection. As spadetail (spt; Griffin et al., 1998) is ubiquitously expressed at sphere stage (4 hours), we used it as a marker for YSL-independent gene expression. The expression of spt was indistinguishable in embryos that had RNase injected into their yolk cells when compared with embryos injected only with dextran (Fig. 1B,C). As a second control, we examined the expression of boz (Koos and Ho, 1998; Yamanaka et al., 1998) in embryos injected with RNase at oblong stage (3.7 hours), when boz is expressed in both the YSL and dorsal blastomeres. boz is normally expressed only in the dorsal blastomeres at the 1K cell stage (3 hours), then expands to include both the dorsal blastomeres and YSL until sphere stage (4 hours), after which it is restricted to the dorsal YSL (Koos and Ho, 1998; Yamanaka et al., 1998). Whereas boz is observed in both the YSL and blastomeres of un.injected embryos (Fig. 1D), RNase injection into the YSL abolished the expression of boz in the YSL without affecting its expression in the dorsal blastomeres (Fig. 1E). These results, in addition to those examining spt expression, demonstrated that we could selectively eliminate RNAs from the YSL without degrading transcripts in the blastomeres.

**Effects of RNase injection on epiboly**

Previous studies have suggested a role for the YSL in driving epibolic movements (Solnica-Krezel and Driever, 1994; Strahle and Jesuthasan, 1993). As was observed with the microtubule inhibitor nocodazole, RNase injections in the YSL resulted in slow epibolic movements and the blastoderm lifting off the yolk cell (data not shown). These results suggested that one or more transcripts in the YSL are necessary for the
microtubule-driven process of epiboly. In order to ensure that the effects on epiboly did not affect our analysis of mesendodermal markers described below were conducted prior to or at 50% epiboly (5.3 hours), before severe morphogenetic effects of RNase injections were observed.

**RNase rapidly and specifically eliminates RNAs in the YSL**

To establish that RNase injection eliminates YSL RNAs and to determine how quickly this process occurs, we injected RNase and examined the expression of boz at a time when it is restricted to the YSL. Injections were made into the yolk cell of sphere stage (4 hours) embryos and then the embryos were fixed varying times after injection. We found that boz expression was significantly reduced within 10 minutes of injection, and completely eliminated 20 minutes after injection (Fig. 2A-F). This demonstrated that RNAs in the YSL are degraded within 20 minutes of RNase injection.

To examine whether or not RNase injections might have a general effect on protein stability, we injected recombinant GFP protein with or without RNase into the yolk, and examined the embryos from oblong stage (3.7 hours) to 30% epiboly (4.7 hours) for the presence of green fluorescent protein (GFP) by fluorescence microscopy. There was no apparent change in the level of GFP fluorescence over this time period in control injected embryos, and there was no difference in GFP intensity between embryos that were injected with or without RNase (Fig. 2G,H). Since we could not detect injections of five times less GFP, it seemed likely that there was not a significant amount of degradation occurring in the embryos. These results suggest that existing proteins in the YSL are not affected by the injection of RNase, and that any effects of RNase injection on mesoderm induction are due to eliminating existing transcripts in the YSL and/or preventing new transcription.

**Ventrolateral mesendoderm induction requires a YSL RNA between 1K (3 hours) and sphere stages (4 hours)**

We examined the effects of RNase injection at the 1K cell stage (3 hours) on the expression of the pan-mesodermal marker no tail (ntl; Schulte-Merker et al., 1992) and the endodermal marker gta5 (gta5; Rodaway et al., 1999) and found that ntl expression at 30% epiboly (4.7 hours) was almost completely absent, except for a patch of cells on the dorsal side (Fig. 3A,B). Double-label in situ hybridization with the dorsal mesodermal marker goosecoid (gsc; Stachel et al., 1993) confirmed that ventrolateral ntl expression was completely abolished, leaving only a dorsal region where gsc and ntl were co-expressed (Fig. 3C-F). Dorsal expression of ntl could not be eliminated, even upon injection of ten times as much RNase (data not shown). Examination of the endodermal marker, gta5, in RNase injected embryos at 50% epiboly (5.3 hours) demonstrated that ventrolateral endoderm also requires signals from the YSL (Fig. 3G,H). These results demonstrate that induction of ventrolateral mesendoderm requires signals from the YSL.

In order to establish that the elimination of ventro-lateral ntl expression was a specific effect of RNase activity, we repeated the injections with inactivated RNase. Since we were concerned that any RNase inhibitor we would add to the RNase could have its own effects when injected into embryos, we inactivated the RNase using a volatile protein modifier, diethyl pyrocarbonate (DEPC). Overnight treatment of RNase with DEPC at 37°C resulted in the inactivation of RNase, measured in vitro on an RNA substrate (Fig. 3L). Treatment of the RNA substrate with RNase that had been incubated at 37°C overnight without DEPC resulted in its complete degradation (Fig. 3L, lane 3). Moreover, while fresh DEPC results in the degradation of the RNA substrate (data not shown) the DEPC
incubated overnight with RNase had no effect on the RNA substrate (Fig. 3L, lane 2). We found that DEPC-inactivated RNase had no effect on ntl expression when injected into embryos (Fig. 3J). These results demonstrated that the elimination of ventrolateral ntl expression is a result of RNase activity.

Using RNase injections, we have shown that ventrolateral mesendoderm induction requires a signal from the YSL. To determine when this RNA is present in the YSL, we injected embryos with RNase at different times and analyzed the effects on ntl expression. Embryos were injected with RNase at the 1K cell (3 hours), oblong (3.7 hours) and sphere (4 hours) stages, and examined at 50% epiboly (5.3 hours) for ntl expression (Fig. 4A-E). Embryos injected at the oblong stage (3.7 hours) showed a reduced effect compared with those injected at 1K (Fig. 4C). Embryos injected at sphere stage (4 hours) showed normal ntl expression (Fig. 4D,E), demonstrating that an RNA in the YSL is required between 1K cell (3 hours) and sphere stage (4 hours).

**Ventrolateral expression of nodals requires YSL signal**

Since RNase injections into the YSL eliminated ventrolateral expression of the endodermal marker gta5, we wondered if this would be accompanied by a reduction in the expression of the nodal-related factors in the ventrolateral region. We observed that expression of sqt (Erter et al., 1998; Rebagliati et al., 1998) and cyc (Rebagliati et al., 1998; Sampath et al., 1998) was eliminated by RNase injection at the 1K cell stage (3 hours) in the ventrolateral marginal regions, just as ntl was (Fig. 5A-D). Dorsal expression of both sqt and cyc remained in RNase injected embryos, as was observed for ntl, gsc and gta5 in RNase injected embryos (Fig. 3). These results demonstrate that the ventrolateral expression of the nodal-related factors is also dependent upon RNAs in the YSL.

**Stabilized β-catenin can induce dorsal mesoderm independently of the YSL**

The previous experiments established a role for the YSL in induction of ventrolateral mesoderm, but clearly demonstrated that dorsal mesoderm is regulated differently. Since boz has been shown to be required for dorsal mesoderm induction (Fekany et al., 1999), we examined its expression in RNase injected embryos. We observed that boz was expressed in the dorsal blastomeres of RNase injected embryos, and that this expression persisted past the point at which boz expression in uninjected siblings has been restricted to the dorsal YSL (Fig. 6LJ). As described earlier (Fig. 1E), boz expression was not observed in the YSL of RNase injected embryos. These results suggest that induction of boz in the dorsal blastomeres is independent of YSL signals, although the normal temporal regulation of boz requires YSL signals.

In *Xenopus*, stabilization of β-catenin has been shown to be required for the induction of dorsal mesoderm (reviewed in Moon and Kimelman, 1998). In zebrafish embryos, β-catenin accumulates in the dorsal YSL and dorsal blastomeres (Schneider et al., 1996). We hypothesized that the presence of β-catenin in the dorsal blastomeres would allow these cells to activate dorsal mesoderm independently of the dorsal YSL. In order to test this, we treated embryos with LiCl, which has been shown to result in ectopic stabilization of β-catenin throughout the embryo (Schneider et al., 1996) by inhibiting GSK-3β (Hedgepeth et al., 1997; Klein and Melton, 1996; Stambolic et al., 1996). We expected that if ectopically stabilized β-catenin did not require the YSL to induce dorsal mesoderm, embryos treated with LiCl and injected with RNase would still express ectopic dorsal mesodermal markers. As shown previously, LiCl-treated embryos ectopically express the dorsal mesodermal marker gsc throughout the margin (Fig. 6C; Stachel et al., 1993).
We observed that this ectopic expression is largely resistant to injection of RNase (Fig. 6D), just as dorsal expression of gsc is resistant in embryos not treated with LiCl (Fig. 6B).

We also examined the expression of boz in LiCl and RNase injected embryos. As previously described, boz is restricted to the dorsal YSL at 30% epiboly (4.7 hours; Fig. 6E; Koos and Ho, 1998; Yamanaka et al., 1998). In RNase injected embryos, YSL expression of boz at 30% epiboly (4.7 hours) was eliminated (Fig. 6F). At the same stage in LiCl-treated embryos, boz was ectopically expressed throughout the blastoderm and not just restricted to the margin as was seen for gsc (Fig. 6 compare G to C). LiCl-treated embryos, which were also injected with RNase, still show ectopic boz expression throughout the blastoderm at 30% epiboly (Fig. 6H), demonstrating that LiCl-dependent expression of boz does not require YSL signals. As boz is required for the formation of the dorsal mesoderm downstream of β-catenin (Fekany et al., 1999), this agrees with our observations that induction of dorsal mesoderm can occur independently of YSL signals and we conclude that this is mediated by stabilized β-catenin.

**DISCUSSION**

While transplantation experiments have shown that the YSL is sufficient to induce mesoderm and endoderm (Mizuno, 1996; Ober and Schulte-Merker, 1999; Rodaway et al., 1999) whether or not the YSL was required for germ layer formation was not known. As genes encoding key regulatory factors known to be expressed in the YSL are also expressed in the marginal blastomeres, mutants in those genes could not resolve the issue of whether gene expression in the YSL is required, as mutants abolished their activities in both the YSL and marginal blastomeres. Therefore, it was necessary to devise a technique to eliminate gene expression specifically in the YSL in order to demonstrate whether or not it was required. We have accomplished this by injecting RNase into the YSL, and have demonstrated that the YSL is required for ventrolateral mesendoderm induction and the expression of nodal-related factors within the marginal blastomeres, while dorsal mesoderm induction can occur independently of the YSL.

**RNase injections**

In order to address whether or not the YSL is required for germ layer formation, we wished to eliminate the activity of the YSL. Dissection of the blastoderm away from the yolk cell in order to separate it from yolk cell signals was not considered a viable option. The dissection process typically results in loss or damage of the most marginal cells, which are the site of sqt and cyc expression (Erter et al., 1998; Feldman et al., 1998; Gritsman et al., 2000; Rebagliati et al., 1998). This would probably compromise the ability of the explanted blastoderm to induce mesendodermal genes. Therefore, we wanted to eliminate the capacity of the YSL to signal without removing the blastoderm. As we hypothesized that the inducing signal would be zygotic, based on studies in Xenopus, we originally sought to inhibit transcription in the YSL specifically. Since commonly available transcriptional inhibitors are membrane permeable, we chose to inject RNase. As proteins can not diffuse into the blastomeres after the YSL forms (Kimmel and Law, 1985), injection of RNase protein into the YSL would specifically degrade YSL RNAs. Although we have performed injections with RNase A and RNase T1, most of our injections were performed using a DNase-free RNase mix. We found that the RNase must be injected with a carrier to obtain consistent results, and we used biotinylated dextrans as carriers, since this afforded us the option of visualizing the extent of RNase diffusion using biotin-avidin peroxidase staining. We have demonstrated that RNase injections into the yolk cell at the 1K cell stage (3 hours) are limited to the YSL, do not affect ubiquitously expressed genes, and we present data that indicate proteins in the YSL are unaffected. These experiments indicate that injection of RNase to the yolk cell after the YSL forms is an effective technique for eliminating RNA transcripts in the YSL specifically.

**A role for YSL RNAs in epiboly**

We have presented evidence that suggests that injection of RNase into the YSL eliminates RNAs without affecting the stability of proteins. Consistent with studies that suggest a role for the YSL in epiboly, we see the same phenotype in our RNase-injected embryos as in embryos treated with UV or nocodazole, which disrupt microtubules: epiboly is slowed,
and the blastoderm of RNase injected embryos constricts at the margin and lifts off of the yolk cell when control embryos are past 50% epiboly (5.3 hours) (Strahle and Jesuthasan, 1993). The manner in which the blastoderm lifts off of the yolk cell was proposed to be due to both contractile forces in the margin of the blastoderm, which normally facilitates blastopore closure, and a weakening of the yolk cytoskeleton (Strahle and Jesuthasan, 1993). We observe that this process occurs more rapidly in our RNase injected embryos, where the blastoderm lifts off the yolk cell by the time uninjected siblings reach 60-70% epiboly (6.7 hours). We suggest that this is because cytoskeletal elements that are turned over after RNase injection cannot be replaced due to the presence of RNase. This results in a weaker cytoskeleton in RNase-injected embryos than in embryos treated with UV or nocodazole, in which the cytoskeleton can recover to some degree after treatment (Strahle and Jesuthasan, 1993). The similarity between the results with RNase injection, and UV and nocodazole treatment suggests that the effects of RNase injection are limited to the YSL, and that the blastoderm itself can properly undergo morphogenetic processes that are independent of the YSL. In addition, our data indicate that there is one or more RNA in the YSL that are essential for epiboly.

RNA in the YSL is required for ventrolateral mesoderm induction

We have shown that expression of mesendodermal markers and inducing genes requires at least one RNA in the YSL between the 1K cell stage (3 hours) and sphere stage (4 hours). A significant exception is dorsal mesendoderm, discussed below. Moreover, the RNase-sensitive activity induces the expression of sqt and cyc, which are required for endoderm induction (Rodaway et al., 1999). Although sqt; cya double mutants show defects in mesoderm formation during gastrulation, sqt and cyc are not required for ventral mesoderm induction (Feldman et al., 1998). Therefore, the activity must separately induce the expression of ntl, either directly or through an unknown pathway (Fig. 7). Whether or not the RNase sensitive activity is zygotic or maternal is not known, as the injected RNase would eliminate both.

Based on a number of investigations, likely candidates for the RNase-sensitive inducing activity belong to the transforming growth factor β (TGFβ) family of signaling molecules. Overexpression of the TGFβ family member antivin (Thiss and Thiss, 1999), which has been shown to antagonize TGFβ signaling, abolishes mesendoderm induction. Additionally, overexpression of the TGFβ signaling molecule activin, results in the induction of mesodermal genes (Schulte-Merker et al., 1992).

We do not consider activin a likely candidate for our mesendoderm-inducing activity, since a study using an activin cleavage mutant, which must interact with newly translated activin ligand in order to function as a dominant negative, had no effect on mesoderm induction in medaka (Wittbrodt and Rosa, 1994). Since our RNase injection experiments indicated that RNA in the YSL is required for mesoderm induction, the activin cleavage mutant described above should have also eliminated ventrolateral mesoderm if activin transcripts were the targets of the RNase.

We can also exclude nodal-related signals as candidates for the factor. Although sqt and cyc are required for endoderm induction (Rodaway et al., 1999), cyc is not expressed in the YSL, and sqt is only expressed in the dorsal YSL between the 1K cell stage (3 hours) and sphere stage (4 hours). Our data suggest that the RNase sensitive mesendoderm-inducing activity is expressed as RNA throughout the YSL during these stages. In addition, maternal and zygotic mutants in one-eyed pinhead, which is required for nodal signaling, still induce ventrolateral mesoderm (Gritsman et al., 1999), as do sqt; cyc double mutants (Feldman et al., 1998). This indicates that the nodal-related genes do not play a role in the initial induction of ventrolateral mesoderm (Feldman et al., 1998; Gritsman et al., 1999).

In summary, our data demonstrate that there is an unidentified RNA required for mesendoderm induction that is expressed specifically in the YSL at the start of zygotic transcription (Fig. 7).

Dorsal mesoderm induction

Our data demonstrated that dorsal mesendoderm is induced through a different pathway from ventrolateral mesendoderm. We found that dorsal mesendoderm was not eliminated in RNase injected embryos, and that dorsal mesoderm could be induced by LiCl treatment in the absence of a functional YSL. Although we can not rule out the possibility that the effects of LiCl are due in part to changes in phosphoinositide...
metabolism (Ault et al., 1996), since LiCl treatment results in the ectopic stabilization of β-catenin in zebrafish embryos (Schneider et al., 1996a) and since stabilized β-catenin has been shown in Xenopus embryos to activate dorsal genes (reviewed in Moon and Kimelman, 1998), we conclude that the stabilization of β-catenin in the dorsal blastomeres makes them independent of signaling from the YSL. Our results correlate with a number of experiments that contribute to a model in which dorsalizing factors, responsible for stabilizing β-catenin, are localized to the vegetal pole of the oocyte and moved up to the future dorsal side via microtubule transport prior to the 16-cell stage (1.5 hours) (Jesuthasan and Stahle, 1996; Ober and Schulte-Merker, 1999; Yamada et al., 1998). We believe that these dorsalizing factors are delivered to a region that includes both the dorsal YSL and dorsal blastomeres, thus accounting for the ability of the YSL to induce dorsal mesodermal genes in transplantation experiments (Mizuno, 1996; Ober and Schulte-Merker, 1999; Rodaway et al., 1999), and of the blastomeres to induce dorsal mesodermal genes independently of the YSL (our results, Fig. 7). Consistent with this interpretation, ectopic gsc expression was frequently less robust in LiCl-treated and RNase-injected embryos when compared with embryos treated with LiCl alone. This suggests that stabilization of β-catenin in the dorsal YSL also plays a non-autonomous role in the induction of dorsal mesoderm (Fig. 6, compare D with C).

One caveat to our interpretation is that there may be a signal from the dorsal YSL that acts too early for our RNase injections to interrupt. We do not believe this is the case, as we inject RNase as soon as the YSL is formed and have shown that injection of RNase eliminates most of the RNA in the YSL within 10 minutes. In addition, nuclear localized β-catenin has been observed in marginal and non-marginal blastomeres at the 1K cell stage (3 hours) (S. Dougan, A. Schier and W. Talbot, personal communication). These observations suggest that the dorsal blastomeres are capable of activating dorsal mesodermal genes independently of signals from the YSL via stabilized β-catenin.

The expression of all mesendodermal markers examined, sqt, cyc, ntl and gta5, was shown to be regulated differently on the dorsal side than in the ventrolateral margin. Our data suggest that dorsal expression of these genes can be induced downstream of stabilized β-catenin. This has been previously demonstrated for boz (Fekany et al., 1999; Yamanaka et al., 1998), sqt (Shimizu et al., 2000) and ntl (Kelly et al., 1995), and our data suggest it is also true for dorsal cyc and gta5 expression.

**Regulation of dorsal mesodermal genes requires the YSL**

In addition to playing a cooperative role with the dorsal blastomeres to induce dorsal mesoderm, our data suggest that the YSL is required to regulate dorsal mesoderm formation. Although the dorsal blastomeres can induce the expression of boz independently of the YSL, in the absence of YSL RNAs the expression pattern of boz is altered such that it is still expressed in the dorsal blastomeres when un.injected siblings have restricted their expression of boz to the dorsal YSL (Fig. 6LJ). This result suggests that dorsal YSL activity regulates boz expression by inhibiting boz in the dorsal blastomeres during epiboly.

**Evidence for a localized co-factor involved in dorsal mesoderm induction**

While stabilization of β-catenin is required in order to induce dorsal mesoderm, it is not sufficient to do so (Schneider et al., 1996b). It has been previously shown in Xenopus that treatment with LiCl results in ectopic stabilization of β-catenin throughout the embryo, but that dorsal mesodermal markers are restricted to the equatorial regions where the mesodermal layer is normally formed (Schneider et al., 1996b). Similarly, in zebrafish we observed that LiCl-treated embryos show gsc (Fig. 6C), ntl and sqt (data not shown) expression only at the margin even though boz expression is induced throughout the blastoderm (Fig. 6G; Yamanaka et al., 1998). Both sqt and boz have been shown to be downstream of β-catenin (Shimizu et al., 2000), which suggests that a co-factor required for sqt expression is responsible for restricting dorsal mesoderm induction to the margin. We suggest that both the YSL and marginal blastomeres contain a co-factor required for expression of sqt, but not boz (Fig. 7).

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