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The ventral and posterior expression of the zebrafish homeobox gene *eve1* is perturbed in dorsalized and mutant embryos

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SUMMARY

We have identified and characterized zebrafish *eve1*, a novel member of the *Drosophila even-skipped* (*eve*) gene family. *eve1* RNAs are expressed initially in late blastulae with a peak during the gastrula stage, at which time expression is confined to ventral and lateral cells of the marginal zone of the zebrafish embryo. Later, *eve1* transcripts are located in the most posterior part of the extending tail tip. We show that LiCl, known to dorsalize *Xenopus* embryos, has the same effect in zebrafish, resulting in embryos with exaggerated dorsoanterior structures. In LiCl-treated embryos, *eve1* transcripts are completely absent. *eve1* is therefore a marker of ventral and posterior cells.

In the light of its ventroposterior expression domain, the localization of *eve1* transcripts was analysed in *spadetail (spt)* and *no tail (ntl)*, two mutants with abnormal caudal development. In *spt*^{b140} homozygous mutants, there is an accumulation of cells in the tail region, resulting from inadequate migratory behaviour of precursors to the trunk somites. These cells, in their abnormal environment, express *eve1*, emphasizing the correlation between ventroposterior position and *eve1* expression. In homozygous mutant embryos for the gene *ntl* (the homologue of mouse *Brachyury*, originally called *Zf-T*), posterior structures are missing (M. E. Halpern, C. B. Kimmel, R. K. Ho and C. Walker, 1993; *Cell* In press). While mutant and wild-type embryos do not differ in their *eve1* transcript distribution during gastrulation, *eve1* expression is absent in the caudal region of mutant *ntl* embryos during early somitogenesis, indicating a requirement for *ntl* in the maintenance of *eve1* expression during tail extension.

Our findings suggest that *eve1* expression is correlated with a ventral and posterior cell fate, and provide first insights into its regulation.

Key words: *Brachydanio rerio*, dorsoventral specification, *even-skipped*, homeobox, *eve1*, gastrulation, mesoderm, LiCl, tailbud, *spadetail*, *spt*, *Brachyury*, *T*, mutation, *no tail*, *ntl*, zebrafish

INTRODUCTION

Among vertebrates, the zebrafish is particularly amenable to both embryological and genetic studies of early development (Kimmel, 1989; Ho, 1992). We have chosen this teleost to study one pivotal process of vertebrate development: dorsoventral specification. In the zebrafish embryo, the positions corresponding to dorsal and ventral sides are not morphologically distinguishable before gastrulation (Warga and Kimmel, 1990). Later, the convergence of cells to the dorsal side of the early gastrula leads to a conspicuous thickening of the margin: the embryonic shield that marks the dorsal side. Although the huge yolk cell influences cell movements, the gastrulation of the zebrafish embryos possesses several main streams of cell migration commonly found in vertebrates: epiboly, involution and convergence to the dorsal side with extension mediated by cell intercalation (Warga and Kimmel, 1990). As in other vertebrates, involution allows the formation of two layers: the epiblast (composed of cells that have not involuted) and the hypoblast (made of cells that have involuted), whose embryological derivatives are ectoderm and endo/mesoderm, respectively. All these features reinforce the concept of unitary mechanisms in the early embryology of vertebrates and suggest that zebrafish can be used as a model for the studies concerning vertebrate gastrulation.

However, it remains clear that most of our knowledge about vertebrate developmental process comes from data accumulated in the last century on amphibians. In *Xenopus*, the bilateral body plan is first laid down according to an early pattern imprinted on the embryonic mesoderm. This patterning occurs in response to initial inductive signals and is modified in late blastula and gastrula by both ventralizing and dorsalizing cell-cell interactions which take place in a context of complex morphogenetic movements (for recent reviews: Slack et al., 1992; Smith and Howard, 1992; Sive,

1993). At the beginning of gastrulation, the mesoderm is essentially of a ventral type, although a small region of dorsal type mesoderm, the organizer, is present near the dorsal midline. During gastrulation, most cells converge to the dorsal side of the embryo and are thus located in the range of a dorsalizing signal emitted by the organizer. Most embryological studies first focused on this embryonic structure which has powerful signaling properties. This led in particular to the molecular isolation of homeobox containing genes exhibiting an expression correlated with the activity of the organizer region, e.g. the *FKH-1* (Dirksen and Jamrich, 1992), *Xlim-1* (Taira et al., 1992) or *goosecoid* (Cho et al., 1991) genes.

In contrast, no *Xenopus* homeobox gene with an exclusive ventrolateral expression in the marginal zone of late blastula or gastrula has been reported so far. One gene with a divergent homeobox of the POU type (called XLPOU91) was shown to be expressed in ventroposterior regions of early neurulae, but its earlier expression is widespread in the whole gastrula (Frank and Harland, 1992). Interestingly however, a gene (called *Xwnt*-8) coding for a secreted protein is first expressed in late blastulae in all marginal cells except those within the organizer field. Several lines of evidence support the hypothesis that *Xwnt*-8 may ventralize the response of mesodermal cells to dorsalizing signals from the organizer (Christian and Moon, 1993).

At the beginning of this century, Holmdahl distinguished two phases in the development of the vertebrate embryo, the first taking place before and during gastrulation and leading to the establishment of the basic body plan and the second consisting of the development of the caudal region from a posterior cell mass, i.e. the tail bud (Holmdahl, 1925). In vertebrate models, cellular characteristics and embryological origins of caudal structures are variable (May Griffith et al., 1992). In bird and mammals, the tail bud results from the coalescence between Hensen's node and the primitive streak at the end of gastrulation. In amphibians, there is no caudally located aggregate of mesenchymal cells corresponding to those found in amniotes, while in zebrafish such a mass originates from cells located dorsal and also ventral to the yolk plug closure (see below).

This study presents the isolation of a zebrafish gene whose expression overlaps both developmental phases formerly hypothesized by Holmdahl, i.e. a gene that is found expressed in the ventrolateral region of the gastrula and in the posterior region of the embryo during somitogenesis. This gene was called *eve1* and is the first zebrafish gene reported to contain a homeobox related to the one found in *even-skipped*, a *Drosophila* pair-rule gene. In an effort to understand the molecular mechanisms that underly the specification of the ventral and posterior region, we analysed the expression of the zebrafish *eve1* gene in normal, mutant and experimentally perturbed embryos. This gene is the only marker of the ventral region of the zebrafish gastrula reported so far and its study provides a counterpart to investigations concerning dorsal genes such as the recently identified zebrafish *goosecoid* (Stachel et al., 1993; Schulte-Merker et al., unpublished data). In a first set of experiments, early blastulae were subjected to lithium treatments. Our results showed that this treatment respecifies the body plan as it does in *Xenopus*, which was reported independently in a recent paper by Stachel et al. (1993). In *Xenopus*, lithium treatments are known to produce hyper-dorsal development by transforming the entire mesoderm to dorsal mesoderm (Kao et al., 1986; Kao and Elinson, 1988). In a second set of experiments, we studied the expression of *eve1* in two zebrafish mutants isolated in Dr Kimmel's laboratory (Kimmel et al., 1989; Halpern et al., 1993): *spadetail* (*spt*) and *no tail* (*ntl*), which exhibit an abnormal posterior development. In *spt* homozygous mutants (Kimmel et al., 1989), a cellular mismigration during gastrulation leads to an accumulation of cells in the tail bud. The *ntl* mutants exhibit a shortened and disorganized tail, accompanied by an incorrect development of the notochord. This mutant is of particular interest because the *ntl* gene is the zebrafish homologue of the mouse *Brachyury* or *T* gene (Schulte-Merker et al., 1992; Schulte-Merker et al., unpublished data) and because *ntl* and *eve1* show overlapping expression domains, as we show in this study. Such studies constitute an entry point into the interaction network between two genes expressed during gastrulation. This paper thus reports several strategies aimed at unraveling the putative mechanisms regulating *eve1*, which may aid our understanding of the specification of ventral mesoderm in vertebrates.

MATERIALS AND METHODS

Screenings of a late gastrula stage cDNA library

Screening under moderate stringency conditions of a λ ZAP cDNA library from zebrafish gastrula was performed as previously described (Joly et al., 1992). The library was screened with a 240 bp *Sac*II-*Acc*I fragment containing the 3′ side of the homeobox of the *Drosophila even-skipped* cDNA and short flanking regions (Macdonald et al., 1986). Fifteen overlapping clones were isolated from the library, one of which, clone CE-51 was subjected to further analysis and used to screen the library at high stringency. The screen was performed following standard methods (Sambrook et al., 1989) with a DNA probe obtained by *Bam*HI-*Pvu*II restriction digest of clone CE-51 (Fig. 1A). After one round of screening, 32 phage plaques containing putative positive clones were picked up and tested by PCR for the presence of inserts with longer 5′ regions of the *eve1* cDNA. Phage plaques were first resuspended in 100 μl of SM (Sambrook et al., 1989). Phage particules (1 μl SM suspension in 80 μl of distilled H_2O) were disrupted by incubation at 70°C for 5 minutes. The volume was brought to 100 μl with 10 μl of Taq polymerase buffer (660 mM Tris hydroxymethyl aminomethane (Tris-HCl), pH 8.6, 166 mM $(NH_4)_2SO_4$, 67 mM $MgCl_2$, 100 mM β-mercaptoethanol, 0.1% (w/v) gelatin), 5 μl of a dNTP mix (5 mM each), 3 μl of a 0.1 nM solution of appropriate oligonucleotides. After 6 minutes at 96°C, the tubes were brought to the desired annealing temperature (62°C) and 5 units of Taq DNA polymerase (Perkin-Elmer/Cetus) were added. Thermal cycling (repeated 35 times) was: 72°C for 30 seconds, 94°C for 1 minute and 62°C for 1 minute. Two PCRs were performed with one primer in the 5′ region of the *eve1* cDNA (Fig. 1A, sequence: TCGTGAAAGCTGTCCGGTGC), and one of the two primers located on either side of the insert in the Bluescript sequences contained in the λZAP vector, respectively (5′CGACGTTG-TAAAACGACGGCC3′ or 5′CGCCAAGCTCCGGAAT-TAACCC3′).

Nucleic acid extractions and DNA sequence analysis

The dideoxy chain termination method (Sanger et al., 1977) with T7 DNA polymerase (Multiwell sequencing kit, Amersham) was

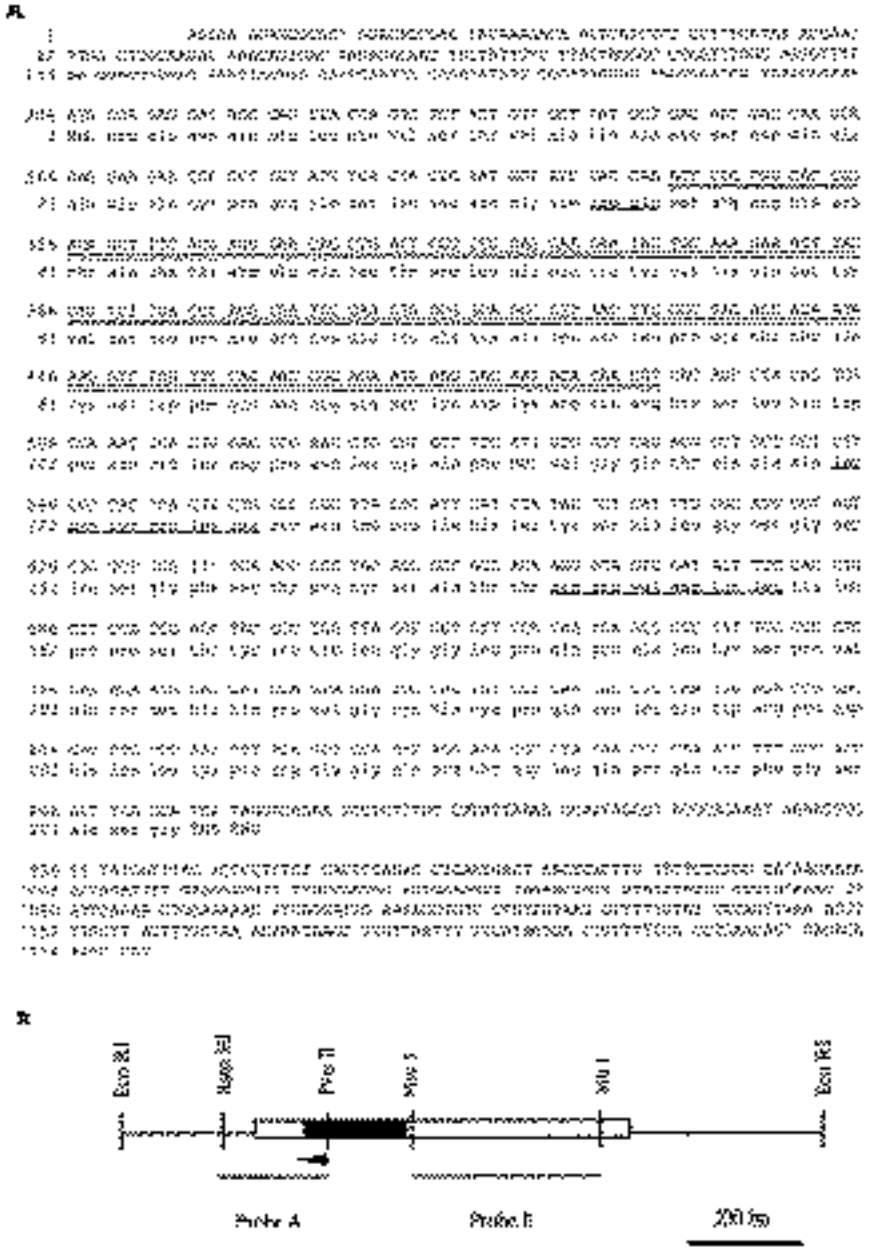


Fig. 1. Molecular characterisation of the zebrafish *eve1* cDNA. (A) Nucleotide sequence and predicted protein sequence of the 1.2 kb *eve1* cDNA. Numbers to the left indicate the base and residue (italics) positions in the nucleotide and predicted protein sequences, respectively. The homeobox is double underlined and the acidic region is dotted underlined. The regions of extended homology to other members of the *even-skipped* family are underlined. (B) Map of the *eve1* cDNA. The position of the two probes and of the oligonucleotide primer used for PCR (arrowhead) are indicated. The open reading frame is represented by a white box and the homeobox is in dark.

carried out on both strands of double strand plasmids using synthetic oligonucleotides.

Total RNA was purified as described (Chomczynski and Sacchi, 1987). Same amounts of RNA were loaded in each lane. Absence of degradation after electrophoresis in agarose gels was checked using EtdBr staining. Subsequent northern blotting was performed according to standard procedures involving a final stringent wash of 20 minutes in 0.1× SSC, 0.1% SDS (Sambrook et al., 1989). Two fragments from *eve1* were used as probes: 'A' which is a 156 bp *Bam*HI-*Pvu*II fragment and 'B' which is a *Msc*I-*Stu*I restriction fragment (335 bp) (Fig. 1B).

Animal accommodation and LiCl treatments

Zebrafish (*Brachydanio rerio*) were maintained as described (Westerfield, 1989). Embryos were obtained from natural crosses or by in vitro fertilization, incubated at 28.5°C and staged (Warga and Kimmel, 1990). h means 'hours after fertilization'. Mutant lines were *spt*^{b140}, *ntl*^{b160} and *ntl*^{b195}.

For LiCl treatments, 'Fish water' (60 mg/l commercial sea salt ('Wimex, Germany) in deionized water) was always used as the embryo medium (Westerfield, 1989). Following in vitro fertilization (Westerfield, 1989), embryos were placed on agarose-coated Petri dishes and manually dechorionated. LiCl (0.3 M) was applied at the 32-cell stage for 30 minutes at 20°C. After thorough washing, embryos were allowed to develop at 28°C.

In situ hybridization

Fragments A and B were subcloned in a T3/T7 promoter-containing pBS vector (Stratagene). After appropriate linearisation of the plasmids, sense and anti-sense RNA probes were generated using bacteriophage T3 or T7 RNA polymerase. In situ hybridization to 8 μm paraffin sections of embryos was done as previously described (Joly et al., 1992).

Hybridization of whole-mount embryos was done according to established procedures (Püschel et al., 1992; Schulte-Merker et al., 1992). Digoxigenin (DIG)-labelled RNA-probes were synthesized according to Boehringer's specifications. In addition, the probe was passed through a Sephadex G-50 column (Pharmacia) before

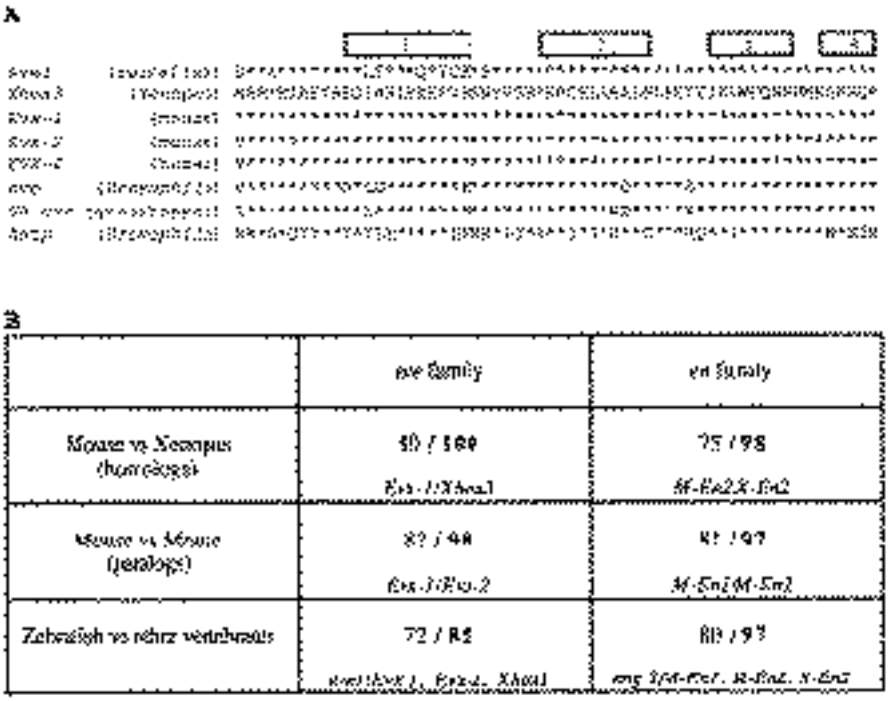


Fig. 2. (A) Sequence comparison between the homeodomain of *eve1* with that of the previously reported *even-skipped* family members. Names of genes and of organisms (between brackets) are given on the left. *Antennapedia* homeodomain is also shown. Amino acids are in single letter code. The *Xhox3* sequence is given in the second line and only residues diverging from the *Xhox3* sequence are represented in other lines. Position of the α-helices within the homeodomain are drawn at the top (Otting et al., 1988).
(B) Nucleotide and amino acid differences between homeobox sequences. Results of pairwise sequence comparaisons are expressed as the arithmetic means of the percentage of identical residues at the nucleotide or amino acid (bold) levels. Sources: *Xhox3* (Ruiz i Altaba and Melton, 1989). *Evx-1* (Bastian and Gruss, 1990). *Evx-2* (Dush and Martin, 1992). *EVX-2* (D'Esposito et al., 1991). *eve* (Macdonald et al., 1986). *GH-eve* (Patel et al., 1992). *Antp* (Garber et al., 1983). *eng 2* (Ekker et al., 1992). *M-En1* and *M-En2* (Joyner and Martin, 1987). *X-En2* (Hemmati-Brivanlou et al., 1991).

ethanol precipitation and fractions were analysed on an agarose gel for the presence of the purified probe. Embryos were subjected to a proteinase K treatment for the following times: 30 seconds for embryos before the shield stage, 45 seconds for gastrula, 90 seconds for 5- to 10-somite embryos and 3 minutes or more for older embryos. Embryos were then treated as described (Schulte-Merker et al., 1992). Photographs were taken using embryos in a filtered solution of 1.5% methyl cellulose in PBS (Westerfield, 1989), which facilitates the orientation of young embryos. Alternatively, the specimen were dehydrated in methanol and mounted in a 2:1 mixture of benzylbenzoate:benzylalcohol (Schulte-Merker et al., 1992).

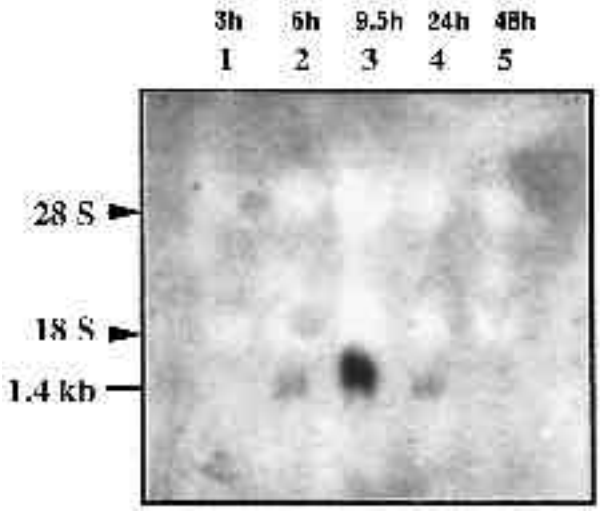
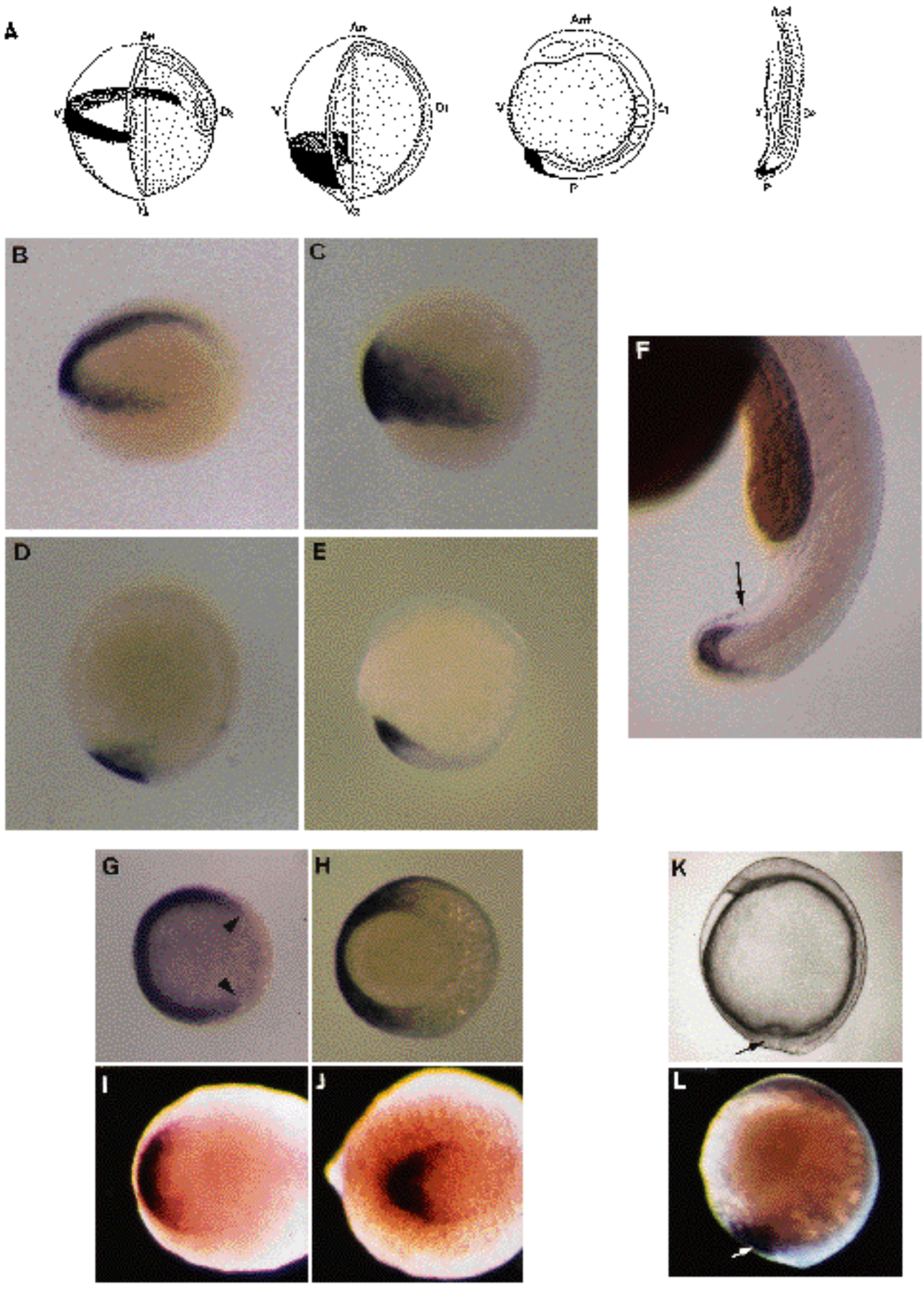


Fig. 3. Temporal expression of *eve1* in zebrafish embryos as detected by northern blot hybridization. Total RNA (20 μg) from different embryonic stages (hours after fertilization indicated upon lanes number at the top) were run in each lane and hybridized with probe 'A' (see Methods). Exposure time was 3 days. Arrowheads indicate the positions of ribosomal RNA.

Whole-mount immunohistochemistry

Antibody staining was performed mainly as described (Hemmati-Brivanlou and Harland, 1989; Schulte-Merker et al., 1992). Whole-mount embryos previously hybridized with a DIG-labelled probe

Fig. 4. Localization of *eve1* RNA in early embryos. (A) Sketch drawings with the distribution of *eve1* RNA shown in dark. From left to right: shield stage (6 h), 90% epiboly (9 h), 5-somite embryo (12.5 h), caudal region of a 24 h embryo. Side views with half dorsal hemisphere removed for the gastrula stages. Animal or anterior poles are at the top. Dorsal sides on the right. Abbreviations: An, animal pole; Vg, vegetal pole; Ant, anterior pole; P, posterior pole; Dl, dorsal side; V, ventral side. (B-J,L). Whole-mount in situ hybridizations using a *eve1* probe. Cells expressing *eve1* appear in dark purple after an alkaline-phosphatase chromogenic reaction. (B-F). Side views of embryos with the same orientation as in A. (B). 30% epiboly (4.7 h) embryo slightly inclined to see the whole arc of positive cells from the vegetal pole. (C). 60% epiboly (7 h). (D). 100% epiboly (9.5 h). (E). 5-somite embryo (12.5 h). (F). 24h embryo. Positive external cells are located around the extremity of the tail (arrow). (G-J). Views from the animal pole in (G), from the vegetal pole in (H) and from the posterior pole in (I,J). Dorsal side on the right. (G). 30% epiboly stage, the positive signal covering more than 270° of arc (limits indicated by arrowheads). (H). 80% epiboly (8.5 h) stage, positive cells in the ventral half of the gastrula. (I). 1-somite embryo (10.5 h). (J). 10-somite embryo (15 h). (K). Micrograph of a living embryo (9.5 h). Side view. Vegetal pole is at the bottom. The dorsal tail bud is on the right from yolk-plug closure. Ventral cells (arrow) converge with their dorsal counterparts. (L). Embryo at the same stage and with the same orientation as in (K). The signal (arrow) forms a plaque ventral to the yolk plug closure and to the dorsal tail bud.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were used without refixation before antibody staining. Embryos were incubated overnight at 4°C with a 10^4-fold dilution of an anti-*ntl* antiserum. A biotinylated secondary antibody (Vector laboratories) was used at a dilution of 1:1000, also overnight at 4°C. Detection was carried out at room temperature using the Avidin/Biotin ABC system (Vector Laboratories) according to manufacturer's instructions. When a satisfactory signal was obtained, the reaction was stopped by PBS washes and a 20 minute postfixation in 10% formaldehyde in distilled water (pH 7 after equilibration with Na_2CO_3). After several washes in PBS, the specimens were treated for observation as described for the embryos hybridized with DIG probes. Embryos to be sectioned were refixed overnight at 4°C in 10% formaldehyde. 10 μm paraffin sections were cut and mounted using standard procedures (Joly et al., 1992).

RESULTS

Isolation and structure of cDNA sequences homologous to the *Drosophila even-skipped* (*eve*) homeobox

A zebrafish cDNA library prepared from late gastrula RNA was screened under moderate stringency conditions with a probe containing the *Drosophila eve* homeobox (Macdonald et al., 1986). The isolated clone with the longest insert (CE-51) was completely sequenced. A second stringent screening of the gastrula library was performed with a probe from the 5′ region of CE-51 (see Fig. 1 and Materials and Methods). Two new clones (CE-13 and 91) were isolated and the sequence of 133 nucleotides located upstream from the 5′ end of CE-51 was determined. The compilation of sequences from this upstream region and CE-51 is shown in Fig. 1A. The *eve1* composite cDNA was found to be 1230 bp (Fig. 1A). CE-13 and 91 have an in-frame stop codon in front of the putative start of translation. Conceptual translation of *eve1* beginning at this AUG yields a small protein of 213 aa. Noticeably, the mRNA (1.4 kb) (see below and Fig. 3) and the putative protein coded by the *eve1* gene are approximately half the size of the corresponding products of other vertebrate *even-skipped* genes (Ruiz i Altaba and Melton, 1989; Bastian and Gruss, 1990; D'Esposito et al., 1991; Dush and Martin, 1992).

The homeobox encoded is clearly not a *hox/Antp* type homeobox but is related to the fly *even-skipped* homeobox (Fig. 2A). The corresponding gene is thus referred to as *eve1*. The *eve1* homeodomain is quite distinct from all other known homeodomains of the *even-skipped* family (Fig. 2A, B): less than 75% of the nucleotides and 85% of the amino acids of the *eve1* homeodomain are identical to each of the *eve* type homeodomains, respectively. This is for example in contrast with the close phylogenetical identity observed among the other vertebrate genes of the *even-skipped* family (Fig. 2B), or among other families of homeoboxes such as the *engrailed* family (Fig. 2B) or the *caudal* family (Joly et al., 1992). Divergent residues between vertebrate and *Drosophila even-skipped* homeodomains are scattered along this region (Fig. 2A). Most divergent positions in the *eve1* homeodomain are found within the N terminus, at positions known to be variant in the different classes of homeoboxes (Scott et al., 1989). However, the phenylalanine at position 20, which is found in all *eve* homeodomains and in more than 80% of the homeodomains reported (Chouard et al., 1990), is replaced by a tyrosine in the *eve1* homeodomain (Fig. 2A).

The primary structure of Eve1 putative protein differs greatly from other vertebrate *eve*-type proteins. Whereas long homologous stretches extending for a total of about 100 residues are found in the C-terminal part of mouse *Evx-1* and *Evx-2*, human *EVX-2* and *Xenopus Xhox3* (Dush and Martin, 1992), the putative Eve1 protein shows no extensive structural similarity with the other members of the *even-skipped* family. When conservative changes are included, only two short amino acid stretches (underlined in Fig. 1A) of the Eve1 protein are conserved in other vertebrate *eve* proteins. Long alanine or glutamine repeats found in the coding frame of all putative proteins of the *eve* family are absent in the Eve1 predicted protein (Fig. 1A); however, a proline-rich region (16%) is present in the Eve1 putative protein as well as in its invertebrate homologs (Patel et al., 1992). Finally, no evidence for conservation at the N-terminal extremity was found when the Eve1 putative protein sequence was compared to the other proteins of the *eve* family, except for a short region of 6 acidic amino acids upstream from the homeodomain (Fig. 1A).

Developmental expression of *eve1*

We have assayed staged embryos for the presence of *eve1* transcripts by probing northern blots with two probes (A and B, Fig. 1B), corresponding to both sides of the homeobox. Fig. 3 shows the result obtained after hybridization with probe 'A' (same result with probe 'B'). The *eve1* gene gives rise to transcripts of a single size (1.4 kb). At the earliest stage studied, i.e. midblastula transition (3 h), no signal is observed (Fig. 3, lane 1). Low levels of *eve1* transcripts are first detected at the embryonic shield stage (6 h) (Fig. 3, lane 2) and a sharp peak of expression occurs around the end of epiboly (Fig. 3, lane 3). After 24 h, when embryos have about 30 somites, transcripts are easily detected (Fig. 3, lane 4). However, RNA extracted from 48 h embryos yields no signal (Fig. 3, lane 5).

Asymmetrical onset of *eve1* expression along the prospective dorsoventral axis

The spatial pattern of *eve1* expression during embryogenesis was studied using in situ hybridizations. A [^{35}S]UTP-labelled antisense RNA probe ('A', 156 nucleotides long) was hybridized to sections and a digoxigenin (DIG)-labelled RNA probe ('B', 335b long), was used with whole-mount embryos. Similar patterns of expression were obtained using both techniques and both probes. Midblastulae (3 h, 1000 cells) and blastulae at the dome stage (4.3 h, Warga and Kimmel, 1990) failed to yield any signal above background when hybridized with antisense probes ([^{35}S] or digoxigenin-labelled). The first signal was detectable at 30% epiboly (4.7 h) by whole-mount in situ hybridization, the technique that appeared the most sensitive (Fig. 4B). At this stage, the blastoderm has the shape of a cup inverted over approximately one third of the yolk cell (Kimmel et al., 1990). The blastoderm is radially symmetrical around the animal-vegetal axis. *eve1* transcripts are localized in the most vegetal zone of the blastoderm, at the margin. The territory of expression has the shape of a horse shoe, and is thus clearly not radial (Fig. 4B,G).

eve1 expression is ventrolateral during gastrulation and posterior during somitogenesis

Gastrulation is marked by the beginning of involution, which starts almost simultaneously all around the blastoderm margin and can be easily detected as a thicker ring of cells, called the germ-ring (Warga and Kimmel, 1990). As during earlier stages, *eve1* RNA is localized in cells closest to the vegetal pole, i.e. the area where cells are involuting (Fig. 4A,C).

Shortly after, cells converge to the dorsal side of the embryo to form a conspicuous thickening of the margin: the embryonic shield. Direct examination as well as section data clearly showed that *eve1* RNA was absent from the dorsal region and restricted to ventral and lateral regions of the embryo (not shown).

During gastrulation, the pattern of *eve1* expression evolves following three main characteristics that were already detectable in late blastula undergoing epiboly.

Firstly, positive cells for *eve1* follow the direction of epiboly, i.e. move to the vegetal pole. They are always located in the most vegetal part of the blastoderm, near the margin (Fig. 4C). When observed from the lateral side of embryos, the second characteristic of the *eve1* expression pattern is that the signal first appears as a narrow line at 30% epiboly, while it represents a gradually wider crescent as gastrulation proceeds. The third characteristic is the progressive restriction of *eve1* RNA to the ventral side of the embryo. While at 30% epiboly, expression covers more than a 270° of arc (Fig. 4G), it is restricted to approximately half the embryo at 80% epiboly (Fig. 4H) and finally ends up as a plaque of cells located ventral to the yolk plug closure (Fig. 4I,L). Such a restriction of expression along the dorsoventral axis is striking and its relationship with ventralization or dorsalization occurring in gastrulae will be discussed.

As shown in Fig. 4K, cells ventral to the position where the yolk plug closes, participate in the tail bud formation, which extends ventrally, past the position of yolk plug closure. It thus appears that in zebrafish, the caudal region originates from both ventral and dorsal cells. Examining *eve1* stainings in stages close to the yolk plug closure indicates that the caudal pattern of expression observed after the end of epiboly (Fig. 4E) corresponds to the ventral pattern seen during late epiboly stages (Fig. 4D,L).

Complexity of the pattern of expression of *eve1* in the tail bud

The tail bud of fish embryos contains a domain of undifferentiated cells, which give rise to a wide range of derivatives. Few histological markers are available in this region. A vesicular structure, the Kupffer's vesicle, is found in the ventral portion of the cell mass, near the yolk syncytial layer (YSL). According to Hisaoka and Firlit (1960), it is located beneath the 'axial cord' during the process of separation of the tail bud from the yolk mass at the 15-somite stage. Around this stage, the axial cord consists of an outer portion, which will form the nerve cord, and an inner portion, which will develop into the notochord (Laale, 1985).

After 10.5 h, when the first somite furrow appears, the plaque of ventral *eve1* staining takes the shape of a wide arc with a fainter signal at both extremities (Fig. 4I). In 10-somite embryos, the distribution observed in whole-mount in situs consisted of a prominent mass of caudal staining with two anterior extensions (Fig. 4J). Histological analysis revealed that the anterior extensions correspond to epiblast cells (Fig. 5C,D). One additional region is also labelled. It consists of a small number of cells located caudal to the Kupffer vesicle (Fig. 5A,B). These cells give rise in 24 h embryos to a row of cells located from the tip of the tail to the position of the future opening of the anus behind the extremity of the caudal yolk (Fig. 4F, see arrow).

As somitogenesis proceeds, positive staining progressively narrows possibly as the result of cell convergence to the embryo axis. After 24 h, cells expressing *eve1* form a bend at the tip of the tail, with the number of cells exhibiting staining being much reduced (Fig. 4F).

The *eve1* RNAs are thus present in the tail following a complex pattern in constant evolution during somitogenesis. They were no longer detected in 30 h embryos, a few hours after the end of somitogenesis. The *Drosophila even-skipped* gene, as well as its vertebrate homologs, *Xhox3* and *Evx-1*, are expressed in the nervous system during neurogenesis (Macdonald et al., 1986; Ruiz i Altaba and Melton, 1989; Bastian and Gruss, 1990). Despite an extensive search for *eve1* neuronal expression in 24 h to 1-week-old embryos, no signal was detected using in situ hybridizations on whole-mount or sectioned embryos.

eve1 RNAs are absent in LiCl-treated embryos

Lithium is able to respecify profoundly the body plan of various invertebrate or vertebrate organisms. Particularly, brief exposure of *Xenopus* premidblastula embryos to LiCl leads to a great enhancement of dorsoanterior structures (Kao et al., 1986; Kao and Elinson, 1988). In this context, we have examined the effects of short and early LiCl treatments on the patterning of the zebrafish embryo.

Several durations of treatments in 0.3 M LiCl were tried, starting at the 32-cell stage blastula. Embryos treated for variable times (5 and 15 minutes) later exhibited several phenotypes, ranging from normal embryos to hyperdorsalized ones (see below), with a few embryos showing intermediate phenotypes. In contrast, after a longer exposure (30 minutes), all embryos exhibit a very abnormal development.

Examples of 16 h (14-somites) LiCl-treated zebrafish embryos are given in Fig. 6B-D. Their abnormalities clearly mimic the ones previously observed for hyperdorsalized *Xenopus* and zebrafish embryos (Kao and Elinson, 1988; Stachel et al., 1993). Representative abnormalities include the development of a brain and a heart without more ventroposterior structures (Fig. 6B), radial development marked by a constriction of putative heart tissues (Fig. 6C, note the large size of the constriction shown. Embryos with abnormal blastopore closure would be expected to display a more tightly constricted yolk as exemplified in Stachel et al., 1993) or by an external structure, already characterized by Kao and Elinson (1988) in *Xenopus* and called 'proboscis' (Fig. 6D).

eve1 is expressed in the ventral region of normal zebrafish blastulae and gastrulae. We studied its expression in zebrafish embryos treated with 0.3 M LiCl for 30 minutes at the 32-cell stage. These LiCl-treated embryos exhibited

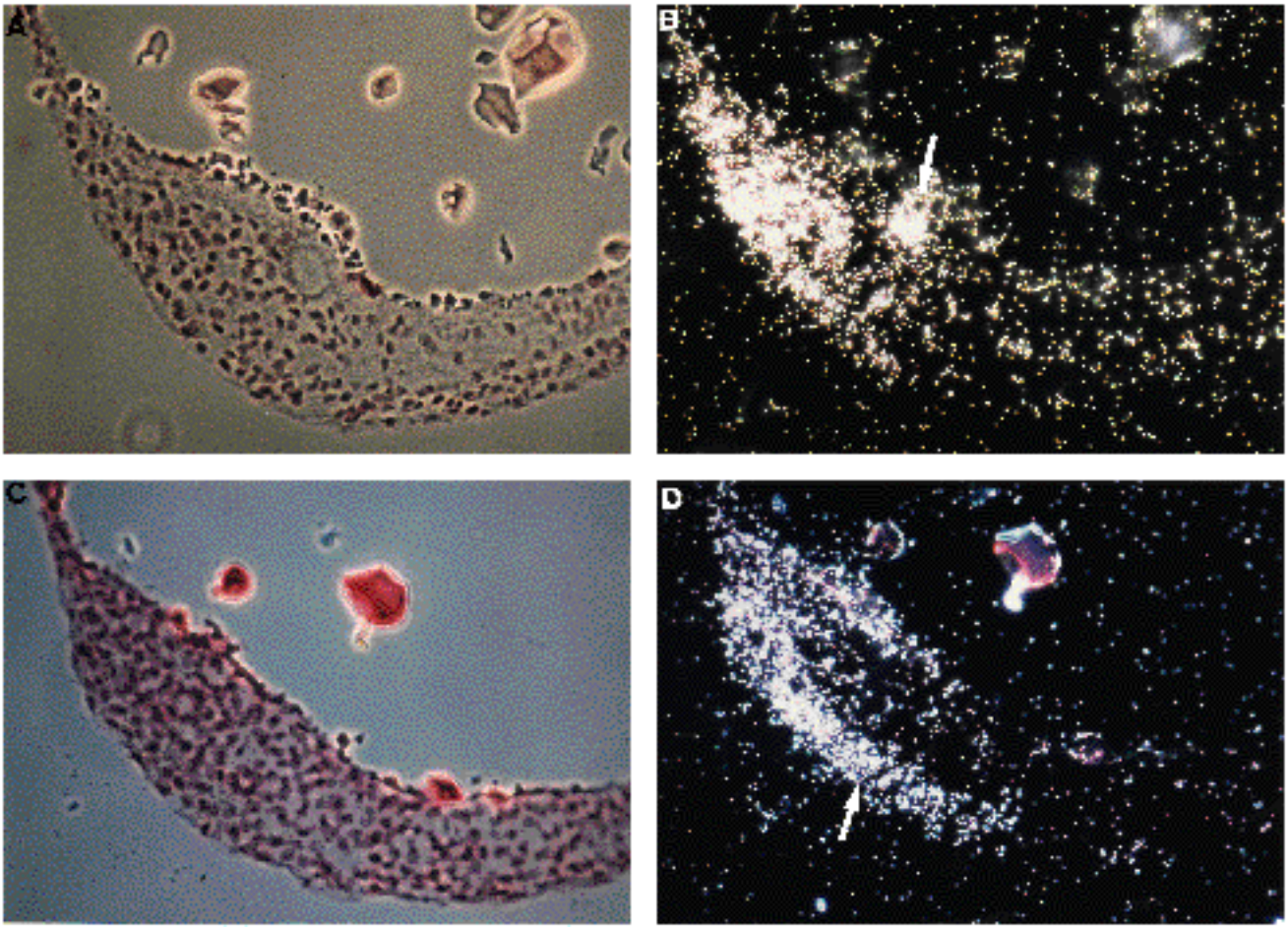

Fig. 5. Expression of *eve1* in 10-somite embryos (14 h). (A). Bright-field section of a sagittal section passing through the Kupffer's vesicle. Yolk has mostly disappeared during the manipulations. (B). Corresponding dark-field view. RNA are located at the tip of the tailbud on the left. A small mass of positive cells (arrow) are located behind the Kupffer's vesicle (which is negative). (C). Bright-field view of a neighbour section (one section thickness is intercalated between the section shown in A,B and this one). (D). Dark-field view of the region seen in (C). *eve1* signal forms an extension in an external cell layer (arrow).

abnormal epiboly movements and this seemed to be correlated with an enlargement of the YSL (refer to Fig. 6G, an abnormally large external YSL, as compared to control embryos (not shown) can be seen at the level of the arrow, beneath blastoderm cells). During the gastrulation of control embryos, presumptive notochord cells move towards the animal pole, while at the same time, they are undergoing convergent extension (Kimmel et al., 1990). Those cells can be detected using an antibody against the Ntl protein as a marker (Schulte-Merker et al., 1992 and Fig. 6E). This antibody recognizes nuclei of deep cells (DEL) (Schulte-Merker et al., 1992 and Fig. 6E), nuclei corresponding to EVL cells (Schulte-Merker et al., 1992) and to 'detached cells', a small group of gastrula cells that segregate from the dorsal margin of the blastoderm and move vegetally at the same level as the EVL (A. E. Melby and C. B. Kimmel, personal communication).

In LiCl-treated embryos, nuclei positive for the Ntl protein were observed all around the blastoderm margin (Fig. 6F,G). A circle of nuclei located at the level of the external YSL was also identified (see arrow in Fig. 6G). No staining was observed in cells closer to the animal pole. This suggests that in LiCl-treated zebrafish embryos, migration of notochord cells towards the animal pole has not begun at that time. In contrast, an extensive migration is observed in normal embryos. Whether this is due to mere retardation or to complete absence of migration in these LiCl-treated embryos is impossible to decide here. However, more anterior cells of the prechordal plate are known to involute and migrate towards the animal pole as detected using *goosecoid* RNA as a marker (Stachel et al., 1993; Schulte-Merker et al., unpublished data). Thus, our data are likely to reflect a delay in the migration of notochord precursors.

Treatment of zebrafish embryos at the 32-cell stage with LiCl dramatically repressed the expression of *eve1* mRNA (Fig. 6F). No cell positive for *eve1* transcripts was observed in any of the treated embryo. Kao and Elinson (1988) demonstrated that the enhanced dorsoanterior phenotype results from an overcommitment of the whole mesoderm of the *Xenopus* marginal zone to dorsoanterior mesoderm. The fact that zebrafish *goosecoid* expression is radialized by early lithium treatments (Stachel et al., 1993) supports the suggestion that such an event occurs after LiCl treatments of zebrafish embryos. Our data suggest these treatments are

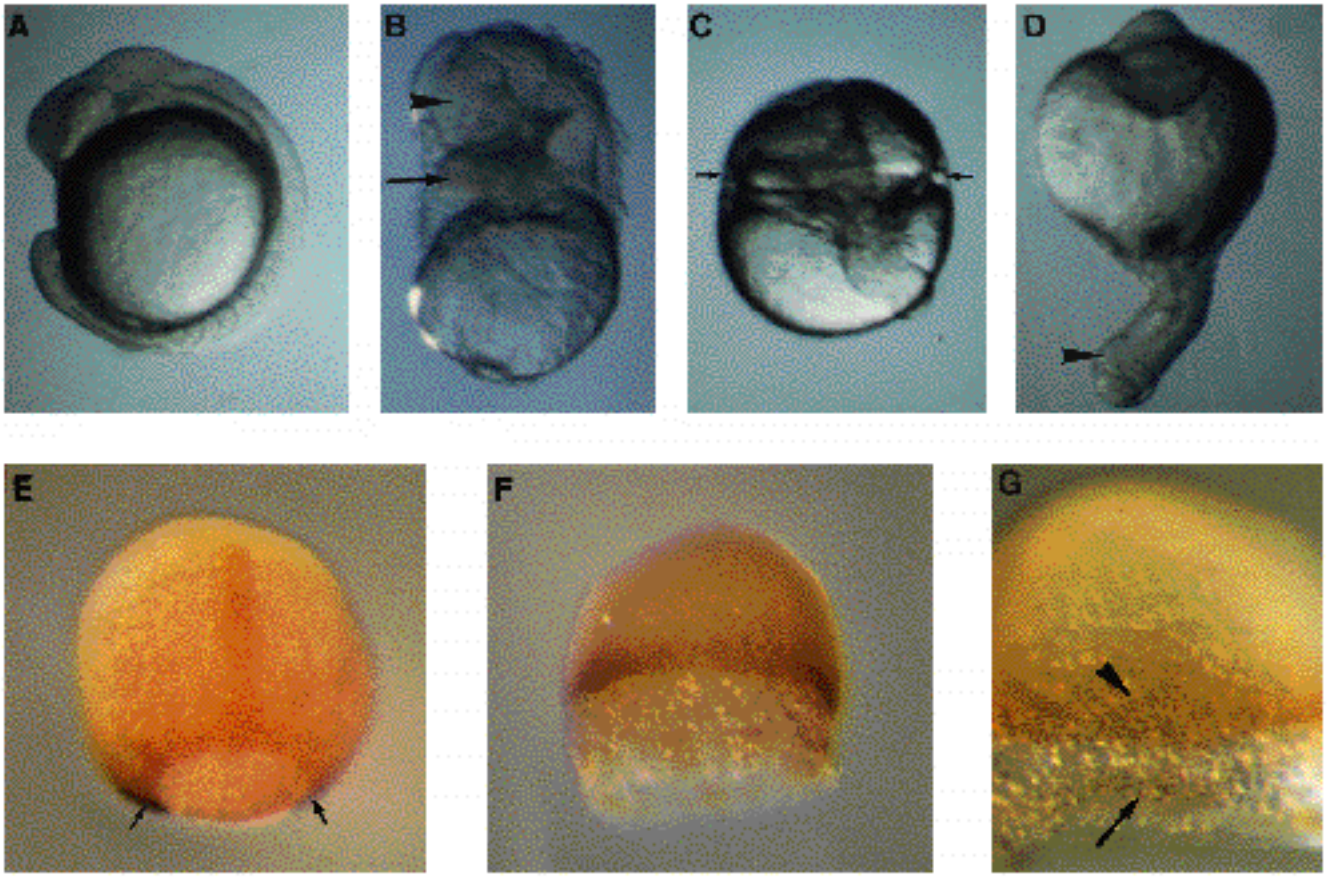

Fig. 6. Treatments of blastula stage embryos withLiCl. (A-D) Micrographs of living embryos at 16 h. (A). Side view of a control embryo at the 14-somite stage. (B-D) Phenotypes of embryos treated at the early blastula (32-cell) stage with 0.3 M LiCl for 30 minutes. (B) Embryo with structures located atop yolk, morphologically identified to brain (arrowhead) and heart (arrow). (C) Embryo with a radial constriction (arrows) possibly indicating heart development. A remnant of axis is visible. (D) Radial proboscis embryo with an external proboscis indicated by an arrowhead. (E-G) Immunodetection of the Ntl protein and whole-mount in situ using a *eve1* probe. (F,G). Embryos were treated as in B-D and fixed at 9 h. (E) The positive nuclei for Ntl are visible in notochord precursors and around the margin. The ventral *eve1* purple signal (arrows) is hardly visible in this embryo viewed from the dorsal side, animal pole at the top. (F,G) Embryo treated with LiCl. Detail in G. Animal pole up. Yolk was accidentally removed during staining experiments probably as a result of a greater fragility of the embryo. In treated embryos, the external YSL is larger than in untreated embryos. There is no staining for *eve1*. The Ntl-positive nuclei are located at the margin (arrowhead in G) and in detached cells (arrows in G).

also accompanied by the absence of ventroposterior *eve1* messengers, while the expression of the so-called 'pan-mesodermal' *T(ntl)* gene is maintained (Dawid, 1992).

Expression in *spadetail* (*spt*) mutants

The phenotype of the recessive lethal *spadetail (spt)* mutation can first be observed by late epiboly, when cell movements occur incorrectly (Kimmel et al., 1989). Prospective trunk mesodermal precursor cells, located along the lateral margin of the blastoderm, inappropriately enter the prospective tail region (Ho and Kane, 1990). After the completion of epiboly at 10 h, this abnormal 'addressing' leads to the accumulation of cells ventral to the location of yolk plug closure. Hence, in *spadetail* mutant embryos, there is an increase in the number of cells remaining during gastrulation in the domain of wild-type *eve1* expression. In this new developmental context, what is the distribution of *eve1* messengers?

We first examined gastrula stage *spt* embryos (from 60 to 90% epiboly) in order to detect early defects in *eve1* expression even before any *spt* phenotype can be detected under the dissecting microscope. We thus stained a high number of offspring of two heterozygous fishes by whole-mount in situ hybridizations (not shown). No heterogeneity in either the quantity or the regionalization of *eve1* messengers was observed, although about one fourth of the embryos were expected to be homozygous mutants.

The beginning of tail bud swelling in mutant embryos allows the recognition of the homozygous mutant phenotype and the staining of homozygous mutants and other embryos in two separate batches. The enlargement of the tail bud in the *spt* mutants at the 5-somite stage is accompanied by a global increase in the *eve1* signal (Fig. 7A). The same observation can be made on 22 h embryos (Fig. 7B), and the larger size of the tail bud allows more precise observations concerning the distribution of *eve1* transcripts: the axial domain of expression, which makes a turn at the tip of the tail bud, is greatly lengthened and cells of the whole caudal mass are positive (Fig. 7B).

These data support the conclusion that, during gastrula-

tion, cells that are incorrectly addressed to the tail bud respond by maintaining *eve1* gene expression at roughly the same level as tail bud cells normally located in wild-type embryos.

eve1 expression in the *no tail* (*ntl*) mutant and in comparison with Ntl protein distribution

Homozygous *ntl* mutant embryos lack a differentiated notochord and all posterior structures (Halpern et al., 1993). The *ntl* phenotype is due to a mutation in the homologue of mouse *Brachyury* (Schulte-Merker et al., 1992; Schulte-Merker et al., unpublished data). The study of the effects of this caudal mis-organization on the posterior expression of *eve1* was particularly interesting, because Cunliffe and Smith (1992) have demonstrated that *Xenopus Brachyury* (*Xbra*) RNA injections caused marked induction of *Xhox3* expression, a *Xenopus* gene with an *even-skipped* type homeobox. We report that *eve1* expression is disrupted in *ntl* homozygous mutants undergoing somitogenesis (see below) and that *ntl* and *eve1* expression domains overlap.

The careful comparison of both patterns of expression was done as a first step in the analysis of the interactions possibly occurring between these two genes. In zebrafish, the *ntl* gene is first expressed at the margin in late blastulae slightly before the *eve1* gene (Schulte-Merker et al., 1992). In late blastula (50% epiboly), both *ntl* and *eve1* genes are strongly expressed in wild-type embryos. We have detected no different *eve1* RNA/*ntl* protein abundance that would correlate with the positions of DEL cells near the surface (EVL) or close to the deeper layers (YSL) of the embryo (not shown). At the germ-ring stage (Fig. 8A), positive cells for the Ntl protein were detected among involuted cells while detectable *eve1* RNAs remained confined to epiblast. Such a distribution was confirmed by the examination of embryos at 60% epiboly (Fig. 8B); although, in ventrolateral regions of zebrafish gastrulae, the hypoblast is only one or two cells thick and is made of mesenchymal cells migrating to the dorsal side of the embryo, the positive *eve1* signal was clearly restricted to an outer cell layer identified as the epiblast, while the Ntl protein was also present in some of the inner cells, although at a lower level than in the epiblast (Fig. 8B). This is in accordance with Schulte-Merker et al. (1992) who reported that recently involuted cells of the ventrolateral region progressively turn off the production of the Ntl protein. In late gastrulae, thinness of ventral hypoblast makes an unambiguous detection of the restriction of *eve1* expression to the epiblast (90% epiboly, Fig. 8C) difficult, while in the vegetal region of the gastrula, the Ntl protein is definitely present in both epiblast and hypoblast at this stage.

Interestingly, we also noticed that, at all gastrula stages analysed at the histological level (ranging from 50% epiboly to 90% epiboly stages), the most animal positive cells for *eve1* RNA were negative for the Ntl protein with the types of detection used (Fig. 8A-C). Conversely, it was clear that the *ntl* gene is expressed in some dorsal and involuted cells where *eve1* RNAs are never found. During gastrulation, both domains of *eve1* and *ntl* expression thus overlap but only partially. This is emphasized during later development, as seen in 5-somite embryos, where posterior cells for *eve1* occupy a more posterior or epiblastic domain than positive Ntl nuclei (histological analysis not shown).

Whole-mount hybridizations were also performed using the progeny from a *ntl*/+×*ntl*/+ cross. Homozygous mutant embryos fail to give a signal when immunolocalization of the Ntl protein is done, which allows the determination of the genotype of embryos. At the shield stage, no segregation of the *eve1* staining was observed although the Ntl staining showed that the population of siblings was composed of heterozygous/wild-type embryos and homozygous mutants (not shown). At 80% epiboly, no heterogeneity in the *eve1* signal was detected in correlation with the absence or presence of the Ntl protein (not shown). At the 5-somite stage, however, *eve1* RNA stainings produced in homozygous *ntl* mutants were dramatically lower than in wild-type embryos: 5-somite stage mutants completely lacked *eve1* staining or, in a few cases, just possessed very few faintly stained cells at the position of the positive cell mass in the wild-type embryos (Fig. 7C). Studies on 24 h embryos also showed that *eve1* RNAs are completely absent from homozygous *ntl* mutants at this stage (Fig. 7D).

It thus appears that the disorganization of the posterior region seen in *ntl* homozygous mutant embryos is correlated with a marked decrease in *eve1* expression during somitogenesis.

DISCUSSION

Relationships among the *eve* genes

We describe here the isolation and characterization of *eve1*, a zebrafish gene whose homeobox is very similar to that of the *Drosophila even-skipped* gene (Fig. 2A).

The comparison between the patterns of expression of the zebrafish *eve1*, mouse *Evx-1* (Bastian and Gruss, 1990; Dush and Martin, 1992) or *Xenopus Xhox3* (Ruiz i Altaba and Melton, 1989) shows that these *eve* homeobox genes are expressed in the posterior region during early somitogenesis. It was proposed by Patel et al. (1992) from the results of expression studies of *eve*-type genes in mice, *Xenopus* and grasshopper that *even-skipped* might have been involved in the posterior regionalization of a common ancestor to vertebrates and arthropods. This newly isolated zebrafish *even-skipped* homeobox gene reinforces this hypothesis.

When the domains of expression are compared more carefully, many characteristics of *eve1* expression differ from the expression of other vertebrate *eve*-type genes. It appears that the zebrafish *eve* RNAs are restricted to ventrolateral regions of the gastrula and to the caudalmost region of the embryo undergoing somitogenesis. In contrast, the *Xhox3* protein is found on both dorsal and ventral sides of the *Xenopus* gastrula (Ruiz i Altaba et al., 1991) and the *Evx-1* RNAs are found in large territories of the posterior region including the mouse hindlimb buds (Dush and Martin, 1992). We have not detected any *eve1* secondary wave of expression in the central nervous system, in contrast with what was previously observed in mice and *Xenopus* (Bastian and Gruss, 1990; Ruiz i Altaba et al., 1991; Dush and Martin, 1992).

Given its divergent structure (see Results) and different

type of expression, it seems possible that *eve1* represents a distinct member of the vertebrate *eve* family. *eve1* could result from a particular evolution in teleost fishes linked with the peculiarities of gastrulation and tail bud ontogenesis, and/or from duplications already observed in other families of zebrafish homeobox genes (*engrailed* family, Ekker et al., 1992). Alternatively, it is conceivable that a gene with more identical structures and expression also exists in other vertebrates. While *eve1* isolation could have been favored in the zebrafish by the screening of a cDNA library of late gastrula stage embryos, when *eve1* expression is particularly strong (Fig. 3, lane 3), no such strategy was applied in the other vertebrates (Ruiz i Altaba and Melton, 1989; Bastian and Gruss, 1990; Dush and Martin, 1992). However, it will also be important to learn more about the numbers and characteristics of *even-skipped* genes in zebrafish.

eve1 expression in wild-type and *spadetail* (*spt*) zebrafish embryos is ventral and posterior

Interestingly, we observed an initial asymmetry in *eve1* expression along the prospective dorsoventral axis of the zebrafish late blastulae (Fig. 4A,B). To our knowledge, no morphological marker is available at this stage for the dorsoventral position. A short time later, when the shield becomes visible and acts as a marker of the dorsal side, *eve1* RNAs are located at the margin, with the exception of an arc of cells centered at the dorsal midline (Fig. 4C). Based on this expression pattern and on the results reported by Stachel et al. (1993) suggesting that dorsal cues are present in the zebrafish embryo before the onset of gastrulation, we propose that the territory of *eve1* expression is located at the ventral and lateral sides of the margin of zebrafish pregastrulae. This could be achieved in response to early inductive signals, some allowing a correct regionalization along the dorsoventral axis and others activating or restricting the expression to the margin. It was shown recently that *goosecoid* transcripts, which are first localized from the margin towards the animal pole, are secondarily restricted to the margin, possibly due to signals similar to the ones that trigger *eve1* expression (Stachel et al., 1993).

Morphogenetic movements in the zebrafish gastrula are globally directed and non-random (Ho, 1992). For instance, some epiblast cells of the ventral region, positive for *eve1* RNA, have the property to follow the direction of epiboly without converging to the dorsal side, thereby staying on the ventral side (Kimmel et al., 1990). Those cells, located ventrolaterally, may turn on the expression of *eve1* at the margin of late blastulae and then stay close to one another during gastrulation. It is noticeable that, during gastrulation, the width of the crescent of *eve1*-expressing cells increases (Fig. 4A-C) and this may be linked to the flattening and expansion of the blastoderm occurring during epiboly, partly driven by radial intercalations of deeper lying cells into more superficial positions within the epiblast (Warga and Kimmel, 1990; Ho, 1992). Alternatively, the extension of the staining pattern could be due to the recruitment of new cells into the *eve1*-expressing population.

However, a clear restriction in the distribution of *eve1* mRNA takes place during gastrulation, which is correlated with the dorsoventral position of a cell: the *eve1*-positive cells form an arc centered at the ventral midline and the angle covered by this arc progressively decreases during gastrulation (Fig. 4G,H). The fate-map established by Kimmel et al. (1990) indicates that lateral cells, located in the domain of *eve1* expression at the margin later give rise to trunk somites. *eve1* RNAs are clearly not found in these structures, thereby stressing the progressive restriction of *eve1* expression to the most ventral cells, which give rise to the most posterior cells. If it is assumed that similar mechanisms are at work in the dorsalization of the zebrafish mesoderm as has been shown in *Xenopus* (Lettice and Slack, 1993; Smith et al., 1993), *eve1* could be negatively regulated by dorsal factors, and the progressive restriction of *eve1* would mark the process of dorsalization. In order to assay whether *eve1* responds dynamically to modifications in dorsoventral patterning and more particularly to an ectopic regionalization of dorsal-like structures, the effect of lithium on the expression of *eve1* was analysed (see below).

Cell transfer experiments such as those performed by Schulte-Merker et al. (1992) could show whether *eve1* expression during gastrulation is triggered by a cell-autonomous mechanism or is the result of population effects. In this respect, the modified expression which is correlated with the *spadetail* mutation favors the hypothesis of a regulative *eve1* expression in the ventral region of gastrulae. After 12.5 h or 22 h, supernumerary cells of the tail bud express *eve1* suggesting that their incorrect addressing to the tail bud during gastrulation contributes to the maintenance of *eve1* expression.

Another restriction in *eve1* expression is correlated with involution movements: *eve1* expression is found in epiblast but not in hypoblast cells of early gastrulae. Although this contention is hard to document, particularly in ventrolateral regions of hypoblast, due to its thinness on sections, data obtained from sections of early gastrula hybridized with DIG probes show that *eve1* RNAs are present only in those cells of the margin that have not involuted (Fig. 8A,B). This gene thus constitutes an example of a negative regulation linked with the involution process. Such regulation was also reported for *Zf-cad1* (Joly et al., 1992) and *ntl* (Schulte-Merker et al., 1992), although for this latter gene the decrease in RNA and protein amounts is more progressive and is only observed in ventrolateral cells of the gastrula. The mechanism of this down-regulation is unknown at present, but it may be relevant that a study on another teleost (*Barbus conchonius*) has shown the existence of a dye-coupling between hypoblast and YSL (Gevers and Timmermans, 1991), suggesting that regulatory signals may be exchanged between the two tissues. These expression studies show that cellular involution is accompanied by major genetic switches, which may in turn participate in the commitment of marginal cells to mesoderm, a process that is clearly not achieved before gastrulation (Ho, 1992).

Finally, the expression of *eve1* during gastrulation appears to be dynamic. If all marginal cells are thought to involute during gastrulation, this would imply that involuting cells continually leave the *eve1*-expressing population and new cells would then be recruited into the population. Alternatively, the territory of expression at the onset of gastrulation could also be heterogenous, with positive and negative cells coexisting, thus accounting for the

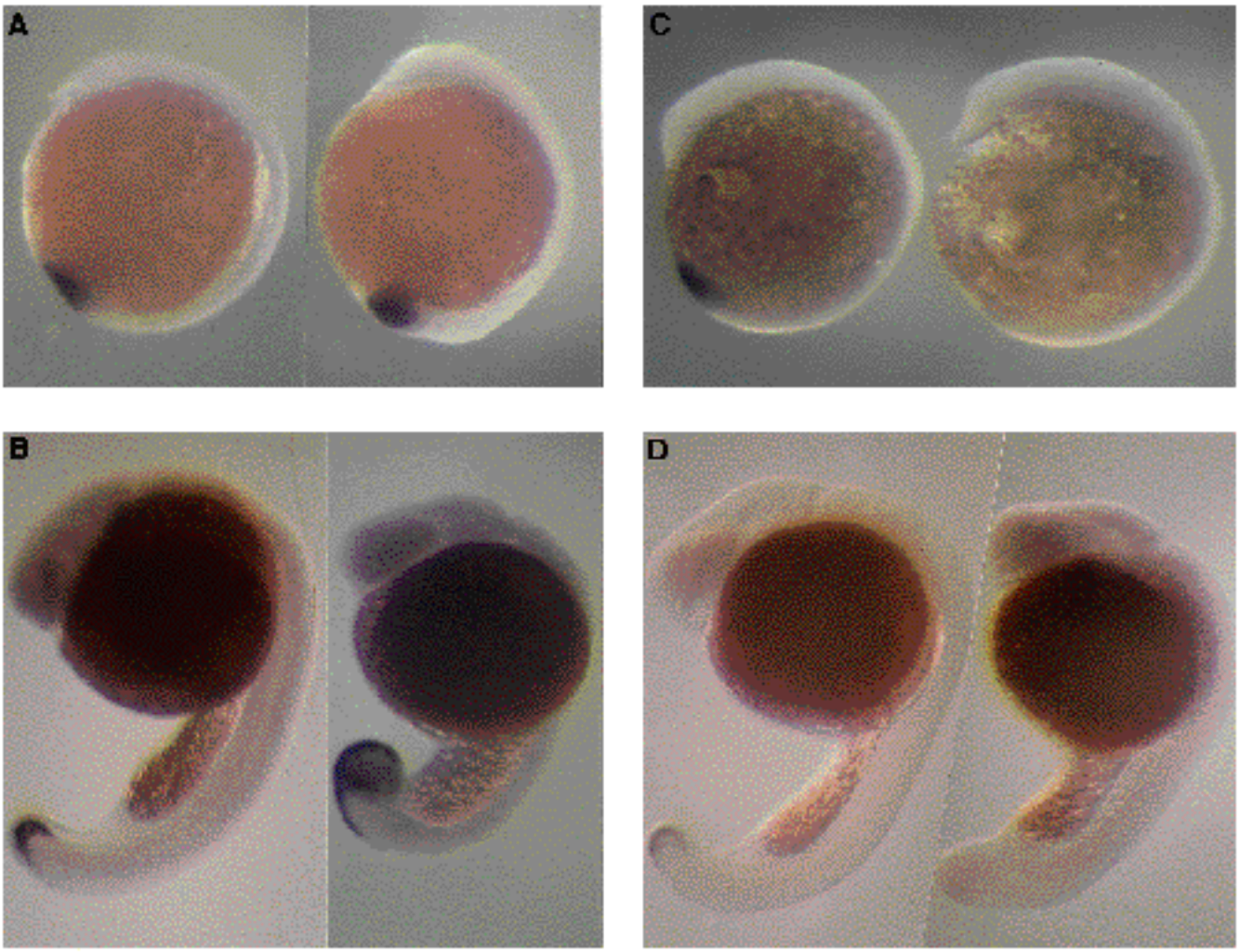

Fig. 7. In situ hybridizations of *eve1* in wild-type and mutant embryos (whole mounts). Wild types are on the left of each picture and homozygous mutants on the right. Anterior poles at the top. Dorsal sides on the right. (A,B) *spadetail* mutation. (A) 12.5 h embryos (5-somite stage for the wild type). Beginning of abnormal tail bud swelling can be seen at the bottom of the embryo on the right. (B) 22 h embryo. In *spt* homozygous mutants, *eve1* staining covers the whole swollen tail bud and anterior extension is longer than in wild-types. (C,D) *no tail* mutation. (C) 5-somite stage (12.5 h). No signal is observed in the mutant embryo. (D) 24 h embryos. Signal begins to fade in the wild-type embryo but is completely absent in the homozygous mutant.

ectoderm/mesoderm overlap of the fate map (Kimmel et al., 1990). It would then be possible that cells that initially express *eve1* maintain expression and fail to involute while those that do not express *eve1* involute. This situation would be reminiscent of the formation of mesoderm in the chick embryo: a similar heterogeneity has been demonstrated (using different markers) in the blastodisc prior to the formation of the primitive streak (Stern and Canning, 1990).

However, it appears that the down-regulation of *eve 1* in marginal cells, their inwheeling and the beginning of their migratory behaviour are intricate phenomena. One stimulating hypothesis would then be that, in *spt* mutants, more marginal cells than in normal embryos might express *eve1* in correlation with their failure to involute, to converge to the dorsal side, or to establish contacts with the YSL. It is too early to assess whether *eve 1* just marks these changes or plays an active role but it is interesting to note in this context that the *Xenopus goosecoid* gene is able to induce a migratory behaviour to the dorsal cells (Niehrs et al., 1993).

eve1 is repressed in dorsalized embryos treated with lithium

Our morphological examination of zebrafish embryos after LiCl treatments suggests that lithium induces exaggerated dorsoanterior structures, as documented in *Xenopus* or zebrafish (Kao and Elinson, 1988; Stachel et al., 1993).

Data concerning *ntl* expression further reinforces the similarity of LiCl effects in zebrafish and *Xenopus*. In untreated gastrulae, the anti-Ntl serum gives a staining in the notochordal precursor cells, which undergo intense convergent extension (Schulte-Merker et al., 1992 and Fig. 6E). In LiCl-treated embryos, fixed when the control embryos are at the late gastrula stage, Ntl-positive cells are confined to the margin, i.e. no Ntl-positive cell is detected near the animal pole (Fig. 6F). This suggests that in zebrafish, convergent extension movements are perturbed. In *Xenopus*, Kao and Elinson (1988) also observed that a massive internal or external extension of the circle of mesoderm mantle surrounding the blastopore begins at the end of gastrulation in

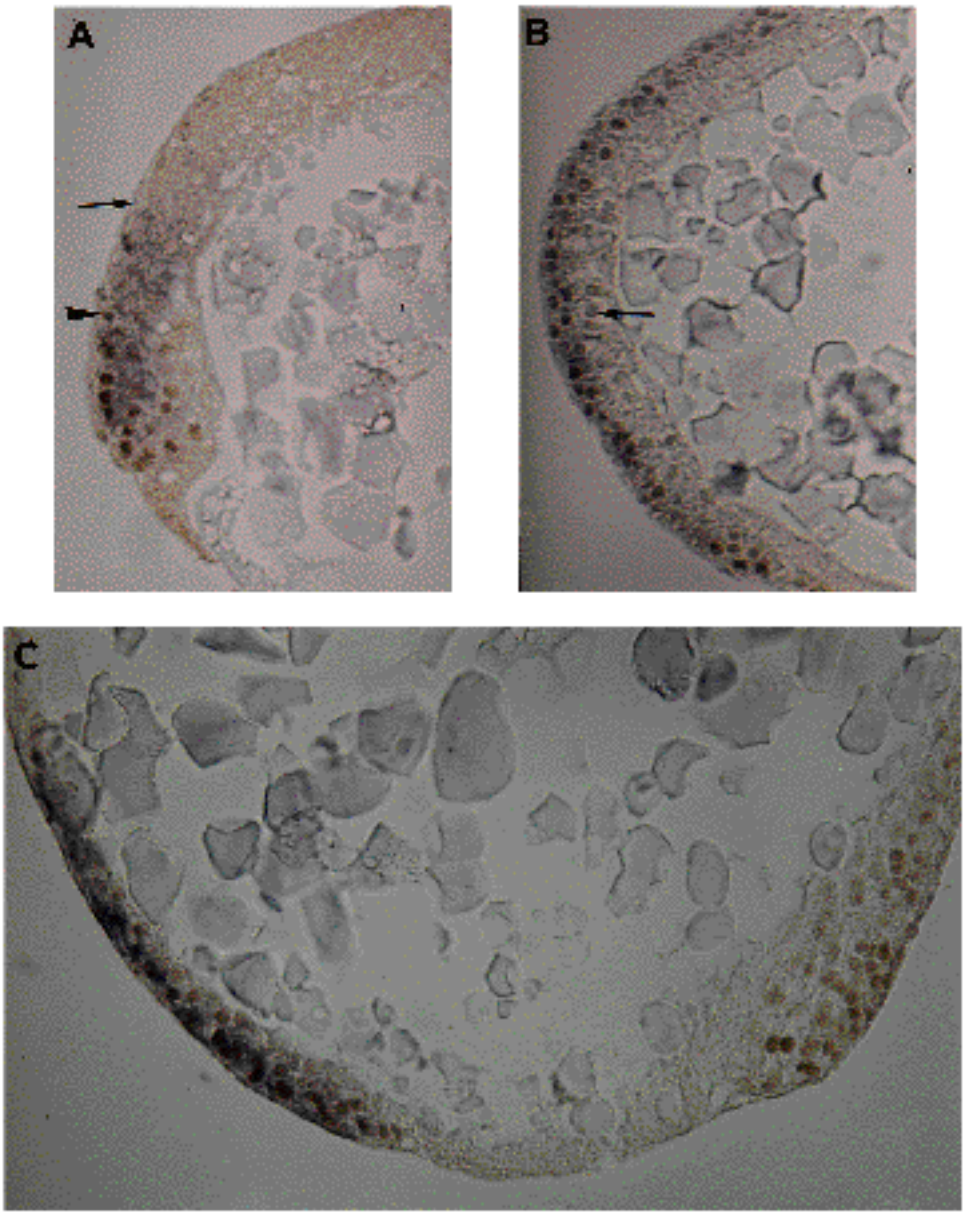

Fig. 8. Sections of embryos stained for *eve1* and Ntl. The *eve1* staining appears as a dark purple cellular coloration after whole-mount in situ hybridizations using the chromogenic alkaline phosphatase reaction, while antibody stainings for Ntl give a brown color in nuclei subsequent to a peroxidase detection. (A) Sagittal section through a germ-ring stage embryo. The extents of *eve1* or Ntl staining are indicated by an arrow and an arrowhead, respectively. (B) Ventral region of a shield stage embryo. Equatorial section. Epiblast is positive for *eve1* and Ntl. In hypoblast, there is no *eve1* staining. One nucleus, faintly stained for Ntl, is indicated by an arrow. (C) Sagittal section through the vegetal region of a 90% epiboly embryo. Ntl staining is on both sides of yolk plug, *eve1* staining is ventral, on the left side.

treated embryos. This abnormal movement is delayed when compared to the normal dorsalwards convergent extension movement, which starts earlier in untreated *Xenopus* gastrulae (Kao and Elinson, 1988).

Finally, in lithium-treated embryos, the *ntl* protein, which is present in mesoderm, is indeed maintained in a circle of marginal cells, suggesting that these cells retain a mesodermal characteristic (Fig. 6F,G). In contrast, the putative respecification of the marginal mesoderm leads to a strong repression of *eve1* expression suggesting that *eve1* RNA distribution reproduces the absence of ventrolateral mesoderm in LiCl-treated embryo (Fig. 6F,G). These data are in accordance with those described in Stachel et al. (1993) who suggested that radial expression of the *goosecoid* gene around the margin of lithium-treated zebrafish embryo is linked with the respecification of the ventral marginal zone to a more dorsal fate.

ntl participates to the maintenance of *eve1* expression in the tail bud

The expression of *eve1* was also studied in *no tail* homozygous mutant embryos. A major observation was that, during gastrulation, *eve1* expression is not disrupted in homozygous mutant embryos that completely lack functional Ntl protein. This suggests that the onset of *eve1* expression is independent of a molecular pathway involving *ntl*. This is also in agreement with the fact that *ntl* is not the only gene necessary for the specification of the mesoderm, as some somitic mesoderm forms in the absence of the mouse *T* protein (Chesley, 1935).

It has also been suggested that mesoderm formation in mice is biphasic (Yanagisawa et al., 1981; Hermann, 1991). A first phase does not require the product of the *T* gene, while a second phase during which posterior mesoderm forms depends critically on a normal activity of *T*. In this

context, it is striking that a dramatic decrease in *eve1* transcripts is observed in *ntl* mutant embryos at the 5-somite stage (Fig. 7C). The *ntl* gene thus appears to regulate *eve1* during tail bud morphogenesis. Our results, together with those reported by Cunliffe and Smith (1992) concerning the interaction between *Xenopus Brachyury* and *Xhox3*, support the conclusion that *Brachyury* genes have an evolutionarily conserved function in the organization of the posterior region, possibly mediated by the regulation of other transcription factors of the *even-skipped* family.

Finally, our results bear upon the relation between the distribution of the Ntl protein and *eve1* RNA. At all stages studied, some *eve1* transcripts are found in epiblast or tail cells where the Ntl protein is absent (Fig. 8A-C). This is consistent with the fact that, during gastrulation, early *eve1* expression does not depend on a molecular pathway involving a product of the *ntl* gene. Although in normal embryos, *eve1* expression can be detected in cells that do not express *ntl*, *eve1* expression in those cells is down-regulated when *ntl* is mutated. This raises the possibility that the regulation of *eve1* by *ntl* in the tail bud of the zebrafish embryo may be indirect. Whether, in zebrafish, the *ntl* gene acts autonomously in the morphogenesis of ventroposterior mesoderm is unknown at present. Both the *ntl* and the *T* mutations autonomously cause failures in the differentiation of zebrafish or mouse notochord precusors (Halpern et al., 1993; Rashbass et al., 1991). However, *ntl* regulatory pathway might very well be different in this respect in the tail bud. *eve1* regulators or targets have now to be determined more precisely in order to assign a role to this gene in the complex hierarchy of spatial determinants involved in early vertebrate development. Moreover, *eve1* appears as a new tool to study the mechanisms involved in ventral patterning of mesoderm and tail bud morphogenesis.

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The sequence reported in this manuscript has been registered within the EMBL Database Library under the following accession number: X71845.

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