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dino and *mercedes*, two genes regulating dorsal development in the zebrafish embryo

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SUMMARY

We describe two genes, *dino* and *mercedes*, which are required for the organization of the zebrafish body plan. In *dino* mutant embryos, the tail is enlarged at the expense of the head and the anterior region of the trunk. The altered expression patterns of various marker genes reveal that, with the exception of the dorsal most marginal zone, all regions of the early *dino* mutant embryo acquire more ventral fates. These alterations are already apparent before the onset of gastrulation. *mercedes* mutant embryos show a similar but weaker phenotype, suggesting a role in the same patterning processes. The phenotypes suggests that *dino* and *mercedes* are required for the establishment of dorsal fates in both the marginal and the animal zone of the early gastrula embryo. Their function in the patterning of the ventrolateral mesoderm and the induction of the neuroectoderm is similar to the function of the Spemann organizer in the amphibian embryo.

Key words: zebrafish, dorsalization, mesoderm patterning, neural induction, *dino*, *mercedes*

INTRODUCTION

Studies in amphibian embryos have revealed that induction and patterning of the mesoderm and neuroectoderm is a multi-step process that starts before gastrulation and requires distinct maternal and zygotic inductive activities (for review see Kimelman et al., 1992; Sive, 1993). Four different signaling activities have been described: two mesoderm inducing signals emanate from vegetal cells which themselves do not contribute to the mesoderm (Nieuwkoop, 1973). One signal from the ventrolateral vegetal hemisphere induces ventral mesoderm in most of the marginal zone, and the second signal originating from dorsal vegetal cells induces the dorsal most mesoderm. The dorsal mesoderm itself generates a third, zygotic signal that converts the initially ventrally specified mesoderm to a whole spectrum of intermediate fates. According to more recent studies, this dorsalizing activity is counteracted by a fourth, ventralizing signal from the ventral mesoderm (Köster et al., 1991; Dale et al., 1992; Jones et al., 1992; Graff et al., 1994; Maeno et al., 1994; Suzuki et al., 1994; Schmidt et al., 1995; Hawley et al., 1995; Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995).

Both the dorsal vegetal cells of blastula embryos (the Nieuwkoop center; Gimlich and Gerhard, 1984), and the dorsal mesodermal cells of early gastrula embryos (the Spemann organizer; Spemann and Mangold, 1924), induce the formation of a secondary axis when transplanted to the ventral side of an embryo. From these experiments, two activities can be assigned to the Spemann organizer during normal development: dorsalization of the mesoderm of the ventrolateral marginal zone and neural induction in the ectoderm of the dorsal animal zone.

Several aspects of early dorsoventral patterning appear to be conserved among vertebrates. An activity similar to that of the Spemann organizer has been found in the dorsal mesoderm of early fish gastrula embryos, the shield (Luther, 1935; Ho, 1992), and in the chick and the mouse node (Kintner and Dodd, 1991; Beddington, 1994). In addition, the embryos of the different vertebrate species have similar fate maps (Kimmel et al., 1990; Hogan et al., 1994), and homologous mesodermal marker genes are expressed in similar patterns (Beddington and Smith, 1993; Kane and Warga, 1994). Thus, early patterning processes might be similarly regulated in the different vertebrates.

In order to identify genes that are required for dorsoventral patterning, we have used a genetic approach, by screening for randomly induced zygotic mutations that lead to visible embryonic phenotypes in the zebrafish (Haffter et al., 1996). Dorsalized mutants are described in the accompanying paper of Mullins et al. (1996). Here, we describe four ventralized

zebrafish mutants defining two genes, *dino* and *mercedes*. The analysis of the altered expression patterns of various marker genes before, during and after gastrulation suggests that in *dino* and *mercedes* mutants, patterning processes reminiscent of those initiated by Spemann's organizer in amphibia are perturbed.

MATERIALS AND METHODS

Fish maintenance and photography

Mutations were induced and zebrafish were maintained, mated and raised as previously described (Mullins et al., 1994). Embryos were kept at 28°C and staged according to Kimmel et al. (1995). For photography, embryos were dechorionated and mounted in 5% methylcellulose in E3 medium (Westerfield, 1994). Older embryos were anesthetized with 1/10 volume of 0.2% MESAB (Sigma).

In situ hybridization and immunostaining

Immunostainings were carried out as described elsewhere (Schulte-Merker et al., 1992) with slight modifications: the blocking, but not the incubation solution was supplemented with 100 μM biotin, the incubation solution of the secondary antibody was supplemented with 1% normal goat serum (Vector) and the secondary antibody was preincubated for 2 hours with zebrafish embryos.

In situ hybridization was carried out as described elsewhere (Schulte-Merker et al., 1992) with the following modifications: hybridization and all washes were carried out at 65°C. Washes were as follows: 1× 20 minutes in 5× SSCT, 50% formamide; 2× 20 minutes in 2× SSCT, 50% formamide; 1x 10 minutes in 2× SSCT, 25% formamide; 1× 10 minutes in 2× SSCT; 2× 30 minutes in 0.2× SSCT; 1× 10 minutes in PBST. Stained embryos were fixed for 2-6 hours at room temperature in 4% paraformaldehyde/PBS, transferred to methanol and stored at 4°C in benzylbenzoate/benzylalcohol 2:1. For a better visualization of the morphological structures, in some cases embryos were transferred to xylene and photographed in Permount (Fisher Scientific).

Acridine orange staining

Acridine orange is a membrane permeable aromatic derivative that becomes fluorescent inside acidic lysosomal vesicles, thereby preferentially staining dying cells (Abrams et al., 1993).

Live embryos were dechorionated and incubated in 2 mg/ml acridine orange in E3 medium for 1 hour at room temperature. After 2 washes, staining was analyzed under fluorescent light and images were captured using a cooled CCD video camera (Photometrics LTD). As a positive control, embryos homozygous for a mutation causing general necrosis were stained in parallel. These embryos always showed a strong and ubiquitous staining, whereas in wild-type embryos only very few isolated cells were positive (data not shown).

Synthesis of mRNA and injection of embryos

The plasmid pSP64T-xBMP-4 (Dale et al., 1992; Jones et al., 1992) was linearized with *Xba*I, and capped mRNA was synthesized and injected into wild-type embryos at the 1-4 cell stage (approximately 25 pg per embryo) as described by Hammerschmidt et al. (1996).

RESULTS

Genetics of *dino* and *mercedes*

Four mutants with a ventralized phenotype were isolated that define two genes, *dino* and *mercedes*. *dino* (*din*) mutant embryos have a small head and a large tail (Fig. 1B,M,Z). *mercedes* (*mes*) embryos display a characteristic duplication of the ventral tail fin (Fig. 1Q, see also accompanying paper of

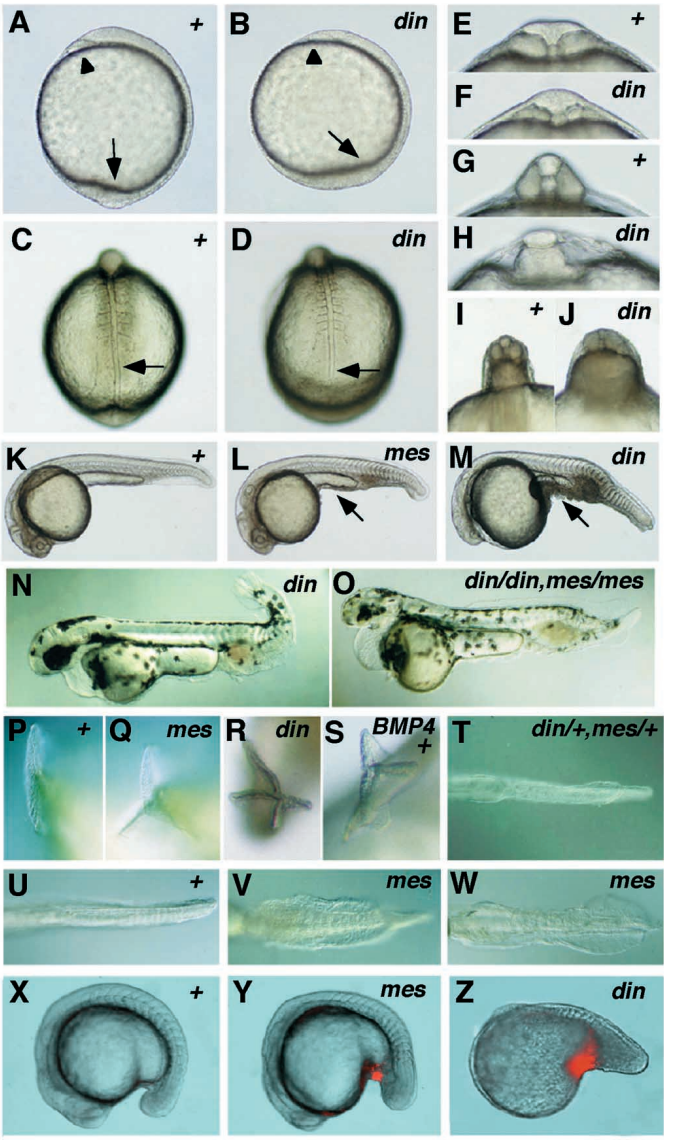

Fig. 1. Morphological characteristics of the *dino* (*din*^{tm84}) and *mercedes* (*mes*^{tm305}) mutant embryos. Wild-type embryos are indicated with '+'. (A,B) Tailbud stage, lateral view: the pillow is marked with an arrowhead, the tailbud with an arrow. (C,D) 8-somite stage, dorsal view: the end of the notochord and the corresponding position in the wild-type embryo are marked by an arrow. (E,F) 3-somite stage, optical cross section at the level of the 2nd somite: the anterior somites of the mutant are smaller and the neural tube is of normal size. (G,H) 8-somite stage, optical cross section at the level of the 6th somite: the notochord is lost and the somites are fused in *dino* mutants with a strong phenotype. (I,J) 15-somite stage, optical cross section at the level of the 13th somite: the posterior somites are larger and the neural tube is smaller in *dino* mutants. (K,L,M) 24 hours, lateral view: cells at the ventral side of the yolk extension are marked with an arrow. (N,O) 48 hours, lateral view: note the smaller head in the *din mes* double mutant. (P-S) 36 hours, posterior view of tail fin; (S) wild-type embryo injected with *Xenopus Bmp-4* mRNA. (T-W) 36 hours, ventral view of tail fin: (V) *mes*^{tm305}/*mes*^{tm305} embryo from heterozygous mother; (W)*mes*^{tm305}/ *mes*^{tm305} embryo from homozygous mother. (X-Z) Lateral views of (X) wild-type, 20-somite stage embryo; (Y) *mes*^{tm305}, 20-somite stage embryo; (Z) *din*^{tt250}, 15-somite stage embryo, showing acridine orange staining. Apoptotic cells (red) can be detected ventral of the forming yolk extension in *din* and *mes* mutant embryos.

van Eeden et al., 1996). For both genes, two independent alleles were isolated. The *dino* alleles *din*^{tm84} and *din*^{tt250} are both recessive lethal and of similar strength. The *mercedes* alleles are both recessive viable. The embryonic phenotype of *mes*^{tm305} is somewhat stronger than that of *mes*^{tz209}. *mes*^{tm305} maintains its tail fin phenotype to adulthood, while homozygous *mes*^{tz209} adults appear normal.

Intercrosses of *din* and *mes* heterozygotes gave only a partial complementation: a fraction of the embryos exhibited a partial ventral tail fin duplication which was weaker than the phenotype of homozygous *mes* mutants (see below, Fig. 1T). The fraction of these embryos was higher when the mother was *din*/+ (23%; 47/207; 3 crosses) than when it was *mes*/+ (6%; 25/391; 3 crosses). This phenotype was not observed when *din*/+ females were crossed to wild-type males (0/139; 2 crosses). All of the mutant embryos with the partial ventral tail fin duplication that were grown to adulthood were shown to be *din*/+, *mes*/+ double heterozygotes: intercrosses lead to a segregation of *din* and *mes* which is typical for 2 factor crosses, with a new double mutant category in the expected proportion (7.7%; 54/699; 4 crosses (expected 6.25%)). The double mutants could be clearly distinguished from *din* and *mes* single mutants because of their stronger phenotype (see below). The segregation pattern of *din* and *mes* in these crosses suggests that they are not partially complementing alleles of one gene. This conclusion is further supported by segregation analysis of outcrosses from *din*/+, *mes*/+ transheterozygotes, which shows that *din* and *mes* mutations reside at unlinked loci (data not shown).

The partial non-complementation and the observation that the same embryonic structures are affected in both mutants suggests that *din* and *mes* are involved in the same patterning processes. The observation that the penetrance of the partial ventral tail fin duplication in *din*/+, *mes*/+ transheterozygotes is stronger when the mother is heterozygous for *din* suggests that *din* may also have a maternal function. Nevertheless, conclusive evidence will require the generation of embryos from a homozygous *din* mutant female germ line. In the case of *mes*, homozygous *mes* females can be grown to adulthood and tested for maternal effects. When homozygous *mes* females were crossed with homozygous or heterozygous *mes* males, the homozygous mutant offspring showed, on average, a stronger phenotype than in crosses between heterozygous parents (see below, Fig. 1V,W), while the *mes*/+ embryos were normal. Thus, both *mes* and *din* may have a maternal function in addition to their zygotic function.

Morphological characteristics of *dino* and *mercedes* mutant embryos

Morphological differences between *din* mutant embryos and their wild-type siblings become obvious at the tailbud stage (Fig. 1A,B). The head region of *din* mutant embryos appears much thinner, while the posterior region is broader and thicker than in wild-type embryos (Fig. 1A-D). The tailbud emerges in a more anterior position than in wild-type embryos, and many more cells are found ventral to the tailbud. Although the head region is much smaller, the prechordal plate or pillow, where the hatching gland cell precursors are located (Kimmel et al., 1990), appears normal (Fig. 1A,B).

There is an alteration in the size of somites along the axis of *din* mutant embryos. The first somites in the anterior trunk region are smaller than those in wild-type embryos. In an optical cross section through the 3rd somite of a 3-somite stage *din* embryo, the somites are about 20% of the normal size (Fig. 1E,F). However, the size of somites progressively increases along the anteroposterior axis. Somites 6-10 are of similar size to those of wild-type embryos, while more posterior somites are larger (Fig. 1I,J). Posterior somites are often fused in the dorsal midline (Fig. 1G,H).

Disruptions in notochord development are also regularly

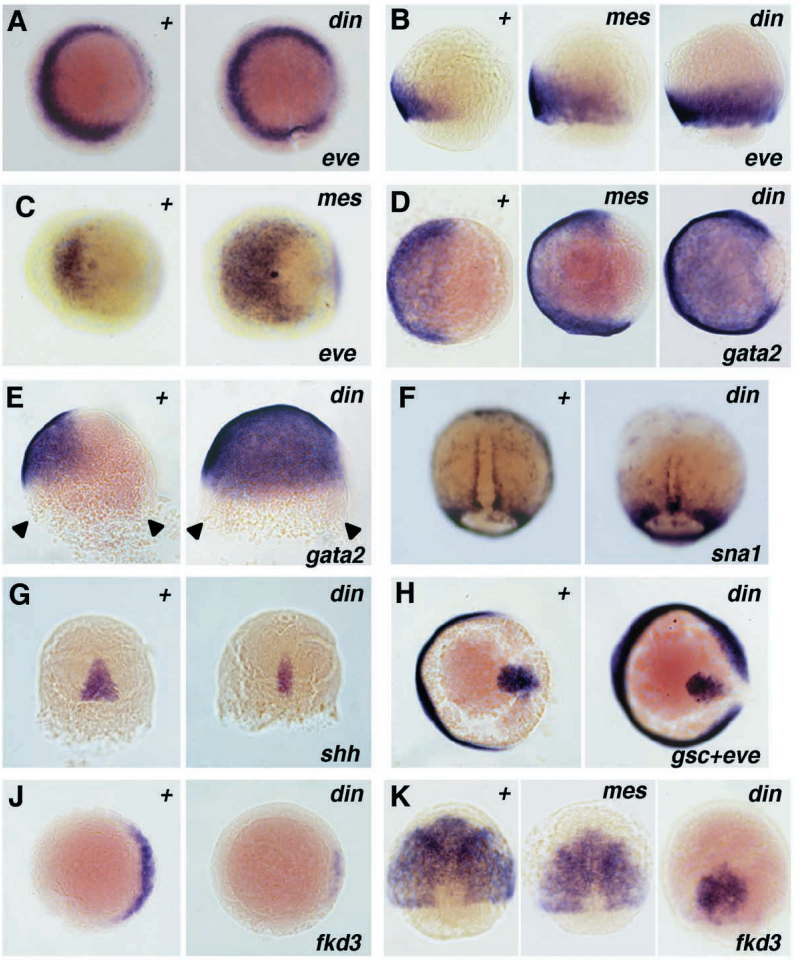

Fig. 2. Altered gene expression in *dino* (*din*^{tm84}) and *mercedes* (*mes*^{tm305}) mutants during gastrulation, detected by whole- mount in situ hybridization. Expression of: (A) *eve1*, at 40% epiboly, animal view, dorsal right; (B) *eve1*, at 70% epiboly, lateral view, dorsal right; (C) *eve1*, at tailbud stage, vegetal view, dorsal right – the more pronounced staining reflects the expression of *eve1* in a group of cells posterior to the Kupfer's vesicle (Joly et al., 1993); (D) *gata2*, at 70% epiboly, animal view, dorsal right; (E) *gata2*, at 70% epiboly, lateral view, dorsal right – the margin of the blastoderm is indicated by arrowheads; (F) *sna1*, at 80% epiboly, dorsal view; (G) *shh*, at 70% epiboly, dorsal view; (H) *gsc* and *eve1*, at 70% epiboly, animal view, dorsal right; (J) *fkd3*, at shield stage, animal view, dorsal right. (K) *fkd3*, at 70% epiboly, dorsal view.

seen in *din* mutants. In most cases, the notochord is of normal size in the anterior part of the body axis and absent in the posterior part of the trunk, beginning at somites 10-20 (Fig. 1C,D). In some mutant embryos, the notochord is continuous and goes all the way to the tailbud, however, it is thinner in the posterior region.

A second quite variable feature of *din* mutants is the size of the head in older embryos and larvae. All mutant embryos have a much smaller head with smaller eyes at 24 to 36 hours post-fertilization (Fig. 1K,M). In some mutants, however, the head phenotype becomes progressively weaker at later stages, and eventually, an almost normally sized head is formed (Fig. 1N). The spinal cord is of about normal size in the trunk (Fig. 1E,F) but is clearly smaller in the tail of all mutant embryos (Fig. 1I,J).

More consistent are the later phenotypes of ventral and posterior structures. Mutant embryos contain many more cells on the ventral side of the tail than wild-type embryos (Fig. 1K,M). In the region posterior to the anus, two or three parallel ventral tail fins are formed instead of one (Fig. 1P,R). Often, the fins form further distal branches leading to a tree-like appearance (not shown). A very similar ventral tail fin phenotype (Fig. 1S) is often (41%, 59/142) found in wild-type embryos injected with approximately 25 pg of synthetic mRNA encoding *Xenopus* Bone Morphogenetic Protein-4 (Bmp4), a signaling molecule of the TGF-β family which has been shown to have ventralizing activities in *Xenopus* embryos (Köster et al., 1991; Dale et al., 1992; Jones et al., 1992; Schmidt et al., 1995). Other ventral and posterior tissues are also enlarged in *din* mutant embryos, e.g. the blood islands, which contain many more cells (Fig. 1N), and a population of cells anterior to the anus on the ventral side of the yolk extension. These cells look brownish and form a rough surface rather than a normal epithelium (Fig. 1K,M). Acridine orange staining revealed a high degree of cell death in this region of *din* mutants at the 15-somite stage (Fig. 1Z), while no significant cell death was detected in the same location in 20-somite stage wild-type (Fig. 1X) and 2-somite stage *din* mutant embryos (not shown). No increased cell death has been found in dorsal or anterior regions of mutant embryos.

The phenotype of *mercedes* mutant embryos is similar to that of *din* mutants, but less severe. As with *din* mutants, *mes* mutant embryos can be identified morphologically at the tailbud stage, when the posterior part of the embryo is broader and thicker than in wild-type embryos (see Fig. 2C). In 1-day old *mes* mutant embryos, the head and the notochord appear more or less normal, and the sizes of the somites are only slightly altered, while significantly more cells are found in the blood islands and on the ventral side of the yolk extension than in wild-type embryos (Fig. 1K,L). As in *din* mutants, apoptotic cells are found on the ventral side of the yolk extension (Fig. 1Y). At later stages of development, two parallel ventral tail fins are formed (Fig. 1Q). The duplication of the ventral tail fin begins in the region just posterior to the anus and can extend to the tip of the tail (Fig. 1V,W). The fraction of *mes* homozygous embryos with a complete ventral fin duplication (extending to the tail tip) is greater when the mothers are *mes/mes* homozygous than when the mothers are *mes*/+ heterozygotes (from mes^{tm305}/mes^{tz209} mothers: 38% of mutant embryos, 255/673, 10 crosses; from mes^{tm305}/+ mothers: 24% of mutant embryos, 143/603, 16 crosses; note that mes^{tm305} is the stronger mes allele). In the *din*/+*; mes*/+ double heterozygous embryos mentioned above, the ventral tail phenotype is weaker. The two ventral branches are narrower than in homozygous *mes* embryos, and the tail fin duplication often is just visible at its posterior boundary. Fig. 1T shows the embryo with the strongest phenotype that was observed among the analyzed 47 *din*/+,*mes*/+ double heterozygotes.

The *din*/*din*, *mes*/*mes* double mutants show a significantly stronger phenotype than *din* single mutants over the entire length of the body axis, with even more blood cells and multiple ventral tail fin branches (up to 8) in the posterior region of the embryo, and a strong and persistent loss of head structures (Fig. 1O). As in *din* single mutants, however, the hatching glands and the anterior part of the notochord are normal.

Altered dorsoventral gene expression patterns before and during gastrulation of *dino* and *mercedes* mutants

In order to examine whether the altered organization of the body axis of *dino* and *mercedes* mutant embryos is preceded by a shift of positional information, we performed in situ hybridizations using marker genes which are expressed in a dorsoventral-specific manner before and during gastrulation.

eve1, a zebrafish homeobox gene related to the *Drosophila* pair rule gene *even skipped* (Joly et al., 1993), is expressed in ventrolateral marginal cells. In wild-type embryos, *eve1* expression becomes progressively restricted to the ventral half of the embryo, while in *din* and *mes* mutants, it maintains a broader expression, which is first apparent at 40% epiboly (Fig. 2A) and more obvious at 70% epiboly, when the *eve1* expression domain spans about 180° of the margin in wild type, 240° in *mes* mutant, and 320° in *din* mutant embryos (Fig. 2B). At the end of gastrulation, the *eve1*-positive cells are located in the posterior region of the embryo, which in the mutants is strongly enlarged (Fig. 2C for *mes*; compare with Fig. 1D for *din*). Similarly, the hematopoietic transcription factor gene, *gata2* (Detrich et al., 1995), which is normally transiently expressed in the ventral half of the animal region is expressed in a much broader and stronger fashion in the mutants (Fig. 2D,E). Together, the *eve1* and *gata2* expression patterns indicate that in both the marginal and the animal zone of *din* mutant embryos, ventral fates expand into more dorsal regions before and during gastrulation. Taken together, analysis of paraxial and axial markers (*sna1*, *sonic hedgehog* and *goosecoid*) indicates that dorsal cell fates are reduced.

sna1, a zebrafish homologue of *Drosophila snail*, is expressed in involuting cells in ventrolateral regions of the germ ring and in the presomitic mesoderm (Hammerschmidt and Nüsslein-Volhard, 1993). In *din* mutants at 80% epiboly (Fig. 2F), the paraxial expression domains of *sna1* are slightly smaller and shifted to more dorsal regions.

sonic hedgehog (*shh*) is normally expressed in the form of a triangle in the presumptive notochordal cells of the axial mesoderm at 70% epiboly (Krauss et al., 1993). In *din* mutants, the *shh* expression domain is of normal width in the anterior part of the notochord anlage, but becomes progressively narrower than in wild-type embryos in more posterior regions (Fig. 2G), while in *mes* mutants, no significant alteration in the *shh* expression pattern has been observed (not shown).

goosecoid (*gsc*), which is expressed on the dorsal side in

early involuting cells of the presumptive prechordal plate (Stachel et al., 1993; Schulte-Merker et al., 1994) is expressed normally in most *din* mutants (13/15) before and during gastrulation. Only very few cases (Fig. 2H; also stained for *eve1* mRNA to distinguish wild-type and mutant embryos) show a slight reduction of *gsc* expression. Taken together, the expression patterns of *gsc* and *shh* in *din* mutants show that the loss of dorsal fates is very mild in the early formed anterior mesoderm, but more pronounced in the later formed posterior mesoderm.

The reduction of dorsal anlagen in the presumptive ectoderm of the animal zone is already apparent at the onset of gastrulation, coincident with the expansion of the ventral marginal fates. The forkhead domain gene *fkd3*, which marks presumptive neuroectodermal cells (J. O., unpublished data) is expressed in a much narrower and weaker fashion on the dorsal side in *din* mutants at the shield stage (Fig. 2J) and at 70% epiboly (Fig. 2K). *mes* mutants also display a significant, but less severe reduction in size of the *fkd3* expression domain (Fig. 2K). For both mutants, the reduction is strongest in anterior and lateral regions of the *fkd3* expression domain, the regions where *gata2* is ectopically expressed. At later stages of development, the reduction of the neural tube is anticipated and reflected by the expression of many marker genes, such as *pax2*, which shows that the number of a specific class of interneurons (Mikkola et al., 1992) is reduced to about 50% of the wild-type level in the spinal cord of *din* mutants (Fig. 3E).

Ventral and posterior mesodermal tissues are enlarged in *dino* and *mercedes* postgastrula embryos

Cells from the different marginal positions of the late blastula zebrafish embryo give rise to different mesodermal tissues (Kimmel et al., 1990). Thus, the described shift in the expression of the early marker genes like *eve1* in *dino* mutants might lead to a shift in the relative sizes of the different mesodermal tissues at later stages of development. To examine this notion, we studied the expression pattern of various tissue specific genes after gastrulation.

As markers for the various dorsally and ventrolaterally derived mesodermal tissues (see Fig. 4), we used *fkd2* for the hatching gland cells (J. O., unpublished data), *no tail* (*ntl*) for the notochord (Schulte-Merker et al., 1992), *myoD* for the somitic muscle (Weinberg et al., 1996), *pax2* for the pronephros (formerly called *paxb*; Krauss et al., 1991) and *gata1* (Detrich et al., 1995) for the blood.

Immunostaining of the protein encoded by the forkhead domain gene *fkd2* (J. O. and R. M. W., unpublished data) revealed that the number of presumptive hatching gland cells, derivatives of the prechordal plate, is normal in most *din* mutants (8/10) and only slightly reduced (150 versus 180) in the strongest cases (2/10; Fig. 3A), consistent with the normal expression pattern of *gsc* during gastrulation (Fig. 2H).

Using anti-Ntl immunostaining, we have also quantified the number of notochord cells. A normal number of notochord cells is found in the anterior part of *din* mutant embryos (Fig. 3B). However, the number of notochord cells in the posterior of the body axis, beginning between somites 5 and 10, is reduced. Strong *din* mutants do not have any *ntl*-positive notochord cells in the posterior part of the midline (Fig. 3B), and even in *din* mutants with very weak notochord phenotypes, the number of posterior *ntl*-positive cells is reduced to about 50% of the number in wild-type embryos (not shown).

The anterior somites of *dino* mutants contain much fewer myotomal cells, as revealed by the expression pattern of *myoD* (Fig. 3C). In contrast, posterior somites are larger than in wild-type embryos and are fused in the dorsal midline (Fig. 3C).

A similar enlargement has been found for the expression domain of genes marking ventrally derived mesodermal tissues such as the blood and the pronephros: *gata1* which is expressed in presumptive hematopoietic cells in two lateral stripes in the posterior part of the trunk, shows a twofold broader expression domain in *din* mutants at the 10-somite stage (Fig. 3D). At 22h of development, just before the presumptive blood cells start to migrate anteriorly (Detrich et al., 1995), many more *gata1*-positive cells can be detected in the region of the anus in *din* mutants, indicating the presence of more blood cells. Similarly, the posterior part of the pronephros is enlarged in *din* mutants, as revealed by the expression pattern of *pax2* (Fig. 3E). *mercedes* embryos show a *gata1* expression pattern similar to that of *din* mutant embryos. However, consistent with the weaker phenotype, the expansion of the *gata1* expression domain is not as strong (Fig. 3D).

DISCUSSION

We have described two genes that are required for the dorsalization of early zebrafish embryos, *dino* and *mercedes*. Mutants in *din* and *mes* display an expansion of ventrolateral fates at the expense of dorsolateral fates in both the ectoderm from the animal zone and the mesendoderm from the marginal zone of the late blastula embryo (see fate map in Fig. 4A). In the ectoderm of both *mes* and *din* mutants, the population of ventral tail fin cells (ventral derivatives) is strongly enlarged, while the dorsally derived neuroectoderm is reduced. Consistent alterations are also found in the mesoderm: mutants show an increased amount of blood, and an enlargement of pronephros and posterior somites (ventrolateral derivatives), while the anterior somites and the posterior notochord (dorsolateral derivatives) are reduced. The notion that both *din* and *mes* mutant embryos are ventralized is supported by the finding that wild-type embryos overexpressing the ventralizing signal Bmp4 display a similar enlargement of ventral and posterior structures, including the characteristic duplication of the ventral tail fin.

dino, *mercedes*, and the Spemann organizer

The derivatives of the dorsal most mesendoderm, the anterior notochord and the hatching glands, are unaffected in the mutants. These structures are derived from the shield, the fish equivalent of the Spemann organizer in amphibia (see introduction and Fig. 4B). As illustrated in Fig. 4C, the altered organization of both mesodermal and ectodermal structures in *din* and *mes* mutant embryos could be explained by a reduction in dorsalizing signaling from cells of the Spemann organizer at late blastula and early gastrula stages. Alternatively, it is possible that the phenotype is caused by gain-of-function mutations in counteracting, ventralizing components. These alternative possibilities will be addressed by segregation analysis of ventralizing and dorsalizing mutations.

Dorsoventral pattern is set up before the onset of gastrula-

tion, as evident from a comparison of the *eve1* and *fkd3* expression. In late blastula stages, *eve1* is expressed in most of the marginal zone, excluding only the dorsal most cells, the region of the presumptive organizer. During further development, still before the onset of gastrulation, *eve1* expression normally becomes progressively restricted to more ventral regions of the margin. This suggests that most of the marginal zone of pregastrula embryos is initially ventrally specified, and that the establishment of intermediate mesodermal fates is accomplished in a later process, probably driven by dorsalizing signals spreading from the shield. At the same time as the ventral retraction of the *eve1* expression in the marginal zone, the expression of *fkd3* is initiated in presumptive neuroectodermal cells in an animal region directly adjacent to the shield, suggesting that neural induction in the ectoderm is initiated simultaneously with the dorsalization of the marginal zone. In *din* and *mes* mutants, the repression of the *eve1* expression and the activation of the *fkd3* expression are impaired to the same extent, leading to a perfectly complementary image of their expression domains in an animal view in both wild-type and mutant embryos. This indicates that *din* and *mes* are required for both the dorsalization of the mesoderm and the induction and patterning of the neuroectoderm. The phenotype of the mutants suggests that these two inductions are achieved by one and the same process.

Are additional genes required for the dorsalization of the embryo?

mes mutant embryos display a generally weaker phenotype than *din* mutants. Even in the stronger *din* mutants, the dorsalization of the ventrolateral mesoderm and the neural induction is not completely abolished, as illustrated in Fig. 4C. This could have several reasons. The remaining induction could result from maternally supplied gene products. For both *din* and *mes*, a maternal function is suggested by the observation that the mutant phenotypes are stronger in a genetic situation in which the maternal contribution is reduced. Alternatively, the isolated alleles might be hypomorphic and retain some zygotic activity. Finally, the remaining induction could be accomplished by other signals of the dorsal mesoderm that are partially redundant to *din* or *mes*. *din* and *mes* themselves may be partially redundant, and other genes might have been identified but not yet recognized to be involved in the dorsalizing process because their corresponding mutant phenotypes are different from those of *din* and *mes* mutants.

During the course of development, the morphological consequences of the early pattern defects of *din* and *mes* mutants become progressively weaker. Many *din* mutant embryos develop a relatively normal head at later stages of development, and *mes* mutants are even viable. Such a progressive weakening of the phenotypic strength has been observed in other mutants in which early morphogenetic processes are affected. Maternal *Drosophila* mutants with an altered Bicoid gradient, for example, display severe fate shifts along the anteroposterior axis during gastrulation but may show less pronounced segmentation defects in the larval stages or even be normal (Driever and Nüsslein-Volhard, 1988). Similarly, mutations in the ventral-specific *Drosophila* genes *twist* and *snail* display a completely penetrant dominant reduction of the mesodermal anlage, but are fully viable (C. Nüsslein-Volhard, unpublished observations).

The system regulating early dorsoventral pattern formation

In addition to the ventralized mutants *dino* and *mercedes* described in this paper, several mutations defining at least 6 genes, *snailhouse*, *swirl*, *somitabun*, *piggy tail*, *lost-a-fin* and *mini fin* have been identified that cause a dorsalized phenotype complementary to that of *din* and *mes* mutants (Mullins et al., 1996). The expression of *eve1* is strongly reduced at late blastula stages, and the ventrolateral mesoderm is dorsalized, while more neuroectoderm is formed. These mutants might be defective in ventralizing activities which normally counteract dorsalizing activitives like those encoded by the genes *dino* and *mercedes*.

A similar view has emerged for the mechanisms regulating dorsoventral pattern formation in *Xenopus* and *Drosophila* embryos. Although not confirmed in a loss-of-function situation, dorsalizing activities like those assigned to *dino* have

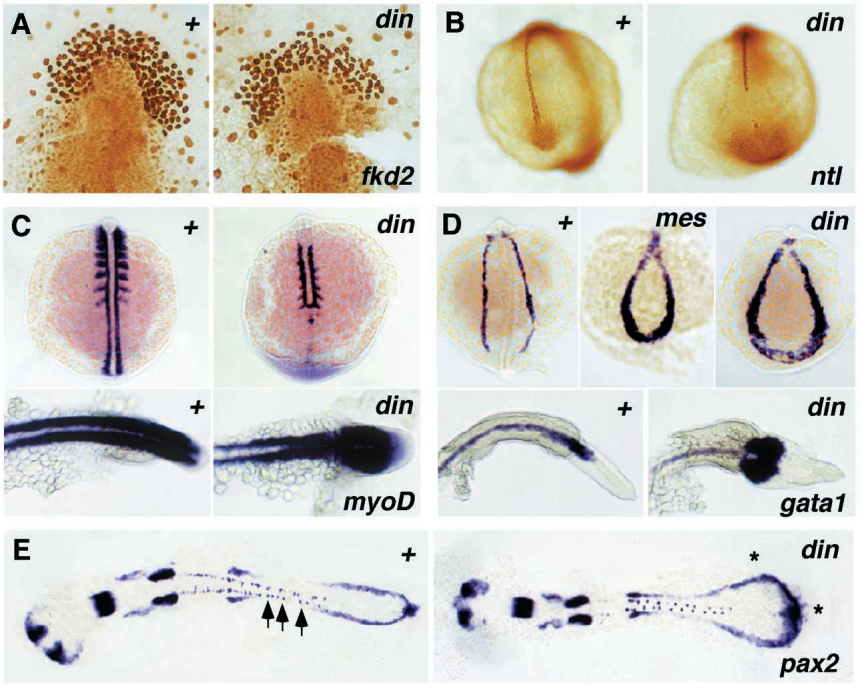

Fig. 3. Altered gene expression in mesodermal tissues of *dino* (*din*^{tm84}) and *mercedes* (*mes*^{tm305}) mutants detected by in situ hybridization (C-E) and immunostaining (A,B). (A) *fkd2*, 10-somite stage, dorsal view of the head region. (B) *ntl*, 10-somite stage, dorsal view. (C) *myoD*: top, 10-somite stage, dorsal view; bottom, 22 hours, ventral view of the tail. (D) *gata1*: top, 10-somite stage, dorsal view; bottom, 22 hours ventral view of the tail. (E) *pax2*, 22-somite stage, dorsal view of a spread embryo, anterior to the left. The pronephric expression domain is indicated by asterisks (in the mutant), the interneurons are indicated by arrows (in the wild type).

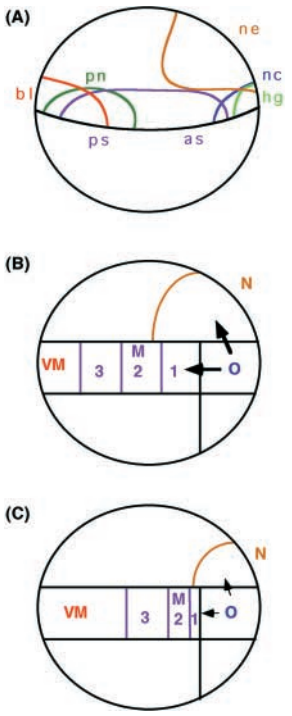

Fig. 4. (A) Fate map of a late blastula wild-type embryo, according to Kimmel et al. (1990) and unpublished results of R. M. W.; dorsal is right, ventral left, animal pole at the top and vegetal pole at the bottom. as, anterior somites; bl, blood; hg, hatching gland; nc, notochord; ne, neuroectoderm; pn, pronephros; ps, posterior somites. (B) Diagram illustrating the inductive function of the shield, the organizer (O) of the fish, induction of neuroectoderm (N) in the dorsal animal zone and dorsalization of the ventral mesoderm (VM) to produce intermediate fates (M1-M3) in the marginal zone. (C) Diagram illustrating that the altered organization of *din* mutant embryos could result from an impaired function of the shield (indicated by the smaller arrows).

been found for three *Xenopus* genes: *noggin* (Smith and Harland, 1992; Knecht et al., 1995), *chordin* (Sasai et al., 1994) and *follistatin* (Hemmati-Brivanlou et al., 1994; Kessler and Melton, 1995, D. A. Melton, personal communication). All three genes encode signaling molecules produced by cells of the Spemann organizer, and all dorsalize the ventrolateral mesoderm and induce neuroectoderm in ectopic expression experiments. In addition, Chordin has been reported to counteract and repress Bmp4 (Sasai et al., 1995), a ventralizing signal generated by the ventral mesoderm (Köster et al., 1991; Dale et al., 1992; Jones et al., 1992). A similar interaction has been described for the structural and functional homologues of *chordin* and *Bmp4*, *short gastrulation* (*sog*) and *decapentaplegic* (*dpp*) in dorsoventral pattern formation of *Drosophila* embryos (François and Bier, 1995; Holley et al., 1995).

It is likely that similar mechanisms are at play in the zebrafish embryo. The observation that the overexpression of the ventralizing signal Bmp4 in wild-type embryos results in defects characteristic of the *dino* mutant phenotype suggests that Dino, similarly to Chordin, might counteract Bmp4-like activities during normal zebrafish development.

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