Mezzo, a paired-like homeobox protein is an immediate target of Nodal signalling and regulates endoderm specification in zebrafish

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SUMMARY

Endoderm specification in zebrafish is mediated by the zygotic transcription factors Bon/Mixer, Faust/Gata5, Casanova and Sox17, whose expression is induced by Nodal signalling. Bon/Mixer and Gata5 require Casanova in order to promote endoderm formation and all three factors act upstream of sox17, but it is not clear whether Casanova acts downstream of or in parallel to Bon/Mixer and Gata5. An additional factor induced at the margin of the blastoderm by Nodal signalling is thought to be required to induce casanova expression. We show that Mezzo, a novel paired-like homeobox protein, may be this missing transcription factor. The homeobox of Mezzo is mostly related to the homeodomain of the Mix-like and Mixer homeoproteins, but Mezzo is distinct from Bon/Mixer, the product of the bonnie and clyde gene. Like bon/mixer, mezzo is expressed transiently in mesendoderm precursors. By analysing the expression of mezzo in various mutants of Nodal signalling, we show that its expression strictly depends on a functional Nodal signalling pathway. By expressing a constitutively active Nodal receptor in the presence of translation inhibitors, we further demonstrate that mezzo, bonnie and clyde, and casanova are all immediate early targets of Nodal signalling, while sox17 requires post-MBT protein synthesis in order to be induced. Overexpression of mezzo mRNA can induce ectopic expression of casanova and sox17 and can also turn on the pan mesodermal marker gene ntl. We show that the function of mezzo is redundant with that of bonnie and clyde and that mezzo RNA can partially rescue bonnie and clyde mutants. Injection of antisense Morpholino oligonucleotides targeted against mezzo into bonnie and clyde mutant embryos abolishes all sox17 expression and aggravates their mutant phenotype. These results highlight the complexity of the transcriptional network operating during endoderm formation. They place mezzo as a new transcription factor with unique properties acting in parallel with bonnie and clyde, faust and casanova in the Nodal signalling pathway that controls specification of mesoderm and endoderm in zebrafish.

Key words: mezzo, Homeobox, Nodal signalling, Endoderm, Mesoderm, sox17, ntl, casanova, faust, mixer, Zebrafish

INTRODUCTION

In zebrafish, endoderm is induced at the vegetal margin of the blastoderm by secreted TGFβ molecules of the Nodal family (Rodaway et al., 1999; Warga and Nusslein-Volhard, 1999; Stainier, 2002). Recent genetic studies have identified a number of loci involved in endoderm formation and epistatic analyses have helped to order them in a molecular pathway (Alexander and Stainier, 1999; Kikuchi et al., 2000; Dickmeis et al., 2001; Reiter et al., 2001; Aoki et al., 2002a). In the current model, the endoderm specification pathway is initiated by activation of serine/threonine kinase receptors, which bind Nodal-type TGFβ ligands. Two Nodal ligands encoded by the cyclops and squint genes are implicated in this first step (Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998). cyclops and squint are expressed in the vegetal marginal region of the blastoderm where endoderm precursors have been mapped. Single mutants lacking the function of either gene still have endoderm but cyclops; squint double mutant lack all endoderm and have little mesoderm demonstrating a functional redundancy between these two factors. The activities of these two Nodal ligands can be inhibited by Antivin, a potent endogenous antagonist of Nodal factors that is induced in a feedback mechanism of the signalling pathway. Overexpression of low doses of Antivin suppresses endoderm formation while higher doses also affect the mesoderm (Thisse and Thisse, 1999; Thisse et al., 2000). TARAM-A, a zebrafish type I TGFβ receptor structurally related to Alk4, is a candidate receptor for Nodal ligands (Renucci et al., 1996; Peyrieras et al., 1998; Aoki et al., 2002b). Endoderm formation also requires the activity of Oep-Crypto proteins which are transmembrane proteins acting as coreceptors for Nodal factors (Zhang et al., 1998). Embryos lacking maternal and zygotic oep transcripts (MZoep) resemble cyclops; squint double mutants and lack all endoderm (Gritsman et al., 1999). Signal transduction from Nodal receptors requires the maternally expressed Smad2 factor, which translocates to the nucleus after phosphorylation (Muller
Several lines of evidence suggest that in addition to bon/mixer and faust/gata5, other unidentified factors induced by Nodal signalling are involved in the regulation of casanova expression (Alexander et al., 1999; Kikuchi et al., 2000; Kikuchi et al., 2001; Reiter et al., 2001; Aoki et al., 2002a). First, bon or faust single mutants as well as bon/faust double mutants embryos still have residual expression of casanova and sox17. Moreover, overexpression of faust or bon alone or in combination is not sufficient to cause ectopic expression of casanova outside the marginal zone and does not restore significant sox17 expression in cyclops/squint mutants. This suggests that another factor normally present at the margin is required to induce sox17 and is lost in Nodal mutants (Kikuchi et al., 2000; Kikuchi et al., 2001). Finally, neither bon nor faust mRNAs are able to induce sox17 or casanova in MZoep embryos. All these observations have led Alexander et al. and Aoki et al. to postulate the existence of an unknown factor induced by Nodal signals at the margin of the blastoderm, which would act at the same level as bon and faust, and would be a positive regulator of casanova expression.

We report the identification of a novel paired-like homeobox protein named Mezzo, which may be the missing factor described above. Mezzo is related to the Mix-like/Mixer homeoproteins and like bon/mixer, mezzo is expressed transiently during zebrafish development. Moreover, we show that mezzo transcripts are restricted to mesendoderm precursors during gastrulation and can be induced by activation of the Nodal/TARAM-A signalling pathway. By analysing the expression of mezzo in various mutants with attenuated or completely defective Nodal signalling, we show that activation and maintenance of this gene strictly depend on a functional Nodal signalling pathway. By expressing a constitutively active form of the TARAM-A receptor in the presence of translation inhibitors we have further demonstrated that mezzo, bon/mixer and casanova are all immediate early targets of Nodal signalling, while sox17 is not. Overexpression of mezzo mRNA can induce ectopic expression of casanova and sox17 and can also turn on the pan mesodermal marker gene ntl. We show that the function of mezzo is redundant with that of mixer. mezzo mRNA can partially rescue bon mutants, and when the function of mezzo and bon/mixer is inhibited, embryos develop without any sox17 expression and fail to form prechordal plate mesoderm, a phenotype that suggests a further reduction of Nodal signalling.

These results place mezzo as a new transcription factor with unique properties acting in parallel with bonnie and clyde, faust and casanova in the Nodal signalling pathway controlling specification of mesoderm and endoderm in zebrafish.

MATERIALS AND METHODS

Isolation of zebrafish mezzo and plasmid constructions
mezzo was identified in a PCR-based screen for new homeobox genes expressed early during zebrafish development. DNA fragments corresponding to conserved regions of paired-like type homeoproteins were amplified from gastrula stage cDNA by RT-PCR using degenerate oligonucleotides (Faucourt et al., 2001). Most of the clones obtained in this screen corresponded to previously described sequences such as bon/mixer, pitx2, mtx1 and mtx2. However, we also isolated a 117 bp cDNA fragment with only partial sequence homology to the homeobox of the Mix-Mixer family. This fragment was subsequently used as probe to screen a zebrafish gastrula cDNA library constructed in Uni-Zap XR (T. Lepage, unpublished). Out of the 12 independent clones isolated, one appeared to contain the largest insert and was entirely sequenced on both strands. The sequence of this 4075 bp cDNA was further confirmed by partial sequencing of
the remaining clones and by sequencing genomic DNA. The Accession Number for the mezzo sequence is AF466189.

Zebrafish strains, embryo manipulation, cycloheximide treatments

Adult zebrafish were maintained at 28.5°C using standard procedures (Westerfield, 1994). Wild-type embryos were collected by natural spawning from the AB strain. Mutant embryos were obtained by intercrossing heterozygous carrier fish identified by random crossing. We used the following mutant alleles: one-eyed pinhead, oepm134 (Schier et al., 1997), cyclopsb16 (Hatta et al., 1991), squins235 (Feldman et al., 1998), fauspm236o (Chen et al., 1996), bonnie and clyde2425 (Kikuchi et al., 2000), casanova265 (Chen et al., 1996), no tail41b (Odenthal et al., 1996) and schmalspur768 (Pogoda et al., 2000).

Note that oepm134 which causes a truncation in the C-terminal hydrophobic domain of the protein, is not a null mutation. cyclops; squint double mutant carriers were identified by backcross in the progeny of a cross between identified cyclops and identified squint fish. As expected, one sixteenth of the embryos produced by intercrossing cyc; sqt carriers displayed the typical double mutant phenotype (Feldman et al., 1998). Adult ZMoe and ZMsqut fish were a generous gift of Frederic Rosa and adult ZMzur fish were kindly provided by Dirk Meyer. bopm2425 embryos were genotyped following the procedure published by Kikuchi et al. (Kikuchi et al., 2000).

In protein synthesis inhibition experiments, cycloheximide was added to dechorionated embryos at the 64- to 128-cell stage to a final concentration of 50 μg/ml. Cleavage in the treated embryos was usually arrested before the 512-cell stage, an indication of the effectiveness of the translation inhibitor.

RNA and oligonucleotides microinjection

For overexpression studies, the coding sequence of mezzo was amplified by PCR using the Pfu DNA polymerase and inserted at the Cld/ Xba sites of pCS2 (Turner and Weintraub, 1994) to generate pCS2-mezzo. Capped mRNA was synthesised from a template linearized with Asp718 using the SP6 mMessage mMachine kit (Ambion). Capped RNA encoding Antivin, TARAM-A*, GFP and NLS β-Gal were synthetised as described (Peyrieras et al., 1998; Thisse and Thisse, 1999; Faucourt et al., 2001). After synthesis, all the capped RNAs were purified on Sephadex G50 columns and quantitated by spectrophotometry.

In control experiments where morpholino oligonucleotides were co-injected with RNA containing the 5’ UTR region of mezzo, both reagents were mixed together and co-injected.

In situ hybridisation

In situ hybridisation was performed following a protocol adapted from Harland (Harland, 1991) with antisense RNA probes and staged embryos. All the riboprobes were used following published protocols.

Radiation hybrid mapping

mezzo was mapped on the LN54 radiation hybrid panel (Hukriede et al., 2001; Hukriede et al., 1999) using the primers 5’ CAGCAACCAATCCGATTTA 3’ and 5’ CAGAGCTTCCCTCCAAACTGC 3’.

RESULTS

Isolation of zebrafish mezzo and structure of the mezzo transcripts

Using degenerate oligonucleotides against conserved segments of homeobox genes of the paired-like family, we isolated a DNA fragment corresponding to a novel sequence. A full-length cDNA was subsequently isolated by screening a cDNA library (Fig. 1). Comparison of the sequence of this cDNA with sequences present in GenBank revealed significant similarity with several transcripts encoding paired-like homeobox proteins (Fig. 1A). However, sequence comparisons did not reveal with certainty a clear orthologue of this transcript (see below), which, outside the coding sequence, did not share significant homology with any other mRNA. This suggested that this cDNA represented a new zebrafish gene and we named it mezzo (for mesendoderm homeobox).

mezzo encodes a homeodomain protein related to the Sebox and Mixer homeoproteins

Analysis of the mezzo transcript sequence revealed an 879 bp single long open reading frame (ORF) (Fig. 1). Starting at the second ATG (see legend of Fig. 1B for a justification of the choice of the initiator codon), the open reading frame is predicted to encode a protein of 293 amino acids. A comparison of the predicted Mezzo protein sequence with other protein sequences present in the PIR NBRF and Swiss Prot databases revealed significant but modest similarities with several other paired-like homeoproteins. The highest similarity was found with the human Sebox (E: 7e-15) and mouse OG9 (E: 4e-13) proteins (Cinquanta et al., 2000). An alignment of these three proteins is shown in Fig. 1A. This alignment shows that the human and mouse proteins are more related one to the other (63% identities) than to Mezzo (32% identities). Moreover, the Mezzo protein (293 amino acids) is significantly longer than the human Sebox (221 amino acids) and mouse OG9 proteins (190 amino acids). Outside the homeodomain, Mezzo and Sebox share limited sequence homology, sequence conservation being found only in the last 25 amino acids. This low conservation between two vertebrate sequences indicates that these genes, while clearly related, are probably not orthologues. When the comparison was limited to the homeodomain region, the highest scores were obtained with the Mix-like (Mml) protein from mouse (Pearce and Evans, 1999) and the Mix1 protein from Xenopus (68% identity) (Rosa, 1989) (Fig. 1C). Slightly lower scores were obtained for the CMIX factor from chick (62% identity) (Peale et al., 1998; Stein et al., 1998), the zebrafish Mixer/Bonnie and Clyde protein (58% identity) (Alexander et al., 1999), and the mouse Sebox/OG9 (57% identity). The C-terminal region of Mezzo does not contain any sequence resembling the acidic domain or the PPNK core sequence present in the Smad interacting motif (SIM) of some Mix-like factors (Germain et al., 2000). An alignment of these three proteins is shown in Fig. 1A. This alignment shows that the human and mouse proteins are more related one to the other (63% identities) than to Mezzo (32% identities).

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Fig. 1. *mezzo* encodes a paired-like homeobox transcription factor related to the Mix-like family. (A) Sequence alignment between zebrafish Mezzo, mouse Sebox and human Sebox. The homeodomain is overlined. Identical amino acids are printed on a black background, similar residues are printed on a grey background. (B) Nucleotide and deduced amino acid sequence of the 5' end of the *mezzo* transcript. The likely potential translation initiation codon is boxed (lower) and the sequence targeted by the morpholino oligonucleotide used in this study is shown. Note that the 5' end of the cDNA contains another in-frame potential initiation codon TGGATGC (top). However, the sequence surrounding this ATG fits poorly with the consensus sequence defined by Kozak ACCATGG. The second potential initiator codon (lower): ACTATGG located 57 bp 3' from the first in the ORF, is in good agreement with Kozak's rules with the most critical residues -3 and +4 being conserved. Furthermore, attempts to isolate longer cDNA clones by rescreening the library with a probe derived from the 5' end of the cDNA gave only clones that started at about the same position as the cDNA described above. Mapping of the 5' end of the *mezzo* transcript by primer extension revealed that the 5' end of the *mezzo* mRNA is located just a few base pairs from the 5' end of the *mezzo* cDNA we isolated. Finally, in the course of overexpression studies, we observed that only the protein initiated at this second ATG has a biological activity (data not shown). (C) Comparison of the Mezzo, Bon/Mixer, Mml, Mix-1 and C-Mix homeodomains. (D) Schematic representation of the *mezzo* gene structure. The homeodomain is shaded and the position of three introns is indicated.

LN54 mapping pannel (Hukriede et al., 1999). The *mezzo* locus resides on linkage group 5, 0.4 cR from *coe2*.

**mezzo expression is restricted to the mesendoderm precursors during gastrulation**

The spatial expression of *mezzo* was analysed by in situ hybridisation on staged embryos. *mezzo* transcripts were first detected by this technique at stage 4 (phase 4) in a small group of cells at the margin of the blastoderm (Fig. 2A,F). As epiboly started, the expression domain of *mezzo* extended over the whole circumference of the margin of the blastoderm (Fig. 2B,G). Expression of *mezzo* was restricted to the blastoderm and no expression was detected in the yolk syncytial layer. A high magnification view of embryos labelled at 50% epiboly shows that the expression domain of *mezzo* extends over six rows of cells, that is over a region that includes the precursors of mesoderm and endoderm (Fig. 2C,H) (Kimmel et al., 1990; Warga and Nusslein-Volhard, 1999). At the shield stage, *mezzo* transcripts are also expressed in the invading axial mesoderm (Fig. 2D,I). After the shield stage, the abundance of the *mezzo* transcripts declined abruptly, only a weak expression is detected in embryos at 60% epiboly and no expression at 70% epiboly or later (Fig. 2E,J). The *mezzo* gene is therefore expressed zygotically during zebrafish development. The early and transient nature of *mezzo* expression was confirmed by northern blot and RT-PCR and no transcripts were detected in RNA extracted from 4-day-old embryos or from adult fish (data not shown).

The temporal and spatial expression profiles of *mezzo* are similar to those of *bon/mixer* (Fig. 2K-O) (Alexander et al., 1999; Kikuchi et al., 2000), which has been shown to be regulated by Nodal signalling.

**mezzo expression is regulated by a TARAM-A/antivin signalling pathway**

To determine if *mezzo* expression is dependent on the Nodal signalling pathway, we made use of TARAM-A*, a constitutively activated form of a type I TGFβ receptor presumed to be a receptor for the Nodal factors (Renucci et al., 1996; Peyriéras et al., 1998; Aoki et al., 2002b). We injected mRNA encoding TARAM-A* into one blastomere of embryos at the eight-cell stage together with mRNA encoding a nuclear β-Gal as a lineage tracer. Injected embryos were allowed to develop up to the 50% epiboly then were fixed in order to

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**Fig. 1**

- **A** Nucleotide and deduced amino acid sequence of the 5' end of the *mezzo* transcript. The likely potential translation initiation codon is boxed (lower) and the sequence targeted by the morpholino oligonucleotide used in this study is shown. Note that the 5' end of the cDNA contains another in-frame potential initiation codon TGGATGC (top). However, the sequence surrounding this ATG fits poorly with the consensus sequence defined by Kozak ACCATGG. The second potential initiator codon (lower): ACTATGG located 57 bp 3' from the first in the ORF, is in good agreement with Kozak's rules with the most critical residues -3 and +4 being conserved. Furthermore, attempts to isolate longer cDNA clones by rescreening the library with a probe derived from the 5' end of the cDNA gave only clones that started at about the same position as the cDNA described above. Mapping of the 5' end of the *mezzo* transcript by primer extension revealed that the 5' end of the *mezzo* mRNA is located just a few base pairs from the 5' end of the *mezzo* cDNA we isolated. Finally, in the course of overexpression studies, we observed that only the protein initiated at this second ATG has a biological activity (data not shown).

- **B** Comparison of the Mezzo, Bon/Mixer, Mml, Mix-1 and C-Mix homeodomains.

- **C** Schematic representation of the *mezzo* gene structure. The homeodomain is shaded and the position of three introns is indicated.

- **D** Temporal and spatial expression profiles of *mezzo* are similar to those of *bon/mixer* (Fig. 2K-O) (Alexander et al., 1999; Kikuchi et al., 2000), which has been shown to be regulated by Nodal signalling.

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analyse the expression of *mezzo*. In addition to the normal expression of *mezzo* at the margin of the blastoderm, *mezzo* expression was strongly induced in the clones of cells inheriting TARAM-A* RNA (Fig. 3B). To inhibit the Nodal signalling pathway, we overexpressed antivin, a potent antagonist of Nodal and Activin in zebrafish (Bisgrove et al., 1999; Thisse and Thisse, 1999). Injection of mRNA encoding Antivin into embryos at the one- to two-cell stage drastically reduced the level of *mezzo* expression at the onset of gastrulation (Fig. 3C). These experiments suggest that *mezzo*, like the other genes acting early in endoderm specification, is regulated by a TGF-β signalling pathway.

**Analysis of mezzo expression in mutants of the Nodal signalling pathway**

To confirm the results above indicating that the Nodal signalling pathway regulates expression of the *mezzo* gene, we examined *mezzo* expression in various mutants with attenuated or completely defective Nodal signalling (Fig. 4; Table 1). We first analysed the level of *mezzo* transcripts in embryos carrying mutations in the cyclops, squint and oep genes, which act early in the Nodal pathway. In cyclops mutants embryos, *mezzo* expression was indistinguishable from control embryos (data not shown). By contrast, in squint mutant embryos, the expression domain of *mezzo* was thinner along the animal-vegetal axis and displayed a gap (Fig. 4C,I). A similar gap in the expression domain of *mezzo* was also observed in embryos lacking zygotic transcripts of *oep* (Fig. 4B,H). Double labelling experiments using a probe for the *chordin* gene, which is still expressed in the dorsal marginal zone in absence of Nodal signalling (Gritsman et al., 1999) showed that this gap corresponds to the dorsal side of the margin where the *squint* ligand is expressed (data not shown). We then looked at *mezzo* expression in cyc;sqt double mutant embryos. In the progeny of crosses between double heterozygotes cyc;sqt, we found that 21% of the embryos showed a dorsal gap in the expression domain of *mezzo*, while 8% had a barely detectable expression (Fig. 4D,J). From the frequency of occurrence of these phenotypic classes, we infer that embryos displaying a dorsal gap are the single *squint* mutants (3/16th) while embryos

**Table 1. Nodal-related signals are required for normal mezzo expression**

<table>
<thead>
<tr>
<th>Mutant background</th>
<th>Embryos showing normal expression of mezzo</th>
<th>Embryos showing altered expression of mezzo</th>
</tr>
</thead>
<tbody>
<tr>
<td>one eyed pinhead</td>
<td>173</td>
<td>80%</td>
</tr>
<tr>
<td>squint</td>
<td>70</td>
<td>76%</td>
</tr>
<tr>
<td>cyclops</td>
<td>105</td>
<td>100%</td>
</tr>
<tr>
<td>cyclops;sqint</td>
<td>184</td>
<td>71%</td>
</tr>
<tr>
<td>casanova</td>
<td>149</td>
<td>100%</td>
</tr>
<tr>
<td>faust</td>
<td>134</td>
<td>100%</td>
</tr>
<tr>
<td>bonne and clyde</td>
<td>72</td>
<td>100%</td>
</tr>
<tr>
<td>bon;faust</td>
<td>294</td>
<td>93%</td>
</tr>
<tr>
<td>MZschmalspur</td>
<td>117</td>
<td>0%</td>
</tr>
</tbody>
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Embryos from heterozygous or homozygous intercrosses of the genotype indicated were analysed at shield stage for the expression of *mezzo*. Examples of altered expression are shown in Fig. 4.
displaying largely absent mezzo expression are the double mutants (1/16th). Similarly, we found that mezzo expression was variably affected in schmalspur (MZsur), the variation correlating with the strength of the MZsur phenotype (Fig. 4E,K). Two maternal genes implicated in Nodal signalling (oeep and schmalspur) are thus critically required for normal expression of mezzo.

To determine if the zygotic genes acting more downstream in the signalling pathway are similarly required, we analysed the expression of mezzo in embryos carrying mutations in the bonnie and clyde, faust, casanova and ntl genes. In all these single mutant backgrounds, expression of mezzo appeared to be normal, suggesting that mezzo is not a downstream target of these zygotic transcription factors. Expression of mezzo in faust;cas double mutant embryos was also indistinguishable from that of control embryos, further indicating that cas does not participate in the regulation of mezzo expression. By contrast, we found that 6% of the embryos derived from a cross between bon;faust double heterozygotes displayed a significant reduction of mezzo expression over half of the circumference of the blastoderm (Fig. 4F,L). This result indicates that, while bon and faust are not individually required for mezzo expression, removing the function of bon and faust does significantly reduce mezzo expression.

Taken together, our analyses of mezzo expression in mutant embryos suggest that transcription of the mezzo gene may be activated by maternal transcription factors in response to Nodal signalling and that Bon/Mixer and GATA5 are subsequently required for sustained expression of this gene.

**mezzo, bon/mixer and casanova are immediate early targets of nodal signalling**

Analysis of the temporal expression of mezzo, bon/mixer, casanova or sox17 shows that all four genes start to be expressed approximately at the same time, sox17 appearing slightly after the others (Alexander and Stainier, 1999; Rodaway et al., 1999; Kikuchi et al., 2000; Reiter et al., 2001; Aoki et al., 2002b). Epistasis experiments have revealed that sox17 probably acts downstream of bon/mixer, faust and casanova during endoderm formation; however, it is not clear whether casanova acts downstream of or in parallel to bon/mixer and faust (Alexander and Stainier, 1999; Reiter et al., 2001; Aoki et al., 2002a; Stainier, 2002). We tested whether mezzo, bon/mixer, sox17 and casanova are transcribed in immediate response to Nodal signalling or if their transcription requires protein synthesis. To achieve this, we injected TARAM-A* into one- to four-cell stage embryos, then treated the injected embryos with the protein synthesis inhibitor cycloheximide (CHX) at the 64- to 128-cell-stage and analysed expression of the different genes by in situ hybridisation at the dome stage. As a control in this experiment, we measured the expression of no tail (ntl), which has already been shown to be an immediate early target gene of Nodal signalling in zebrafish (Vogel and Gerster, 1999). Injection of TARAM-A* induced very robust expression of sox17, ntl, mezzo, bon/mixer and casanova in large patches of cells (Fig. 5C,H,M,R; data obtained with mixer are similar to those obtained with mezzo and are not shown here). By contrast, the response of these different genes after TARAM-A* injection in the presence of translation inhibitors was quite different. Ectopic expression of sox17 was largely suppressed by CHX as expected for a gene acting downstream of casanova, bon/mixer and faust (Fig. 5D,E). By contrast, mezzo (Fig. 5N,O), casanova (Fig. 5S,T) and bon/mixer transcripts were clearly detectable after inhibition of protein synthesis and all three genes behaved in the same manner as the immediate target gene ntl (Fig. 5LJ).

From this experiment, we conclude that transcription of mezzo, bon/mixer and casanova in response to Nodal signalling is probably activated directly by maternal factors. By contrast, sox17 expression is completely dependent on zygotic factors, in agreement with the previous finding that Casanova is necessary for sox17 expression.

**Overexpression of mezzo induces ectopic expression of casanova, sox17 and no tail**

To test the role of mezzo in germ layer specification, we
mezzo in endoderm formation in zebrafish

overexpressed it in early zebrafish embryos. Synthetic mRNAs encoding Mezzo or GFP were microinjected into cleavage stage embryos at the two- to 16-cell stage and the effects were analysed at the morphological and molecular level. Embryos injected with 100 pg of GFP mRNA or with low doses (1-5 pg) of mezzo mRNA developed just like control uninjected embryos. Developmental defects were observed with higher doses of mezzo (50-100 pg) and included reduced convergence and extension movements of the dorsal mesoderm during gastrulation, leading to later defects in neural tube closure. Overexpression of mezzo was also associated with defects in patterning of the axial mesoderm, as evidenced by the presence of U-shaped somites and kinked notochords. More rarely, outgrowths of tissues often located in the trunk and tail regions, were also observed but head development was largely normal (data not shown).

To determine whether the observed morphological defects were the consequence of earlier defects in formation of the mesoderm and endoderm, we examined the expression of early endodermal and mesodermal marker genes in the injected embryos. We injected synthetic mRNA encoding Mezzo into eight- to 16-cell stage embryos and analysed at shield stage the expression of sox17 and casanova (Fig. 6). We found that overexpression of mezzo mRNA induced strong ectopic expression of sox17 and casanova across the animal hemisphere (Fig. 6B,D,E,F). The animal cells ectopically expressing sox17 or casanova did not seem to contribute to the hypoblast but remained in a superficial position in the epiblast. Similar observations have been reported in previous studies on casanova and bon (David and Rosa, 2001; Kikuchi et al., 2001; Aoki et al., 2002a). The ability of mezzo RNA to induce ectopic expression of endodermal markers in presumptive ectodermal cells is remarkable as bon/mixer RNA is apparently not able to induce sox17 expression outside the margin (Alexander and Stainier, 1999; Kikuchi et al., 2000).

To test the ability of mezzo to promote the expression of endodermal marker genes in embryos in which the Nodal signalling pathway is completely inactive, we microinjected mezzo mRNA into embryos derived from MZoep mothers. In the absence of maternal and zygotic oep transcripts, cells are unable to respond to Nodal signals and the expression of endodermal marker genes is abolished (Fig. 6C) (Alexander et al., 1999; Gritsman et al., 1999; Kikuchi et al., 2000). By contrast, all the injected embryos clearly showed ectopic expression of sox17 (Fig. 6G), indicating that mezzo mRNA can partially rescue sox17 expression in MZoep embryos.

Casanova has previously been shown to be largely required for the induction of sox17 by faust/gata5 and strictly required for the induction of sox17 by bon/mixer (Alexander and Stainier, 1999; Reiter et al., 2001; Aoki et al., 2002a). We
Fig. 6. Induction of endodermal marker genes induced by ectopic mezzo in wild-type embryos, MZoep mutants and casanova mutants. (A,E) animal pole views. (B,D,F-H) Side views. Embryos were injected at the 2- to 16-cell stage with 40 pg of GFP (A) or 40 pg of mezzo mRNA and examined at the shield (A,B,E,F) or at 80% epiboly (C,D,G,H) for the expression of sox17 (B,C,D,E,G,H) or casanova (F). Ectopic mezzo induces robust expression of sox17 (B,D,E,G) and rescues -expressing cells in MZoep (G) and induces ectopic expression of sox17 in casanova (H).

Therefore tested the ability of mezzo mRNA to induce expression of sox17 in absence of the casanova gene product. Out of 56 injected embryos, 12 (21%) were identified as homozygous cas embryos by the lack of endogenous sox17 expression in the blastoderm and the upregulation of this gene in the YSL. Six out of the 12 homozygous embryos showed weak ectopic expression of sox17 in small patches of cells, while the other six showed no expression. (Fig. 6D,H). Overexpression of mezzo, like overexpression of faust/gata5, can thus activate low levels of sox17 expression in complete absence of casanova gene function. Nevertheless, in the normal embryo, sox17 expression is abolished in the absence of the casanova gene product. This confirms that Casanova is the most crucial activator of sox17. It also suggests that, although the sox17 gene may receive some direct input from Mezzo and Gata5, the main role of Mezzo, Faust/Gata5 and Bon/Mixer is probably to maintain casanova transcription.

Taken together, these results strongly suggest that mezzo is indeed a mediator of the Nodal signalling pathway that cooperates with bon/mixer and casanova during endoderm specification.

We next examined whether mezzo could promote the expression of other downstream targets of the Nodal signalling pathway, and particularly of mesodermal marker genes such as ntl. We injected mezzo mRNA into one blastomere of embryos at the eight-cell stage, at doses which induced ectopic expression of endodermal markers. Out of 163 embryos injected with mezzo mRNA, 88 (54%) showed an expansion of the ntl territory compared with control uninjected embryos (Fig. 7A,B). The remaining fraction of the injected embryos, however, did not display ectopic ntl expression but instead showed an interruption in the ring of marginal ntl expression. Co-injection of ß-gal RNA as a lineage tracer revealed that when targeted at the margin, high doses of mezzo mRNA could suppress ntl expression in a cell autonomous manner (Fig. 7G). To determine if mesodermal markers were repressed as a consequence of upregulation of endodermal genes, we performed double in situ hybridisation to detect both casanova and ntl after injection of mezzo mRNA. We found that, out of 75 embryos injected into one blastomere at the eight cell stage, 25 showed marginal clones of cells in which casanova was overexpressed while ntl expression was simultaneously repressed (Fig. 7C,H). This result suggests that ectopic expression of mezzo could repress expression of ntl at the margin by inducing casanova, consistent with previous data (Aoki et al., 2002a).

Fig. 7. Effects of mezzo mRNA injection on ntl expression. Overexpression of mezzo induces ectopic expression of ntl in the animal hemisphere and repress endogenous ntl at the margin. (A,B,C,D,E,G,J) Side views and (F,H) animal pole views of embryos at the shield stage (A-D,F-I) or 60% epiboly (E,J). (A,E,F) Control uninjected embryos. (B,G) Embryos injected at the eight-cell stage with 40 pg of mezzo plus ß-Gal RNA. (B) Expansion of the territory expressing ntl caused by injection of mezzo mRNA in the non marginal region. A close examination of these embryos showed the presence of the lineage label outside the margin. (G) Repression ntl expression caused by injection of mezzo in the marginal region. Note the presence of the lineage label in the sector of cells that do not express ntl. (C,H) Two-colour in situ hybridisation showing induction of casanova (brown) and repression of ntl (red) after overexpression of mezzo in the marginal region. (D,L) Injection of mezzo mRNA at the same concentration as above into one central blastomere of embryos at the 16-cell stage induced patches of ntl expression in the animal hemisphere.
Similarly, we found that the expression of *ntl* in the injected embryos at 80% epiboly and during somitogenesis was severely perturbed by *mezzo* overexpression, leading either to a complete absence of the *ntl* in part of the marginal region (30%) or to a patchy and broadened marginal and axial expression of *ntl* (54%) (data not shown). These observations suggest that the developmental defects caused by overexpression of *mezzo* are the consequences of early patterning defects.

Remarkably, when similar doses of *mezzo* mRNA were injected into one internal blastomere of embryos at the 16-cell stage, ectopic *ntl* expression was clearly observed near the animal pole in about half of the injected embryos (*n*=65) (Fig. 7D,I,J). Thus, *mezzo* can induce mesodermal markers in presumptive ectodermal cells. This property distinguishes *mezzo* from *bon/mixer*, which appears to lack mesoderm inducing activity (Alexander et al., 1999) (M. P. and T. L., unpublished).

**Inhibition of mezzo function using antisense Morpholino oligonucleotides**

The experiments described above have shown that *mezzo* is expressed at the right time and at the right place to be a downstream mediator of Nodal signalling. Moreover, the results from ectopic expression of *mezzo* strongly suggest that this gene is involved in specification of the fate of mesodermal and endodermal cells. To determine if *mezzo* function is required for specification of mesendodermal cells, we used a loss-of-function approach using antisense morpholino oligonucleotides (Summerton and Weller, 1997). To first test the effectiveness of this approach in the case of *mezzo*, we constructed an artificial mRNA where the 5′ leader sequence of *mezzo* and the sequence coding for the first 43 amino acids were fused to the coding sequence of the green fluorescent protein to make *mezzo*-GFP mRNA. This *mezzo*-GFP mRNA was injected in embryos alone or mixed together with the antisense morpholino directed towards the 5′ end of *mezzo* (*mezzo*-MO). An unrelated morpholino was also injected at various concentrations as a negative control. All the embryos injected with *mezzo*-GFP mRNA were brightly fluorescent when examined at the shield stage (Fig. 8A) (*n*=50). By contrast, embryos co-injected with *mezzo*-GFP and 12 ng of

<table>
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<tr>
<th>Mutant background</th>
<th>Uninjected</th>
<th>Embryos injected with 24 ng of <em>mezzo</em>-MO</th>
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<tbody>
<tr>
<td></td>
<td>Number of embryos with</td>
<td>Percentage of embryos with</td>
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<tr>
<td></td>
<td>mutant phenotype</td>
<td>mutant phenotype</td>
</tr>
<tr>
<td></td>
<td><em>n</em></td>
<td>mutant phenotype</td>
</tr>
<tr>
<td>one eyed pinhead</td>
<td>12</td>
<td>50</td>
</tr>
<tr>
<td>squint</td>
<td>3</td>
<td>43</td>
</tr>
<tr>
<td>MZsquint</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>faust</td>
<td>7</td>
<td>35</td>
</tr>
<tr>
<td>casanova</td>
<td>13</td>
<td>62</td>
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*mezzo*-MO increases the penetrance of *squint* and MZsquint.
mezzo-MO did not show any fluorescence (n>100) when examined at the same stage indicating that the morpholino oligonucleotides efficiently reduced translation of the mezzo-GFP mRNA (Fig. 8F). We then microinjected this oligonucleotide at various concentrations into wild-type embryos. Even at high doses (up to 12 ng), injection of mezzo-MO did not perturb development of the injected embryos and the expression of marker genes such as ntl and sox17 was normal in these embryos (data not shown).

The lack of effects of mezzo-MO suggested that in wild-type embryos, another factor was able to compensate for the absence of mezzo function. To address this possibility, we tested the effects of inhibiting mezzo function in embryos lacking the activity of various genes acting in the Nodal pathway (Table 2). We first injected mezzo-MO into embryos derived from heterozygous squint parents. The squint mutation is incompletely penetrant and typically 5-15% of the progeny of squint parents manifests a cycloptic phenotype at 24 hours. We selected pairs of fish in which the penetrance of the mutation was reproducibly low (near 5%). Injection of mezzo-MO in the progeny of such fish caused a significant increase (near 20%) in the percentage of embryos displaying a cycloptic phenotype (Table 2). A similar experiment was performed using MZsquint embryos and again, a significant increase (from 60% to 100%) in the penetrance of the phenotype was observed after injection of mezzo-MO. We then extended this analysis to the oep mutant and to mutants in downstream targets of the Nodal signalling pathway such as bon, faust and cas. Injection of mezzo-MO in Zoep, faust and cas mutant backgrounds increased neither the percentage of mutant embryos nor the severity of the phenotype. By contrast, injection of mezzo-MO in the progeny of heterozygous bon parents produced a striking result. About 13% of the injected embryos showed a cyclopaia very similar to that caused by inactivation of squint or oep (n=262) and most of these cyclopic embryos also displayed a cardiac phenotype suggesting that only bon homozygous embryos were affected. We confirmed that the cyclopic embryos were homozygous bon mutants by determining their genotype by PCR (Kikuchi et al., 2000). Injection of a control antisense MO did not perturb development of the bon embryos demonstrating that this phenotype was specific to the mezzo-MO (Fig. 8B).

In the case of cyclops, squint or oep mutants, cyclopaia has been correlated with the early loss of goosecoid (Stachel et al., 1993; Thisse et al., 1994) and later of hgg1 (Vogel and Gerster, 2000), two genes expressed in the prechordal mesoderm cells during and at the end of gastrulation. We analysed the expression of these markers in embryos derived from bon/+ parents injected with mezzo-MO. Expression of goosecoid appeared normal in most of the injected embryos when examined at the shield stage; however, in about 20% of these embryos, expression of goosecoid was drastically reduced or lost at 70% epiboly (Fig. 8C,H; n=36) in good agreement with the morphological observations. Similarly, expression of hgg1, a late marker of prechordal mesoderm, was strongly reduced in the cyclopic embryos at 24 hours (Fig. 8D,I). These results confirm that the cyclopaia caused by the lack of both Bon and Mezzo proteins is associated with the loss of prechordal mesoderm precursors during gastrulation.

We also analysed sox17 expression in bon mutants injected or not with mezzo-MO. In homozygous bonm425 embryos, expression of sox17 is reduced but not abolished (Kikuchi et al., 2000). Typically, about 20 sox17-expressing cells are still present, suggesting that additional factors are involved in regulating sox17 expression (Fig. 8E). Inhibition of mezzo function in bon mutant embryos eliminated all residual sox17 expression (Fig. 8J; n=25/82). This results shows that the activities of Mixer and Mezzo are both required for specification of the normal number of sox17-expressing cells.

In summary, inhibition of mezzo function using an antisense oligonucleotide increases the penetrance of the squint and MZsquint mutations and increases the severity of the phenotype caused by the lack of bon/mixer, leading to defects in endoderm and prechordal plate development similar to those observed in the oep, cyclops and squint mutants.

**Overexpression of mezzo partially rescues the phenotype of bon mutants**

To test if overexpression of mezzo could compensate for the lack of Mixer in bon mutants, we microinjected mezzo mRNA into bon mutant embryos. Bon mutant embryos have a drastic reduction of casanova and sox17 expression during gastrulation and, typically, 25% of the embryos later develop with two well separated hearts primordia because of improper specification of the endoderm (Kikuchi et al., 2000) (Fig. 9B,E,H; Table 3). By contrast, when mezzo mRNA was microinjected into the progeny of bon/+ parents, the percentage of embryos showing a cardia bifida was consistently around 15% and some embryos displayed a morphologically abnormal but single heart chamber (Fig. 9C). This suggested that injection of mezzo mRNA had allowed some mutant embryos to develop with a single heart. In one experiment, out of 77 injected embryos, only 12 embryos displayed a typical cardia bifida at 35 hours (15%). Out of the 65 remaining embryos, 15 had a single but abnormal heart. We genotyped those 15 embryos and found that six (7.8%) were in fact homozygous bon mutants. The other nine embryos were heterozygous bon/+ in which mezzo overexpression had probably caused abnormal heart morphogenesis.

We conclude that overexpression of mezzo can partially rescue heart morphogenesis in bon homozygous embryos. Because the cardia bifida phenotype of bon mutants is a

<table>
<thead>
<tr>
<th>Injected RNA</th>
<th>Average number of sox17-expressing cells</th>
<th>Phenotype</th>
<th>Wild type</th>
<th>Single abnormal heart</th>
<th>Cardia bifida</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Normal/increased 1-25 25-100</td>
<td></td>
<td>64</td>
<td>23 (26%)</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>mezzo</td>
<td>Normal/increased 1-25 25-100</td>
<td></td>
<td>191</td>
<td>41 (15%)</td>
<td>48 (17%)</td>
</tr>
</tbody>
</table>
and endodermal gene expression in these embryos. Analysis of mezzo may have also partially rescued cells, the partial rescue of cardiac fusion caused by overexpression of Fig. 9.

(A-C) Rescue of heart morphogenesis after injection of mezzo mRNA into bon mutant embryos monitored by expression of nkx2.5 at 30 hours. The failure of heart primordia to migrate and fuse in the midline in bon mutants (B) was partially restored by overexpression of mezzo (C). (D-I) Dorsal views of embryos at 80% epiboly, showing expression of endodermal markers in wild-type embryos (D,G), bon mutant embryos (E,H) and bon mutant embryos injected with mezzo mRNA (F,I). Embryos were injected with 20-40 pg of mezzo mRNA at the one- to four-cell stage and examined for expression of sox17 (F) or casanova (I). The number of sox17- or casanova-positive cells is significantly increased in homozygous bon mutants injected with mezzo mRNA (F,I) compared with control uninjected mutant embryos (E,H). bon mutant embryos were genotyped by PCR.

consequence of abnormal specification of the endodermal cells, the partial rescue of cardiac fusion caused by mezzo suggests that ectopic mezzo may have also partially rescued endodermal gene expression in these embryos. Analysis of sox17 and casanova expression in homozygous bon mutants injected with mezzo mRNA confirmed that mezzo partially rescued the expression of these two endodermal marker genes in bon mutant embryos (Fig. 9F-I). Taken together, these results suggest that the function of Mezzo is partially redundant with the function of Bon/Mixer during endoderm specification.

DISCUSSION

Mezzo as a downstream mediator of the Nodal signalling pathway

Endoderm specification in zebrafish is mediated by the transcription factors Bon/Mixer, Faust/Gata5 and Casanova, whose expression is induced by Nodal signalling. The involvement of other factors had been anticipated from previous work showing that the activities of bon/mixer and faust/gata5 are not sufficient to restore endoderm formation in absence of Nodal signalling (Alexander and Stainier, 1999; Kikuchi et al., 2000; Dickmeis et al., 2001; Reiter et al., 2001; Aoki et al., 2002a). This study describes the characterisation, expression pattern and functional analysis of a novel zebrafish gene that fulfils several of criteria expected for such a factor. mezzo encodes a homeobox transcription factor with sequence similarities to the human Sebox and to the Mix-like and Mixer homeoproteins. The mouse Sebox gene was characterised recently as a paired-like homeobox gene not closely related to any other homeobox gene. In mouse, Sebox is expressed in maturing oocytes as well as in epidermis, brain and liver in newborn and adult mice. By contrast, we found that zebrafish mezzo is expressed early and transiently in mesoderm and endoderm precursors. The expression pattern of mezzo is much more similar to the expression pattern of the early zygotic Bon/Mixer and Mix/Bix genes, which have been shown to participate in the processes of germ layer specification in zebrafish and Xenopus. The significance of the sequence similarities between Sebox and mezzo genes is unclear.

By injecting mRNA encoding TARAM-A* and Antivin we showed that mezzo, like mixer and the mix-like genes, is regulated by TGFβ signals. Furthermore, we showed that mezzo expression is reduced in sqrt and oep mutants, and completely lost in cyc;qrt double mutants. Finally, we found that the activity of the maternal transcription factor Schmalspur/FoxH1 is required for the normal expression of mezzo, although we do not know whether this factor is required for initiation or maintenance of mezzo expression.

An important finding was obtained by analysing the effects of translational inhibitors on the expression of mezzo and of bon/mixer, casanova and sox17 after overexpression of TARAM-A*. We were able to show that the transcriptional activation of mezzo, bon/mixer and casanova in response to activation of the Nodal pathway occurs in absence of post-MBT protein synthesis. By contrast, activation of sox17 was found to require zygotic factors, consistent with the previous finding that sox17 acts downstream of casanova in the process of mesendoderm specification in zebrafish. These results suggest that mezzo, bon/mixer and casanova are, like ntl, immediate downstream targets of maternal factors activated by Nodal signalling.

By analysing mezzo expression in bon/faust double mutants, we also uncovered a requirement for zygotic transcription factors for the maintenance of mezzo expression. The picture emerging from these experiments is that mezzo expression is regulated by maternal factors activated by Nodal signals but that accumulation of mezzo transcripts at the beginning of gastrulation also requires an interplay between zygotic Bon/Mixer and Faust/Gata5 transcription factors.

Role of mezzo in endoderm determination

Recently, Alexander and Stainier (Alexander and Stainier, 1999) proposed a model of endoderm formation based on overexpression experiments and mutant analysis. In this model, they postulated the existence of a putative zygotic gene they named X which would be expressed at the margin under the
control of Nodal signals and would be required in parallel with mixer and gata5 for endoderm formation. The arguments for postulating the existence of additional factor are the following: first, although in reduced number compared with controls, sox17-expressing cells are still present in bon or faust mutants, as well as in bon/faust double mutants. Second, co-injection of bon/mixer and gata5 RNAs in MZoeP mutant embryos does not restore the normal number of cells expressing sox17, indicating that additional factors induced by Nodal signalling are required for this rescue to occur. Third, overexpression of mixer and/or gata5 in normal embryos does not activate sox17 expression outside the territory of the margin, suggesting that a co-factor present only in cells of the margin restricts spatially the activity of bon and/or gata5. Aoki and colleagues reached the same conclusion by testing the ability of bon/mixer to induce casanova in Nodal-deficient embryos (Aoki et al., 2002a). Mezzo is a good candidate for such a factor: it is expressed early and transiently in the same cells as mixer and gata5 and its expression is directly controlled by Nodal signalling. Most importantly, ectopic expression of mezzo induces ectopic expression of the endodermal markers sox17 and cas even near the animal pole. The ability of Mezzo to induce expression of endodermal markers in presumptive ectodermal cells is remarkable compared with the inductive ability of Mixer, which is restricted to the marginal zone (Alexander and Stainier, 1999; Kikuchi et al., 2000).

Other early zygotic factors, in addition to Mezzo, might participate with Mixer and Gata5 in endoderm specification. One candidate is the homeobox-containing gene pitx2. Like gata5, pitx2 is expressed transiently in the four vegetal most rows of cells of the margin at the late blastula stage (Faucourt et al., 2001). Moreover, the early expression of pitx2 is strictly dependent on a functional Nodal signalling pathway as is the late expression in the left lateral plate mesoderm during determination of left/right asymmetries (Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998; Campione et al., 1999). In Xenopus, inhibition of pitx2 function using a chimeric Engrailed-Pitx2 fusion suppresses sox17 expression in presumptive endoderm and blocks the response of cells to Nodal signalling. However, overexpression of pitx2 in zebrafish does not promote expression of endodermal marker genes but instead promotes prechordal plate markers genes (Essner et al., 2000; Faucourt et al., 2001) (M. P. and T. L., unpublished). Other factors that might participate in the regulation of casanova might be orthologues of the Bix homeobox genes which in Xenopus are transcribed in an immediate-early response to Nodal signalling (Tada et al., 1998; Ecochard et al., 1998).

**Role of mezzo in mesoderm specification**

The processes of endoderm and mesoderm specification are intimately linked in vertebrates and both germ layers require a functional Nodal signalling pathway (Dale, 1999; Kimelman and Griffin, 2000; Whitman, 2001). The bon/mixer and milk/Bix genes have been mainly studied in the context of endoderm specification, although they may also participate in mesoderm formation as the Bix genes are capable of inducing ventral mesoderm at low doses and goosecoid at high doses (Tada et al., 1998). Our study suggests a requirement for Mezzo and perhaps bon/mixer in both development of endoderm and mesoderm. The main evidence supporting this conclusion is that mezzo, in addition to its ability to promote endodermal gene expression, is also an inducer of the pan mesodermal gene marker ntl. These observations are consistent with the spatial and temporal expression pattern of mezzo. mezzo transcripts are present in the first six rows of cells at the margin of the blastoderm, a region that has been shown to contain progenitors of both mesodermal and endodermal lineages. Interestingly, the Bix genes in Xenopus are first expressed in both mesoderm and endoderm before becoming restricted to endoderm during gastrulation (Ecochard et al., 1998). An additional indication of roles for mezzo and bon/mixer in mesoderm formation is that inhibition of mezzo function in bon mutants interferes with goosecoid expression and causes a reduction of prechordal plate mesoderm.

In contrast with the inducing activity of mezzo outside the margin of the blastoderm, we have shown that this homeobox gene can repress ntl expression when overexpressed in marginal cells and that this repression is associated with upregulation of casanova. Many studies in zebrafish and in Xenopus have documented mutual repressive effects of mesoderm and endoderm determination factors, including Mix1 (Lemaire et al., 1998; Latinick and Smith, 1999), Milk/Bix (Ecochard et al., 1998; Tada et al., 1998), Pitx2 (Faucourt et al., 2001), Sox-17 (Engleka et al., 2001) and Casanova (Aoki et al., 2002a). As we have shown that mezzo is an inducer of endoderm determination genes such as casanova and sox17, it is likely that high doses of mezzo lead to repression of ntl in part by inducing casanova.

**Functional redundancy between mezzo and bon/mixer and relation between mezzo, mixer and cas**

Several lines of evidence support the idea that mezzo and bon/mixer are functionally redundant factors. First, mezzo and bon/mixer are expressed at the same time in the same cells. Second, the homeodomains of both genes are structurally related. Third, while bon mutants still express sox17 in a few
cells, injection of mezzo-MO into bon mutants abolishes this residual sox17 expression and results in a phenocopy of the cyclops mutant phenotype. By contrast, injection of mezzo-MO into cyclops, cas or faust mutant backgrounds does not cause such a phenotype. Most importantly, injection of mezzo RNA into bon mutants partially restores expression of endodermal gene markers and rescues heart morphogenesis, which is disrupted in the absence of functional bon/mixer gene product.

Based on our various observations, we would like to integrate mezzo into a model of gene regulation involved in endoderm specification (Fig. 10) inspired by those of Stainier et al. (Stainier et al., 2002) and Aoki et al. (Aoki et al., 2002a). In this model, mezzo stands as an immediate-early target of Nodal/TARAM-A/op signalling, together with bon/mixer and faust/gata5. casanova, which we have also shown to be an immediate-early target of Nod, occupies the same position but requires inputs from Mezzo, Bon/Mixer and Faust to assure continual expression. Finally, in agreement with previous proposals, this model shows that casanova is a crucial node of this network and the most important transcriptional regulator of sox17.

In conclusion, we have identified a novel transcription factor acting in the gene network that regulates specification of the mesendoderm in zebrafish. The function of mezzo is partially redundant with the function of bon/mixer but differs in that mezzo may also participate in mesoderm formation. Future studies should aim at understanding how these different transcription factors interact and what is the molecular basis for the segregation of the endodermal and mesodermal germ layers from common precursors.

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REFERENCES


mezzo in endoderm formation in zebrafish


