Int. J. Dev. Biol. 38: 613-622 (1994)

Original Article

Widespread expression of the *Xenopus* homeobox gene *Xhox3* in zebrafish eggs causes a disruption of the anterior-posterior axis

OUSMANE BARRO[1]*, CLAIRE JOLY[1], HUBERT CONDAMINE[2] and HABIB BOULEKBACHE[1]*

[1]*Laboratoire de Biologie du Développement, Université Paris 7, Paris and*
[2]*Unité de Génétique des Mammifères, Institut Pasteur, Paris, France*

ABSTRACT **The widespread expression of the *Xenopus* homeobox gene *Xhox3* in zebrafish was performed by microinjection of synthetic Xhox3 mRNA into uncleaved fertilized eggs and resulted in embryos displaying varying degrees of anterior-posterior (A-P) axis disruption. The phenotype observed was dose-dependent and showed anomalies, mainly in neural keel development, from microphthalmia to acephaly. Injection of 5 pg to 10 pg of mRNA caused a range of phenotypes in prim 5 stage embryos from normal to acephalic. Anomalies have been categorized according to an index of axis deficiency (Zf-IAD). We further characterized the axis disturbance by analyzing the expression of two genes implicated in axis formation: engrailed *(eng)* and brachyury *(ntl)*. *eng* could not be detected in the muscle pioneer cells of embryos injected with Xhox3. The pattern distribution of brachyury (Ntl) protein is abnormal in Xhox3 injected embryos. Evidence for the conservation of the NH2 terminal region of the Xhox3 protein in frogs and fish is provided by the detection of a nuclear Xhox3-like protein in 24 h zebrafish embryos located in posterior mesoderm tissue. Previous results in *Xenopus* embryo research and the data presented here support the conservation of an A-P patterning mechanism involving the *Xhox3* gene.**

KEY WORDS: *homeobox, even-skipped, Eng, no tail (ntl), pattern formation, anterior-posterior axis, mesoderm, gastrulation, zebrafish embryo*

Introduction

Many genes involved in the control of embryonic development have been isolated in the last few years, mainly in *Drosophila*, and among them, homeobox genes (McGinnis *et al.*, 1984; Scott and Weiner, 1984). This hierarchy of zygotic genes is involved in segment polarity and identity (for recent reviews see Bienz, 1994; McGinnis and Kuziora, 1994). Several groups have identified vertebrate genes homologous to *Drosophila* homeobox genes in humans, mice, chicken, frogs and zebrafish.

However, the mechanisms of their regulation and roles during early embryogenesis are still not clear (Gehring, *et al.*, 1990; Budd and Jackson, 1991). One of several approaches used to discern gene function is ectopic expression. Ectopic expression involving Hox genes was performed in mice, frogs and flies. *Drosophila*, which express transgenes ectopically under the control of an inducible promoter, show homeotic transformations. The general expression of a mouse homeobox gene, *Hox2.2 (HoxB6)* of the antennapedia family, induced the antennapedia phenotype (Malicki *et al.*, 1990). This result is striking since there are only three conserved regions between *antennapedia* and *Hox 2.2*. These regions consist of the homeobox, a hexapeptide upstream of the homeobox and an amino-terminal region. Thus, these 3 regions appear to be sufficient to allow the Hox 2.2 protein to functionally replace the Antennapedia protein.

The *Xhox3* gene is a member of the vertebrate even-skipped *(eve)* related homeobox gene family. *Eve* is a pair-rule gene. At present, *eve* class homeobox genes have been identified in *Xenopus* (*Xhox3*; Ruiz i Altaba and Melton, 1989a), mouse (*Evx1* and *Evx2*; Bastian and Gruss, 1990; Dush and Martin,1992), human (*EVX2*; D'Esposito *et al.*, 1991 and *EVX1*; Faiella *et al.*, 1991), *Cnidarian Acropora formosa* (*eveC*; Miles and Miller, 1992) and recently in zebrafish (*eve1*; Joly *et al.*, 1993 and *eve2*, Duboule, personal communication).

The *Xhox3* gene has 100% homology in the homeobox with the mouse *Evx1* gene. Moreover, the amino-terminal part of Xhox3 and Evx1 protein and all of the carboxy-terminal region are similar. In addition, regionalization of *Xhox3* expression in the early embryo is similar to that of *Evx1* which specifies the posterior region of the embryo shortly after gastrulation. Widespread expression of Xhox3

Abbreviations used in this paper: MAb, Mouse Antibody; IAD, Index of Axial Deficiency; zf, zebrafish; FIV, *In Vitro* Fertilization; CNS, Central Nervous System; HTH, Helix Turn Helix; A-P, Anterior-Posterior.

***Address for reprints:** Laboratoire de Biologie du Développement, U.F. Biologie de l'humain case 7077, Université Paris 7, 2 Place Jussieu, 75251 Paris cedex 05, France. Fax: 33.1.44275653.

0214-6282/94/$03.00

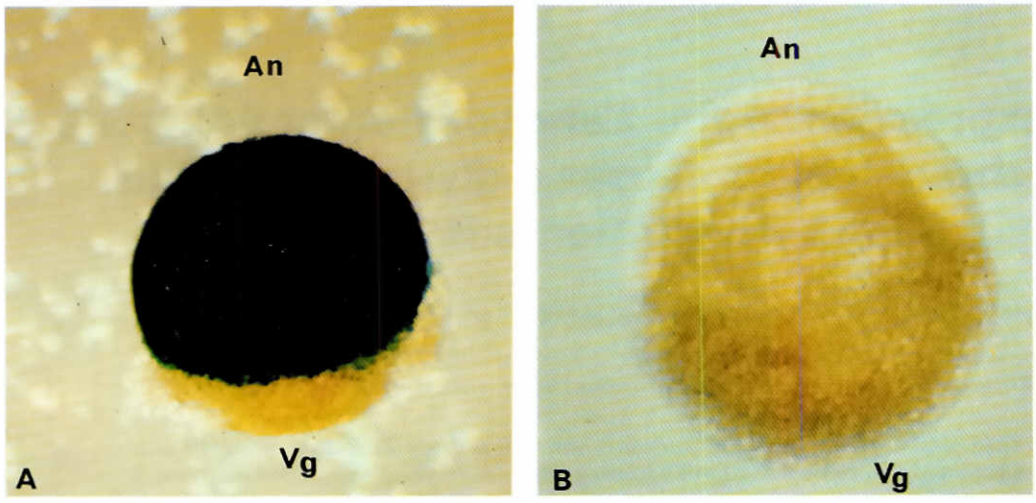
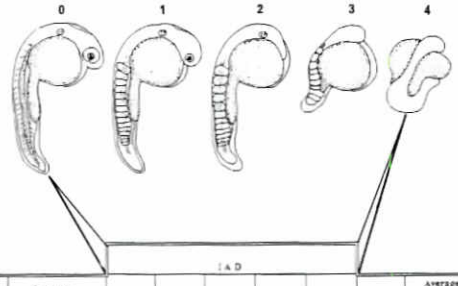


Fig. 1. Whole-mount staining of ß-galactosidase activity. (A) *Embryo injected with 50 pg of LacZ mRNA. The activity was detected in the embryonic cellular cap 6 h after injection at the 50% epiboly stage.* **(B)** *Uninjected embryo. This negative control shows no endogenous ß-galactosidase activity. Side view: vegetal pole is at the bottom. An, Animal pole; Vg, Vegetal pole. Note that the LacZ injected embryos develop normally.*

TABLE 1

CATEGORIZATION OF 24 h ZEBRAFISH EMBRYO (prim5) PHENOTYPES



	Survivors (%)	0	1	2	3	4	Tail⁻	Average I A D
Controls Non injected embryos	226 (96.6)	214	1	4	0	3	4	0.1
Lac Z mRNA (50 pg)	48 (91)	46	0	1	0	0	1	0.04
XHOX3 BEΔ mRNA (12 pg)	208 (91.6)	191	3	1	0	9	7	0.19
XHOX3 mRNA (6 pg)	150 (94.3)	97	15	23	5	4	3	0.62
XHOX3 mRNA (9 pg)	94 (81)	9	7	38	30	5	5	2.17
XHOX3 mRNA (12 pg)	217 (92)	31	19	59	49	37	2	2.28

The lines correspond to different messengers and/or amounts of injected mRNA. The mRNA amounts are placed inside brackets under the name of the injected mRNA. The columns represent the different phenotypes classified according to the IAD: grade 0, normal embryos in all externally visible respects; grade 1, embryos with reduced forehead; eyes smaller than normal (microphthalmic or hemimicrophthalmic) which are sometimes joined or fused; grade 2, embryos with no eyes, otic vesicles still visible; somites broader than normal, and slight trunk reductions; grade 3, embryos with no otic vesicle(s) present; somites present in trunk, broader than normal; severe trunk reduction; grade 4, embryos with anterior-posterior axis very disrupted, no somites present; Tail⁻, embryos show a slight bend in the axis on the tail or they have a severely reduced trunk, ranging from a truncated tail to a small vestigial axis closely attached to the head. Somites are visible (short axis). The average IAD was calculated according to Scharf and Gerhart (1983) by dividing the sum of the products of each grade and the respective grade number by the total number of embryos for each experiment.

in anterior regions, obtained by microinjection of Xhox3 mRNA in frog embryos (Ruiz i Altaba and Melton, 1989b, 1990), results in a variety of cephalic anomalies. It has been suggested that the *Xhox3* gene may be involved in establishing A-P cell identities during pattern formation in the axial mesoderm (Ruiz i Altaba and Melton, 1989b).

In recent years, the zebrafish embryo has emerged as a system for studying vertebrate development because it provides an excellent opportunity for genetic analysis (Streisinger *et al.*, 1981; Kimmel, 1989; Hatta *et al.*, 1991b; Driever *et al.*, 1994), cell lineage analysis (Kimmel and Warga, 1987; Strehlow and Gilbert, 1993), and *in vivo* observation (phenotypic analysis) due to its transparency. This is especially important during gastrulation (Warga and Kimmel, 1990), a key stage during which the A-P axis is specified. To determine the existence and potential role in A-P patterning of the Xhox3-like protein in zebrafish, we ectopically expressed frog *Xhox3* in zebrafish embryos.

We perfected a new and faster method to remove the chorion of the zebrafish embryo which allows the microinjection at the one cell stage. Injection of the LacZ messenger gene into zebrafish embryos exhibited the stability, efficient translation, and widespread distribution of injected mRNA during development. Injection of

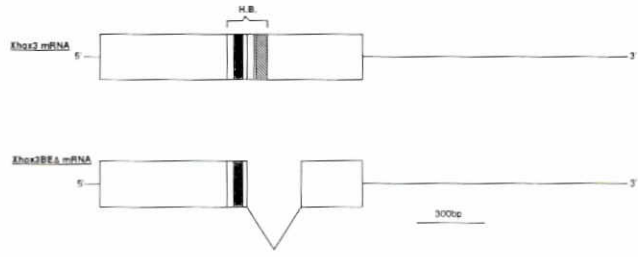


Fig. 2. Representation of pcXhox3 and pcXhox3 BEΔ transcripts. *Two mRNAs (Xhox3 and Xhox3 BEΔ) were injected and result respectively in developmental malformation and normal development; for these two transcripts, protein-coding regions are indicated by open boxes and the non-coding regions by lines. The size-bar corresponds to 300 bp. The black, shaded or diagonally striped boxes in the homeobox region (H.B.) correspond respectively to helix I or II or III and IV. The Xhox3 BEΔ mRNA is aligned with the top Xhox3 mRNA and the deleted nucleotidic region is schematized by the lack of an open box part.*

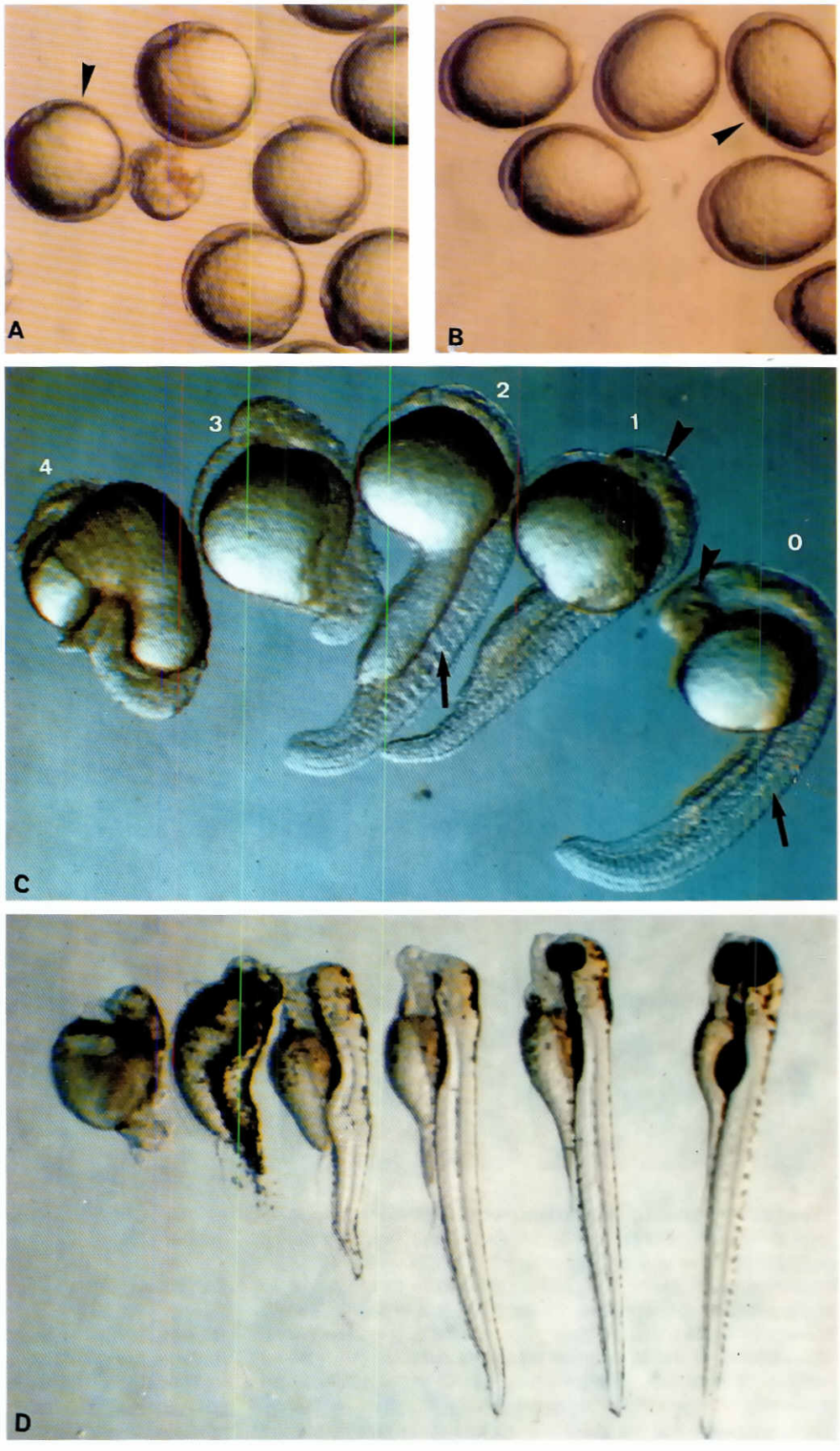


Fig. 3. Phenotype and dose dependence of anterior-posterior axis disruption in Xhox3 mRNA injected embryos. **(A)** *Xhox3 mRNA injected embryos, 9 h after FIV, with an abnormal spherical form with reference to* **(B)** *the ovoid form control uninjected embryos at the same stage as (A), the bud stage. The arrowheads show embryos in* **(A and B)** *that have the same orientation, so they can be easily compared.* **(C and D)** *A series of embryos with a range of axial defects. Embryos were injected with 9 pg of mRNA.* **(C)** *24 h embryos classified according to the Index. The number at the top of each embryo corresponds to the grade of IAD. From right to left: IAD grade 0, control embryo, normal in all externally visible respects. Eyes (arrowhead) chevron-shaped myotomes (arrow). Grade 1, embryo with reduced forehead, eyes smaller (arrowhead) than normal (microphthalmic or hemimicrophthalmic) which are sometimes joined or fused (cyclopic). Grade 2, embryo with no eyes, otic vesicles still visible, somites broader (arrow) than normal, and slight trunk reductions. Grade 3, embryo with no otic vesicle(s) present, somites present in trunk, broader than normal, and severe trunk reduction. Grade 4, embryo with very disrupted anterior-posterior axis, and no somites present. Side views. Anterior at the top and dorsal at the right with the exception of IAD grade 4, dorsal view.* **(D)** *4 day embryos. Some embryos show microcephalia and anophthalmia, the others are more severely affected, exhibiting acephalia and severe trunk reductions associated with reduced neural development.*

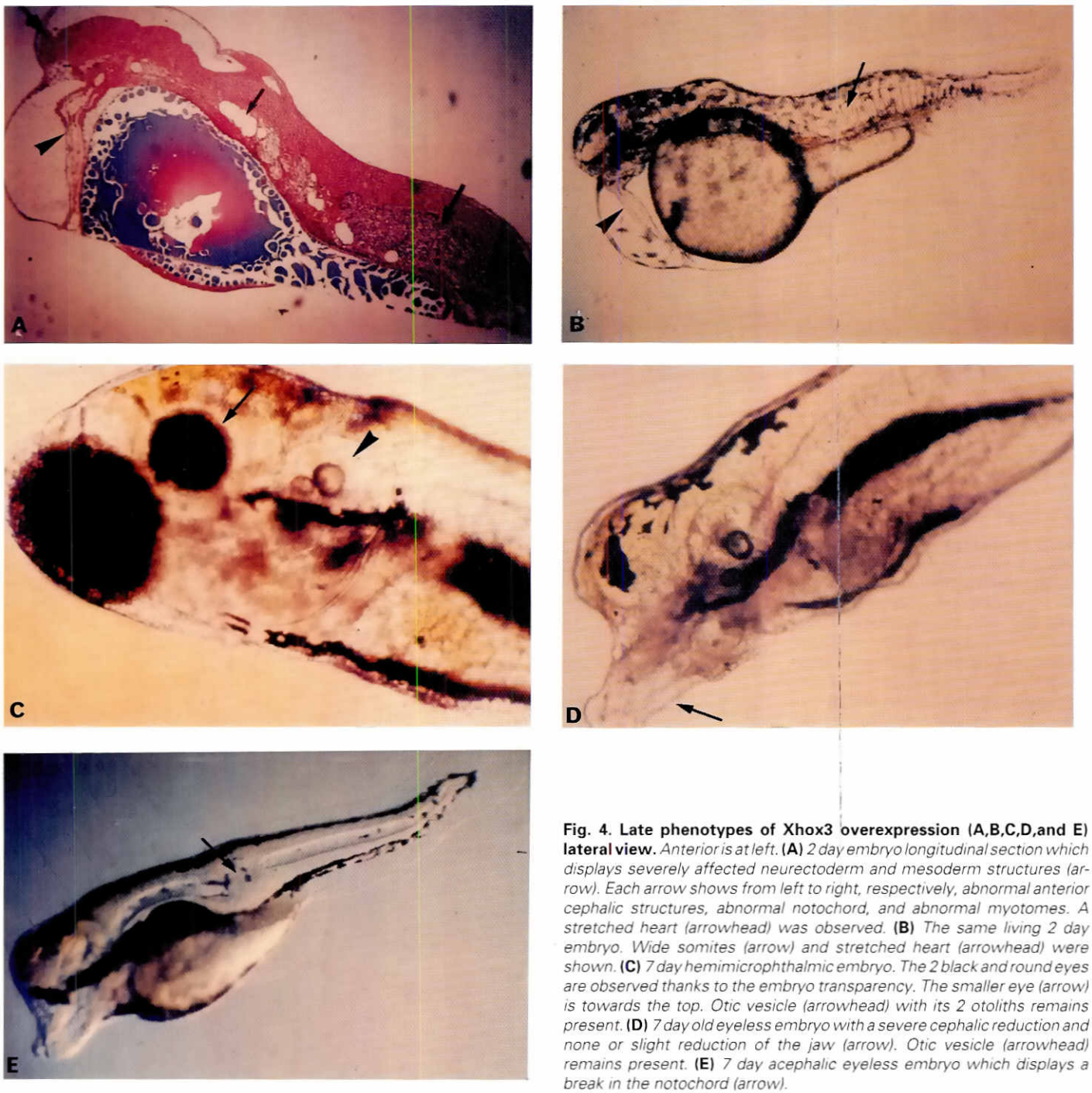


Fig. 4. Late phenotypes of Xhox3 overexpression (A,B,C,D,and E) lateral view. *Anterior is at left.* **(A)** *2 day embryo longitudinal section which displays severely affected neurectoderm and mesoderm structures (arrow). Each arrow shows from left to right, respectively, abnormal anterior cephalic structures, abnormal notochord, and abnormal myotomes. A stretched heart (arrowhead) was observed.* **(B)** *The same living 2 day embryo. Wide somites (arrow) and stretched heart (arrowhead) were shown.* **(C)** *7 day hemimicrophthalmic embryo. The 2 black and round eyes are observed thanks to the embryo transparency. The smaller eye (arrow) is towards the top. Otic vesicle (arrowhead) with its 2 otoliths remains present.* **(D)** *7 day old eyeless embryo with a severe cephalic reduction and none or slight reduction of the jaw (arrow). Otic vesicle (arrowhead) remains present.* **(E)** *7 day acephalic eyeless embryo which displays a break in the notochord (arrow).*

Xhox3 mRNA caused graded axial defects, scored with an index of axial deficiency (zf-IAD) and anomalies in cell migration during gastrulation. The use of Eng antibodies (Patel *et al.*, 1989) as anterior markers (Hatta *et al.*, 1991a) allowed for the classification of the anterior abnormalities obtained. We found that in Xhox3-injected embryos there is a down-regulation of Eng in the somites. The majority of the Xhox3-like protein is detected in posterior somites of zebrafish. The similarity of our results with those previously obtained in frog embryos (Ruiz i Altaba *et al.*, 1989b, 1991) suggests that the molecular mechanisms involved in the regulation of A-P axis formation is conserved in different vertebrates.

Results

Distribution of ß-galactosidase activity in LacZ mRNA injected embryos

The injection of 40-50 pg of LacZ mRNA into uncleaved embryos resulted in a homogeneous distribution of the ß-gal activity at different stages of development. This uniform distribution in-

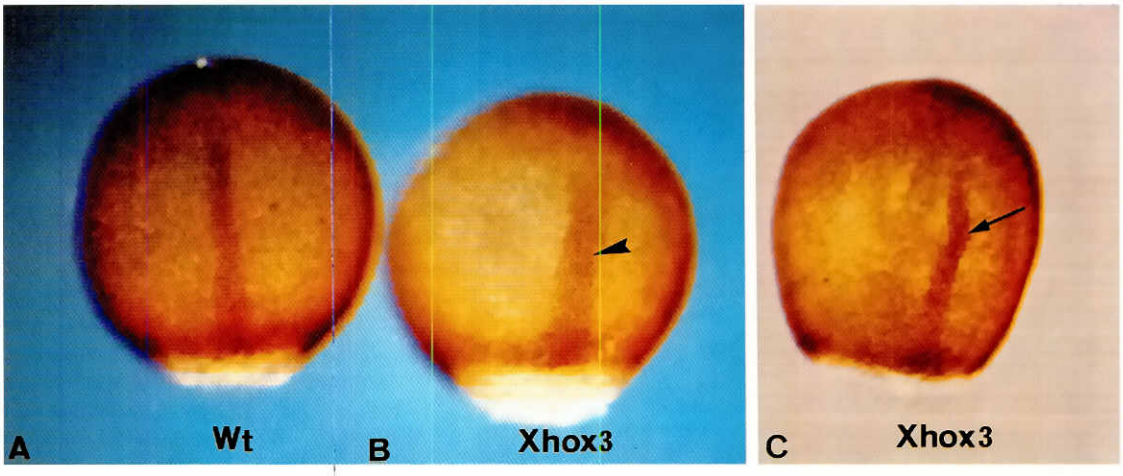


Fig. 5. Whole-mount antibody stainings in 90% epiboly stage embryos using anti-Zf-T antiserum. (A, B and C) *Dorsal views. Anterior is towards the top. At the left control embryo* **(A)** *and at right Xhox3 injected phenotypes* **(B and C)**. *The nuclear antigen is restricted to a few cells at the margin and presumptive notochord cells.* **(B)** *Wide and short band (arrowhead) staining in Xhox3 injected phenotype* **(C)** *undulating band (arrow) in Xhox3 injected phenotype in reference to control embryos.*

cludes the enveloping layer, the deep cells, and the yolk syncytial layer: expression is homogeneous up to 50% epiboly in embryos injected at the one cell stage (Fig. 1A). In the negative control, no ß-galactosidase activity was observed at the same stage (Fig. 1B). This result is similar to those recently obtained by Takeda *et al.* (1994) and suggests that exogenous mRNA are efficiently translated in the early zebrafish embryo.

Heterogeneity of the ß-gal activity was not apparent in gastrula stage embryos but became visible in 24 h embryos following the degradation of ß-gal proteins and/or mRNA. ß-gal activity was found in the yolk cytoplasm and in endoderm cells at the posterior intestine (data not shown). These results tend to favor this approach that produces a homogeneous ectopic distribution of either exogenous or endogenous proteins which play an important role in gastrulation.

96% of the embryos injected with 50 pg of ß-gal mRNA show a normal phenotype. The rest show diverse phenotypes including caudal malformations (Table 1). This percentage of abnormal embryos is comparable to that seen in uninjected embryos, indicating the weak effects of the mechanical action involved in the injection process on embryonic phenotypes. For the most part, these abnormalities could be the result either of the dechorionation which takes place at the time the cytoplasm streams toward the animal pole or of movement of the embryos in Pasteur pipettes.

Xhox3 injection phenotypes

Phenotypic defects

Xhox3 mRNA with an even-skipped-like homeobox (Fig. 2) was injected into one cell zebrafish embryos. The resulting abnormalities were classified according to the index of axial deficiency (IAD) (Table 1). The IAD is adapted from the one proposed by Scharf and Gerhart (1983) in *Xenopus*.

The different phenotypes obtained have been indexed from 0 to 4. IAD grade 0 corresponds to the wild type embryo while IAD grade 4 represents the phenotype with the most disturbed A-P axis. Between these two extremes, intermediate phenotypes are observed (Fig. 3C). In later stages, other morphological criteria can be added to discriminate between the various intermediate embryonic phenotypes, thus broadening the index range, making gradual disruptions more visible (various sizes of eyes or lengths of body axis) (Fig. 3D).

The comparative study of embryonic phenotypes for each of the experiments is greatly facilitated by analyzing the average IAD which reflects the average phenotype observed.

The embryos obtained following injection of 9 pg of Xhox3 mRNA (Table1) displayed no abnormality during the first stages of development. The development of these embryos and the controls proceeded in exactly the same manner. Controls correspond to embryos injected with Xhox3 mRNA from which the helix turn helix (HTH) motifs in the homeobox domain have been deleted while conserving the original open reading frame (Xhox3 BEΔ mRNA) (Fig. 2). At the end of gastrulation (bud stage: 9 h) 70% of the Xhox3 mRNA injected embryos had a spherical shape, (Fig. 3A) whereas the control embryos were oval (Fig. 3B) due to the convergence of cell movements involved in the embryonic axis formation. This was the first morphological abnormality observed among the gain of function Xhox3 phenotypes. For the 24 h test embryos, a range of phenotypes were scored for each experiment. These embryos had an average IAD of 2 which corresponds to eyeless embryos with dramatic forebrain disruption but with visible otic vesicles. In addition, these injected embryos had slightly reduced trunks and broader somites than the control embryos (Fig. 3C2). This was the most common and typical Xhox3 phenotype (Table 1), but we also observed embryos with reduced foreheads and smaller eyes (IAD grade 1) (Fig. 3C1). These phenotypes were more typical in later embryos (Fig. 3D) and showed great disruption of mesodermal derivatives (notochord, muscles) (Fig. 4A,B,E). The group exhibiting most anterior abnormalities (IAD grade 1 and IAD grade 2), which represented 48% of the embryonic phenotypes, did not include otic vesicle deformity. This particular result was observed in every experiment that we conducted (over six hundred embryos).

In approximately 10% of all cases, normal embryos were obtained and no abnormality could be observed (IAD grade 0; Fig. 3C0). 37% of the tested embryos displayed both posterior and anterior abnormalities and could be classed into two types:

1) In the first type (32%), the embryos did not possess otic vesicles and displayed a reduced trunk in which several abnormally broad somites were observed (IAD grade 3) (Fig. 3C3).
2) In the second type (5%), the embryos had severely disturbed anterior-posterior structures and lacked somites altogether (IAD grade 4; Fig. 3C4).

As we observed with the LacZ or Xhox3 BEΔ injected embryos, 5% of the embryos had caudal abnormalities (tail$^-$).

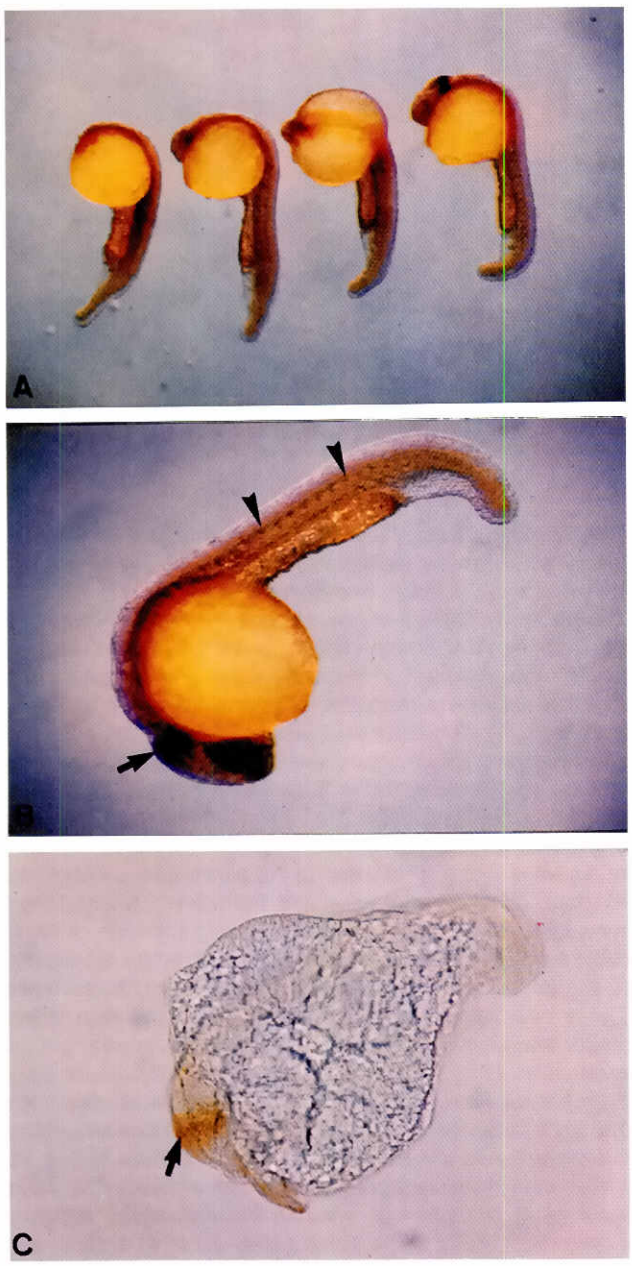


Fig. 6. Expression of *Eng* in the developing central nervous system and muscle pioneers cells. *Lateral* **(A and B)** *and dorsal* **(C)** *views of embryos. Eng proteins are detected by 4D9 antibodies in whole mount embryos. A mes-metencephalic region staining (arrow) is present in all embryos which possess this cephalic structure.* **(A)** *Series of 24 h embryos with a range of axial defects following Xhox3 mRNA injections. Anterior is towards the top. From right to left, control embryo (IAD grade 0) which displays CNS and somitic stainings for details see (B). IAD grade 1, grade 2 which show a weaker brown staining with reference to the control and no muscle pioneer cell staining. The last embryo is also an IAD grade 2 embryo which does not possess mes-metencephalic region or CNS staining. No muscle pioneer cell staining was observed in this embryo.* **(B)** *24 h control embryo, IAD grade 0, which shows mes-metencephalic (arrow) and muscle pioneer cells (arrowhead) stainings. The anterior part towards the bottom.* **(C)** *18 somites stage wild embryo which displays mes-metencephalic area staining (arrow). The anterior part towards the back of the bottom.*

In contrast to Xhox3 BEΔ or LacZ experiments, the injection of 7.5 to 9.4 pg of Xhox3 mRNA caused abnormal development in over 90% of all cases (Table 1), suggesting that the Xhox3 protein is indirectly or directly responsible for these abnormalities. It is clear that the concentration of injected mRNA does not directly cause these abnormalities, because a fourfold higher concentration of LacZ mRNA did not disturb normal development (Table 1).

Xhox3 injected messenger dose effect and malformations

We observed how the abnormalities varied as a function of concentration of Xhox3 mRNA injected. The measure of the dose effect was set by comparing one group of embryos to another.

35% of the embryos injected with 6 pg of Xhox3 mRNA had an abnormal phenotype. These embryos were phenotypically similar to those described by Ruiz i Altaba and Melton (1989b) in *Xenopus* embryos. 70% of these embryos had abnormal encephalic structures in a range from hemimicrophthalmia (Fig. 4C) to anencephalia (Fig. 4D). At higher concentrations of Xhox3 mRNA, more posterior abnormalities become evident.

The different mRNA amounts injected produced the following phenotypes:

1) With 6 pg, 35% of the embryos were abnormal.
2) With 9 pg, 88% of the embryos were malformed and posterior abnormalities appeared.
3) With 12 pg, 86% of the embryos were abnormal and 50% of injected embryos displayed abnormal posterior structures.
4) With 18 pg, 100% of the resulting embryos were abnormal (data not shown).

The comparative analysis of the average IAD shows the dose dependent effect of Xhox3 mRNA; as we increased the amount of mRNA injected from 6 to 12 pg, the average IAD increased from 0.69 to 2.38 (Table1).

The most prominent abnormal phenotypes observed in embryos older than 24 h were the absence of one or both optic vesicles, the lack of encephalic structures, a decrease in melanophore numbers, and a reduction in size of the embryos; these last three phenotypes were also previously described in *Xenopus* (Ruiz i Altaba and Melton, 1989b).

We also observed intermediate phenotypes such as the spectacular hemimicrophthalmic embryos (Fig. 4C).

Detection of Ntl proteins

No tail is the zebrafish homologue of the mouse brachyury *(T)* gene (Schulte-Merker *et al.*, 1994). It was reported that this gene is required for normal mesoderm development and extension of the body axis in zebrafish (Halpern *et al.*, 1993; Schulte-Merker *et al.*, 1994), as it is in mice (Cunliffe and Smith, 1992; Kispert and Herrmann, 1994). In wild-type zebrafish embryos, the Ntl protein production is transient in the cells of the germ ring, but stable in the cells of the presumptive notochord during early embryogenesis (Schulte-Merker *et al.*, 1992). Ntl protein detection allows us to correlate its pattern with the beginning of gastrulation. During this period and notably at the 90% epiboly stage, the whole-mount labeling of zebrafish anti-T protein (Ntl) showed a staining of only a few cells in the margin and presumptive notochord cells in the control embryos (Fig. 5A; Schulte-Merker *et al.*, 1992). The most commonly occurring staining pattern observed, in Xhox3 injected embryos, is an abnormally wider and shorter band of presumptive notochord cells (Fig. 5B). Injected embryos also displayed an undulating band of notochordal cells which represented a second abnormal pattern (Fig. 5C).

Detection of Engrailed homeoprotein in the injected embryos

The whole-mount immunolocalization of Eng in 18 somite embryos (17 h after FIV) and in the 24 h embryos, shows specific staining in the mes-metencephalic area and somitic cells that confirms the nuclear labeling pattern observed by Patel *et al.* (1989) and Hatta *et al.* (1991a) (Fig. 6C,B). A western-blot study using proteins from different species and tissues was performed to test the specificity of the 4D9 antibody (data not shown) (Hatta *et al.*, 1991a).

In the 24 h embryos injected with 12 pg of Xhox3 mRNA, Engrailed-like proteins using 4D9 antibodies (markers of the mes-metencephalic area) were immunolocalized, showing that:

1) The IAD grade 1 to 4 is characterized by an absence of Eng staining in the muscle pioneer cells compared to those of the control embryos (Fig. 6B).
2) The IAD grade 1 and 2 phenotypes show Eng staining only in the mes-metencephalic region. However, this staining is less intense than in controls (Fig. 6A).
3) The IAD grade 3 and 4 phenotypes, acephalic embryos lacking otic vesicles, do not display any Eng staining in the mes-metencephalic cells.

The Eng antibody immunostaining enables us to determine the anterior defects of the different phenotypes and shows specifically that the mes-metencephalic region is affected.

The detection of endogenous Xhox3 proteins

The use of anti-Xhox3 antibodies for whole-mount immunolocalization revealed that most 24 h embryos displayed a specific nuclear staining in somitic structures, forming a gradient along the anterior-posterior axis, with the highest intensity in the posterior region (Fig. 7A). A high staining is also observed in the caudal cellular mass (Fig. 7A).

Sections of the trunk showed that this labeling was present in cells of the sclerotome and myotome (Fig. 7B), with the highest degree of labeling in the sclerotome cells. We also observed staining in the large nuclei of the yolk syncytial layer. In the anterior region of these embryos, the sections showed a slight nuclear labeling of the mesenchymal cells located near the spinal chord and of the nervous tissue in the region just posterior to the hindbrain. With respect to the more anterior sections, no labeling was observed in either the nervous tissue or in the neighboring mesenchymal cells. Posterior sections of the embryos provide an opportunity to observe intense labeling at the periphery of the neural keel located close to the spinal chord and as well as to observe somitic staining.

In conclusion, the intensity of the somitic labeling is the result of a combination of three gradients: anterior-posterior, dermatome-sclerotome and dorso-ventral, with the highest degree of labeling seen respectively in the posterior, sclerotome and ventral part of the embryos.

Discussion

Ruiz i Altaba and Melton (1989b) showed that the injection of Xhox3 mRNA into *Xenopus* embryos had no effect on the migration of mesodermal cells. They proposed that the resulting phenotypes were due to a high degree of Xhox3 expression in presumptive anterior tissues which prevents the determination of anterior mesoderm cells. However, phenotypes with malformed or missing anterior structures could also be obtained in *Xenopus* by injecting trypan blue into the blastocoel of early gastrulae or by irradiating the vegetal pole of fertilized eggs with UV, thus producing abnormal mesoderm migration.

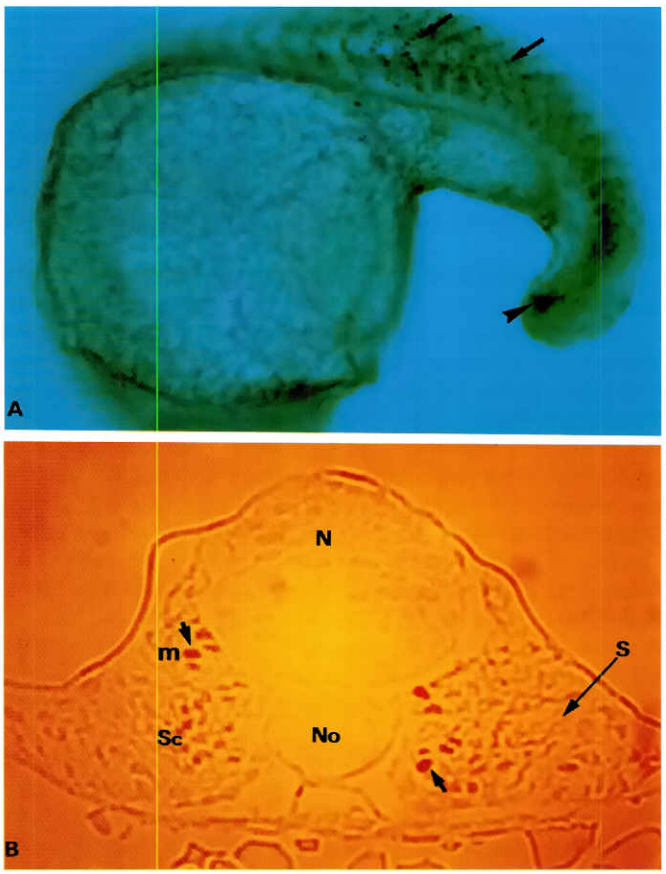


Fig. 7. Localization of Xhox3-like production in a 24 h embryo. (A) *A whole-mount embryo stained with peptide-purified Xhox3 antibodies shows nuclear labeling in the somites (arrow) and the extending tail tip (arrowhead). Xhox3-like protein production occurs mainly in the mesoderm. There is less nuclear staining in the anterior mesoderm versus posterior.* **(B)** *A trunk cross section of the same stage embryo shows nuclear labeling (arrowheads) in myotome (m) and sclerotome (Sc) cells. No: notochord; S: Somite; Nt: Neural keel.*

In zebrafish, an altered morphology of the injected embryos at the bud stage was observed (Fig. 3A). The phenotypes described could be explained by a change in the pattern of ecto-mesodermal cell distribution during gastrulation. Such a hypothesis would explain the round morphology of test embryos in contrast to the characteristic oval form observed in controls which is essentially due to the convergent movements of cells towards the embryonic shield.

The injection of Xhox3 BEΔ, which causes no embryonic abnormalities, allowed us to control the specificity of the effects of the Xhox3 mRNA injections in embryos and indicates that the binding of the Xhox3 homeoprotein to DNA is required for production of the various phenotypes. The zebrafish anti-protein T (Ntl) used as a marker of cells in the margin and of the presumptive notochord cells (Schulte-Merker *et al.*, 1992), allowed us to observe an abnormal distribution of these cells. The abnormally wide and short band presumptive notochord cells (Fig. 5A), supports the hypothesis of cellular migration defects. However, previous observations (Ruiz i Altaba and Melton, 1989b) did not suggest abnormal mesodermal

cell movements during gastrulation in Xhox3 injected *Xenopus* embryos.

In *Drosophila* it was previously shown that the *Eng* genes were an indirect target of the *Eve* products. The *Eng* gene appeared to be repressed in a concentration-dependent fashion (Manoukian and Krause, 1992). Many features of developmental control genes are highly conserved among organisms (Duboule and Dollé, 1989), so the Xhox3 product is likely to indirectly affect Eng genes expression.

Use of the 4D9 MAb showed that Engrailed proteins can be found in many organisms throughout the animal kingdom (Patel *et al.*, 1989). In zebrafish, the first Eng expression to appear during development is a transverse stripe of cells across the brain primordium at the 1-2 somites stages, as previously reported (Njolstad and Fjose, 1988; Patel *et al.*, 1989; Hatta *et al.*, 1991a). In embryos older than the 22 somite stage, a deep furrow forming the midbrain-hindbrain boundary becomes visible in the middle of the staining stripe (Hatta *et al.*, 1991a). At this late stage, a small subset of cells in each somite expresses Eng. These somitic cells have been identified as muscle pioneer cells which are the first muscle cells to differentiate within the myotome and they can be recognized by their characteristic cell shape and their position at the center of the chevron-shaped myotome (Fig. 6B) (Felsenfeld *et al.*, 1991; Hatta *et al.*, 1991a). This subset of cells seems to play an important role in muscle formation in many organisms (Jellies, 1990).

Using the 4D9 antibody which recognized all three gene products (Eng1, Eng2, Eng3) (Ekker *et al.*, 1992), Eng expression in 24 h Xhox3 injected embryos was examined. In the controls, muscle pioneer cells express two of the *Engrailed* genes, *eng1* and *eng2* and cells at the junction between the midbrain and hindbrain express all three *Engrailed* genes (Ekker *et al.*, 1992). In 24 h Xhox3 injected embryos, no Eng products in the somitic cells were detected. This observation suggests that the muscle pioneers fail to develop in trunk segments of injected embryos or that they are present but fail to express Eng. This is likely to be related to the malformed somites in Xhox3 injected embryos. The Xhox3 phenotypes show broad somites without the characteristic chevron-shape and which lack horizontal myosepta as was previously observed with 'spadetail' mutants, spt-1 (b104), (Kimmel *et al.*, 1989) and *no tail* mutants (Halpern *et al.*, 1993). The use of Eng labeling has previously allowed us to observe that there is no *eng* expression within the trunk, and notably in muscle pioneer cells, of embryos bearing the spt-1 mutation which disrupts muscle organization in the trunk (Hatta *et al.*, 1991a). Eng expression in the other body parts of 'spadetail' embryos is normal (Hatta *et al.*, 1991a).

Our Xhox3 results, in addition to spt-1 and no tail results, support the role of the muscle pioneer cells and their expression of Eng in the patterning of body wall muscle including the establishment of myotomal boundaries and the horizontal myosepta.

The weaker staining observed in the mes-metencephalic region of IAD grade 1 and 2 phenotypes (Fig. 6A) with no Eng product within the trunk supports the hypothesis of a down regulation of *eng1* and *eng2* expression by Xhox3 overexpression. In these phenotypes the anterior positive Eng cells may reflect the presence of the Eng3 product (Ekker *et al.*, 1992).

The use of an antibody against the Xhox3 protein N-terminal region which immunoprecipitate the synthetic translation products of Xhox3 mRNAs (Ruiz i Altaba *et al.*, 1991) shows, in zebrafish, a distribution of an endogenous Xhox3-like protein in tissues similar to those observed in *Xenopus*.

The main pattern observed in 24 h embryos is an A-P gradient of nuclear staining in the somitic structures. This result was also observed in *Xenopus* (Ruiz i Altaba *et al.*, 1991) where the pattern most similar to 24 h zebrafish embryos was that of the *Xenopus* early neurula (stage 18) with respect to the lack of anterior Xhox3 protein production. The 33/34 stage *Xenopus* embryo, which is equivalent to the 24 h zebrafish embryo, shows endogenous production of Xhox3 protein in cephalic regions. This previous study (Ruiz i Altaba *et al.*, 1991) is consistent with the bimodal expression of Xhox3 observed. During the early period of Xhox3 expression, the gastrula and neurula stages, transcripts are found in a graded fashion along the A-P axis in the mesoderm and are concentrated at the posterior end. In the late period of expression, the tailbud and tadpole stage, transcripts are concentrated in the anterior nervous system and posterior tailbud.

Our results suggest that the lack of detection of Xhox3-like protein in the cephalic region is likely to correspond to the absence of the late period of Xhox3 expression. Alternatively, translation of the Xhox3 mRNA may produce a protein in which the N-terminal region is no longer accessible to the antibody.

The zebrafish embryo Xhox3-like expression pattern looks like a *Xenopus* Xhox3 pattern with a good conservation of the specific distribution feature of this protein. In the two species, gradual staining was observed in the somitic structures: in anterior versus posterior and dorsal versus ventral regions. In both cases we also observed very low levels of Xhox3 protein in restricted endodermic structures.

These Xhox3 antibody results in combination with the Xhox3 overexpression phenotypes are consistent with the presence of an endogenous zebrafish Xhox3 homologue and support the hypothesis that the mechanisms implicated in embryonic development involving the *Xhox3* gene are highly conserved.

Materials and Methods

Plasmid DNA constructs

pcXhox3: the plasmid used in this experiment was previously described by Ruiz i Altaba *et al.* (1989a). It is a double stranded Xhox3 cDNA which was blunted with T4 DNA polymerase, methylated with EcoR1 methylase, ligated to EcoR1 linkers, and inserted into the pSp70 multicloning site at the EcoR1 site.

pcXhox3 BEΔ was constructed by the deletion of a 243 bp fragment of pcXhox3 using a BSaAI and Eco47 III sites respectively located in the homeobox and 3' of the homeobox.

pT3 ß-gal (nuclear localization signal; nls) is a pbluescript (KS+) (Stratagene) plasmid which contains, in the multicloning site, the reporter gene *LacZ* excised from the pcH11O plasmid (Pharmacia) by Hind III and pst I digestion. The nls sequence was inserted into the Kpn1 site near the ATG codon.

In vitro transcription

The *in vitro* transcription of plasmids was achieved using the mCap mRNA capping Kit according to the instruction of the supplier (Stratagene). Xhox3 and Xhox3 BEΔ mRNA was synthesized by the transcription of HindIII-cut, pcXhox3 and pcXhox3 BEΔ with T7 RNA polymerase. LacZ (nls) mRNA was synthesized by transcription of Pst1-cut pT3 ß-gal (nls) with T3 RNA polymerase.

Egg production and in vitro fertilization (FIV)

Zebrafish were maintained at 28.5°C on a 14 h light and 10 h dark cycle according to Stuart *et al.* (1988). Male and female fish to be mated were kept in separate tanks until the night before pairing. *In vitro* fertilization (FIV) was carried out according to the Streisinger protocol (Streisinger *et al.*, 1981). Modifications were made to collect spermatozoa. They were collected by

pressing the male sides and simultaneously adding cold Hank's buffer. The buffer including the spermatozoa is then picked up and stored on ice at 4°C. Staging of the embryos was based on morphological criteria, following the classification of Westerfield (1994).

Removing the zebrafish chorion

The injection of embryos requires the removal of the chorion by treatment with pronase (Stuart *et al.*, 1988). Here, we report a method using chemical and enzymatic products which is a more effective and rapid way of removing the zebrafish chorion.

Fifteen minutes after the FIV, embryos are less sensitive to manipulations and may be covered by Tyrode Acid solution (pH 2) for 20 to 30 seconds. This first stage of dechorionation enables the chorion to be cleaned and weakened. After approximately ten seconds, the chorion appeared crumpled under binocular loup. Embryos could be left in Tyrode Acid solution for up to 8 minutes and/or up to the early gastrula stage without altering their development. Embryos were rinsed twice with fish water and covered by a pronase E solution (10 mg/ml) Sigma P5147. After five to ten seconds, embryos were observed leaving center and entering into contact with chorion. Perforation of the chorion followed rapidly.

The embryos were poured into a beaker containing about 150 ml of distilled water. After one or two seconds, the embryos sank to the bottom of the beaker. The water was poured off until only 30 ml remained; more water was then added inducing eddies which caused tearing in the weakened chorion which then floated on the meniscus. The embryos remained at the bottom of the beaker.

The water containing the remains of the chorions was then poured off until 30 ml was left at the bottom of the beaker, and more water was added carefully to avoid any weakening in order to rinse the embryos. This process was repeated three times to optimize the dechorionation and to perfect the rinsing of the embryos.

Microinjection of mRNA

Micropipettes were made on a Vertical puller (Narishge PB7) using 1 mm fibre-filled glass capillary tubing (Clark GC 100F-15). The micropipette was held by a micromanipulator (UHL Germany) and connected to a microinjector (Eppendorf 5242) with a teflon tube (inside diameter 1.5 mm, outside diameter 3 mm). Just prior to use, the end of micropipette containing the DNA solution was carefully broken with a blunt instrument to generate a tip approximately 3 µm in diameter (Stuart *et al.*, 1988). The injection experiment was carried out according to the Stuart *et al.* (1988) protocol. The RNA solution (200 to 250 pl) was injected through a continuously flowing pipette: the flow rate was controlled by application of 30-100 Kpa of pressurized nitrogen (99.9%) with a maintained pressure of 4-6 KPa. The estimation of the injected volume was made using phenol red X20 (Sigma) which is added to the injection solution.

Immunostaining of whole embryos

This experiment was carried out according to the Molven *et al.* (1990) protocol.

ß-gal staining

ß-gal staining was performed according to the Westerfield *et al.* (1992) protocol.

Histology

For light microscopy, embryos were dechorionated and fixed by 24 h immersion in aqueous "Bouin" solution. After stepwise dehydration into absolute ethanol, the embryos were rinsed in propylene oxide, subsequently infiltrated in increasing concentrations of Epon 812 in propylene oxide, then embedded in fresh Epon and oriented before polymerization.

Serial 3 µm sections were cut and stained with methylene blue-azurII and basic fuchsin (Humphrey and Pittman, 1974).

Acknowledgments

We wish to thank A. Ruiz i Altaba, M. Westerfield and S. Vriz for stimulating discussion and helpful comments on the manuscript. We thank A.R.A. for the donation of both the pcXhox3 plasmid and antibodies, C.S. Goodman for gifts of engrailed antibody, S. Schulte-Merker for the brachyury antibody, J.S. Joly and P. Nouvel for the pT3 ß-gal (nls) construction and R. Novak for helpful reading of the manuscripts. This work was supported by grants of the "Ministère de l'enseignement supérieur et de la recherche" and "Université Paris 7" to H. Boulekbache and CNRS to H. Condamine.

References

BASTIAN, H. and GRUSS, P. (1990). A murine *even-skipped* homologue, *Evx* 1, is expressed during early embryogenesis and neurogenesis in a biphasic manner. *EMBO J. 9:* 1839-1852.

BIENZ, M. (1994). Homeotic genes and positional signalling in the *Drosophila* viscera. *Trends Genet. 10:* 22-26.

BUDD, P.S. and JACKSON, I.J. (1991). What do the regulators regulate? First glimpses downstream. *Trends Genet. 7:* 74-76.

CUNLIFFE, V. and SMITH, J.C. (1992). Ectopic mesoderm formation in *Xenopus* embryos caused by widespread expression of a brachyury homologue. *Nature 358:* 427-430.

D'ESPOSITO, M., MORELLI, F., ACAMPORA, D., MIGLIACCIAO, E., SIMEONE, A. and BONCINELLI, E. (1991). EVX2, a human homeobox gene homologous to the even-skipped segmentation gene, is localized at the 5' end of HOX4 locus on chromosome 2. *Genomics 10:* 43-50.

DRIEVER, W., STEMPLE, D., SCHIER, A. and SOLNICA-KREZEL, L. (1994). Zebrafish: genetic tools for studying vertebrate development. *Trends Genet. 10:* 152-159.

DUBOULE, D. and DOLLÉ, P. (1989). The structural and functional organization of the murine HOX gene resembles that of *Drosophila* homeotic genes. *EMBO J. 8:* 1497-1505.

DUSH, M.K. and MARTIN, G.R. (1992). Analysis of mouse Evx genes: Evx-1 displays graded expression in the primitive streak. *Dev. Biol. 151:* 273-287.

EKKER, M., WEGNER, J., AKIMENKO, M.A. and WESTERFIELD, M. (1992). Coordinate embryonic expression of three Zebrafish engrailed genes. *Development 116:* 1001-1010.

FAIELLA, A., D'ESPOSITO, M., RAMBALDI, M., ACAMPORA, D., BALSOFIORE, S., STORNAIUOLO, A., MALLAMACI, A., MIGLIACCIO, E., GULISANO, M., SIMEONE, A. and BONCINELLI, E. (1991). Isolation and mapping of EVX1, a human homeobox gene homologous to even-skipped, localized at the 5' end of HOX1 locus on chromosome 7. *Nucleic Acids Res. 19:* 6541-6545.

FELSENFELD, A.L., CURRY, M. and KIMMEL, C.B. (1991). The fub-1 mutation blocks initial myofibril formation in zebrafish muscle pioneer cells. *Dev. Biol. 148:* 23-30.

GEHRING, W.J., MÜLLER, M., AFFOLTER, M., PERCIVAL-SMITH, A., BILLETER, M., QIAN, Y.Q., OTTING, G. and WÜTHRICH, K. (1990). The structure of the homeodomain and its functional implications. *Trends Genet. 6:* 323-329.

HALPERN, M.E., HO, R.K., WALKER, C. and KIMMEL, C.B. (1993). Induction of muscle pioneers and floor plate is distinguished by the zebrafish no tail mutation. *Cell 75:* 99-111.

HATTA, K., BREMILLER, R., WESTERFIELD, M. and KIMMEL, C.B. (1991a). Diversity of expression of *engrailed*-like antigens in Zebrafish. *Development 112:* 821-832.

HATTA, K., KIMMEL, C.B., HO, R.K. and WALKER, C. (1991b). The cyclops mutation blocks specification of the floor plate of the zebrafish central nervous system. *Nature 350:* 339-341.

HUMPHREY, C.D. and PITTMAN, F.E. (1974). A simple Methylene Blue-Azur II Basic Fuschin Stain for Epoxy-embedded tissue sections. *Stain Technol. 49:* 9-14.

JELLIES, J. (1990). Muscle assembly in simple systems. *Trends Neurosci. 13:* 126-131.

JOLY, J.S., JOLY, C., SCHULTE-MERKER, S., BOULEKBACHE, H. and CONDAMINE, H. (1993). The ventral and posterior expression of the zebrafish homeobox gene *eve1* is perturbed in dorsalized and mutant embryos. *Development 119:* 1261-1275.

KIMMEL, C.B. (1989). Genetics and early development of zebrafish. *Trends Genet. 5:* 283-288.

KIMMEL, C.B. and WARGA, R.M. (1987). Cell lineages generating axial muscle in the zebrafish embryo. *Nature 327:* 234-237.

KIMMEL, C.B., KANE, D.A., WALKER, C., WARGA, R.M. and ROTHMAN, M.B. (1989). A mutation that changes cell movement and cell fate in the zebrafish embryo. *Nature 337:* 358-362.

KISPERT, A. and HERRMANN, B.G. (1994). Immunohistochemical analysis of the brachyury protein in wild-type and mutant mouse embryos. *Dev. Biol. 161:* 179-193.

MALICKI, J., SHUGHART, K. and McGINNIS, W. (1990). Mouse *Hox-2-2* specifies thoracic segmental identity in *Drosophila* embryos and larvae. *Cell 63:* 961-967.

MANOUKIAN, A. and KRAUSE, H.M. (1992). Concentration-dependent activities of the even-skipped protein in *Drosophila* embryos. *Genes Dev. 6:* 1740-1751.

McGINNIS, W. and KUZIORA, M. (1994). The molecular architects of body design. *Sci. Am. 270:* 36-42.

McGINNIS, W., LEVINE, M.S., HAFEN, E., KUROIWA, A. and GEHRING, W.J. (1984). A conserved DNA sequence in homeotic genes of the *Drosophila* Antennapedia and Bithorax complexes. *Nature 308:* 428-433.

MILES, A. and MILLER, D.J. (1992). Genomes of diploblastic organisms contain homeoboxes: sequence of *eve* C, an *even-skipped* homologue from the cnidarian *Acropora formosa*. *Proc. R. Soc. Lond. B 248:* 159-161.

MOLVEN, A., WRIGHT, C.V.E., BREMILLER, R., DE ROBERTIS, E.M. and KIMMEL, C.B. (1990). Expression of a homeobox gene product in normal and mutant Zebrafish embryos: evolution of the tetrapod body plan. *Development 109:* 279-288.

NJOLSTAD, P.R. and FJOSE, A. (1988). *In situ* hybridization patterns of zebrafish homeobox genes homologous to Hox-2.1 and En-2 of mouse. *Biochem. Biophys. Res. Commun. 157:* 426-432.

PATEL, N.H., MARTIN-BLANCO, E., COLEMAN, K.G., POOLE, S.J., ELLIS, M.C., KORNBERG, T.B. and GOODMAN, C.S. (1989). Expression of *engrailed* proteins in Arthropods, Annelid and Chordates. *Cell 58:* 955-968.

RUIZ I ALTABA, A. and MELTON , D.A. (1989a). Bimodal and graded expression of the *Xenopus* homeobox gene *Xhox3* during embryonic development. *Development 106:* 173-183.

RUIZ I ALTABA, A. and MELTON, D.A. (1989b). Involvement of the *Xenopus* homeobox gene Xhox3 in pattern formation along the anterior-posterior axis. *Cell 57:* 317-326.

RUIZ I ALTABA, A. and MELTON, D.A. (1990). Axial patterning and the establishment of polarity in the frog embryo. *Trends Genet. 6:* 57-64.

RUIZ I ALTABA, A., CHOI, T. and MELTON, D.A. (1991). Expression of the Xhox3 homeobox protein in *Xenopus* embryos: blocking its early function suggests the requirement of Xhox3 for normal posterior development. *Dev. Growth Differ. 33:* 651-669.

SCHARF, S.R. and GERHART, J.C. (1983). Axis determination in eggs of *Xenopus laevis:* a critical period before first cleavage, identified by the common effects of cold, pressure and ultraviolet irradiation. *Dev. Biol. 99:* 75-87.

SCHULTE-MERKER, S., HO, R.K., HERRMANN, B.G. and NÜSSLEIN-VOLHARD, C. (1992). The protein product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. *Development 116:* 1021-1032.

SCHULTE-MERKER, S., VAN EEDEN, F.J.M., HALPERN, M.E., KIMMEL, C.B. and NÜSSLEIN-VOLHARD, C. (1994). no tail (ntl) is the zebrafish homologue of the mouse T (Brachyury) gene. *Development 120:* 1009-1015.

SCOTT, M.P. and WEINER, A.J. (1984). Structural relationships among genes that control development: sequence homology between the Antennapedia, Ultrabithorax and fushi tarazu loci of *Drosophila*. *Proc. Natl. Acad. Sci. USA 81:* 4115-4119.

STREHLOW, D. and GILBERT, W. (1993). A fate map for the first cleavages of the zebrafish. *Nature 361:* 451-453.

STREISINGER, G., WALKER, C., DOWER, N., KNAUBER, D. and SINGER, F. (1981). Production of clones of homozygous diploid zebra fish *(Brachydanio rerio). Nature 291:* 293-296.

STUART, G.W., Mc MURRAY, J.V. and WESTERFIELD, M. (1988). Replication, integration and stable germ-line transmission of foreign sequences injected into early Zebrafish embryos. *Development 103:* 403-412.

TAKEDA, H., MATSUZAKI, T., OKI, T., MIYAGAWA, T. and AMANUMA, H. (1994). A novel POU domain gene, Zebrafish pou2: expression and roles of two alternatively spliced twin products in early development. *Genes Dev.8:* 45-59.

WARGA, R.M. and KIMMEL, C.B. (1990). Cell movements during epiboly and gastrulation in Zebrafish. *Development 108:* 569-580.

WESTERFIELD, M. (1994). *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Brachydanio rerio).* University of Oregon Press, Eugene.

WESTERFIELD, M., WEGNER, J., JEGALIAN, B.G., DE ROBERTIS, E.M. and PÜSCHEL, A.W. (1992). Specific activation of mammalian *Hox* promoters in mosaic transgenic zebrafish. *Genes Dev. 6:* 591-598.

Accepted for publication: September 1994