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Original Article

X-twi is expressed prior to gastrulation in presumptive neurectodermal and mesodermal cells in dorsalized and ventralized *Xenopus laevis* embryos

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ABSTRACT Early *X-twi* expression has been now investigated from egg laying to the early neurulation stages in *Xenopus* embryos, using both *in situ* hydridization and the more sensitive techniques of RT-PCR. We show that in unfertilized eggs, a decreasing gradient of *X-twi* transcript distribution is observed from animal to vegetative caps. *X-twi* RNA can be weakly detected at stages prior to gastrulation, and with increased intensity from stage 8 onwards. At blastula, *X-twi* transcripts are located towards the animal pole, and as gastrulation begins, they are detected in the developing axial mesoderm and then they accumulate in the sensorial layer of the neurectoderm, the mesodermal layer and in neural crest cells up to late neurula stages. We show, in addition, that in lithium-chloride- and UV-treated *Xenopus* embryos (that are respectively both "anteriorized/dorsalized" and in "posteriorized/ventralized"), *X-twi* RNA is detected in cells in similar positions to those that express *X-twi* in normal embryos. As a whole, our results show that *X-twi* is expressed even when regionalization of the mesoderm is disturbed and raises the question of a putative function of *X-twi* prior to gastrulation.

KEY WORDS: *X-twist , mesoderm, blastula, gastrula*

Introduction

Due to ease of manipulation, *Xenopus* embryos have been widely used as model-system for the study of early developmental processes. Different reviews report the dorso-ventral patterning in *Xenopus* (Sive, 1993), the role of Spemann's organizer (Gilbert and Saxén, 1993) embryonic induction (Howard and Smith, 1993; Slack, 1994) and the gastrulation process (Winklbauer, 1990; Shih and Keller, 1994). Also, models about amphibian gastrulation have been presented (Harger and Gurdon, 1996; and references therein). Regional specification within the mesoderm has also been studied (Dale and Slack, 1987; and references in Zimmerman *et al.*, 1996).

Briefly: at fertilization a cortical rotation occurs that defines, at about 180° from the sperm entry point, the future blastopore region and, consequently along this meridian, the future dorsal midline of the embryo. This event defines the dorso-ventral polarity of the embryo. Mesoderm induction seems to take place during the blastula stage (Jones and Woodland, 1987) and is known to be induced in the whole equatorial marginal zone by animal cells that are responding to signals from the vegetative pole. Both events allow mesoderm regionalization to occur and the Nieuwkoop center to be activated at the 32-cell stage (Nieuwkoop, 1973; Gimlich and Gerhart, 1984; Jones and Woodland, 1987). At this stage in the mesodermal layer, presumptive anterior cells lie at the yet defined dorsal side, and the future posterior cells lie at the ventral side of the embryo. However, in experimental conditions, the establishment of dorsal-ventral polarity and regionalization of the mesoderm are developmental events that can be uncoupled. In UV-irradiated embryos, no cortical rotation occurs (see below), but embryos develop as three-layered cylinders containing mesoderm of a purely ventral character (Gerhart *et al.*, 1991). In lithium chloride treated embryos, the dorsal-ventral axis is not formed and embryos develop into structures that contain mesoderm with a purely dorsal character (Kao *et al.*, 1986).

Abbreviations used in this paper: AP-enzyme, Alkaline Phosphatase; DAI, dorsal anterior index; DEPC, diethy-pyrocarbonate; LiCl, lithium chloride; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; SLN, sensorial layer of neurectoderm; st21, stage 21; UV, ultraviolet light.

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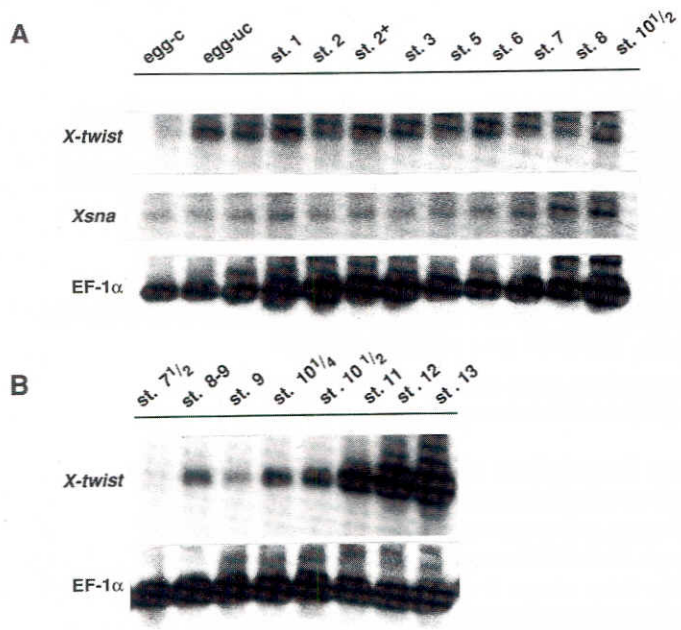

Fig. 1. Temporal early transcript detections of *X-twi, Xsna,* and *EF1-α* by RT-PCR amplifications. (A) *X-twi transcripts are detected as a 350bp fragment in unfertilized eggs, and then in embryos from early stage 1 (st. 1) onwards to gastrula (st. 10 1/2) (40 min exposure time). Xsna transcripts are detected as a 450bp fragment almost at constant level throughout all stages tested from unfertilized eggs to stage 7 embryos (20 min exposure time). EF1-a transcript amplification was used as a control for quantification (20 min exposure time). All those experiments were performed in parallel on the same RNA samples. egg-c : RNA extracted from dejellied eggs. egg-uc : RNA extracted from unjellied eggs.* **(B)** *Detailed analysis of X-twi expression prior to and just after gastrulation stage confirms that X-twi transcripts are significantly detected prior to gastrulation. EF1-α transcript amplification on the same RNA samples is shown below.*

Gastrulation in other species has been studied using different methods. In *Drosophila*, genetic studies opened the field towards the molecular characterization of those genes that are expressed at that developmental stage (see Reuter and Casal, 1994 for a review). Thus, *twist* and *snail* are indispensable for correct gastrulation: homozygous *twist* (and *snail)* mutant embryos that display the strongest phenotype do not gastrulate properly, do not form mesoderm but die only at the end of embryogenesis (Simpson, 1983; Grau *et al.*, 1984; Alberga *et al.*, 1991). The *twist* gene is expressed before gastrulation in all presumptive endomesodermal cells, and after gastrulation in cells of the mesoderm layer and its derivatives (Thisse *et al.*, 1987,1988). *twist* function is required for the expression of *snail* (Ip *et al.*, 1992) and the expression of both *twist* and *snail* is required for full mesoderm establishment (Leptin, 1991). Regionalization of the mesoderm occurs only after gastrulation (Bate and Baylies, 1996 for a review). Twist from *Drosophila* was shown *in vivo* to act as a transcriptional activator for early mesoderm-specific genes (Leptin, 1991; Ip *et al.*, 1992; Taylor *et al.*, 1995). It does not prevent somatic myogenesis (Baylies and Bate, 1996), but functions to subdivide the mesoderm (Dunin-Borkowski *et al.*, 1995). In addition, high Twist levels in cells that normally contain low levels of *twist* protein disturb the development of those cells (Baylies and Bate, 1996).

In vertebrates, the murine *M-twist* gene (Wolf *et al.*, 1991) and the *X-twi* sequence from *Xenopus* (Hopwood *et al.*, 1989) present high similarities with that of the *Drosophila twist* gene, but their expression in mesodermal cells, was only described as appearing after the onset of gastrulation. The mouse homolog of *snail (Sna)* is expressed throughout post-implantation development (Nieto *et al.*, 1992, Smith *et al.*, 1992). For the *Xenopus Snail (Xsna)* gene, the capacity to synthesize its RNA was detected at stage 8, with a bulk of zygotic transcription observed in the marginal zone of stage $10^1/_2$ embryos (Sargent and Bennett, 1990). Studies on the relationship among *Xsna*, *Xtwi* and *Xbra* around stages $10^{1/2}$ and 12 have been reported by Essex *et al.* (1993). The developmental defects of the homozygous murine embryos bearing the *twist-null* allele pointed towards a function of *M-twist* after gastrulation (Chen and Behringer, 1995). And *M-twist* heterozygous mutant new-born mice present skull and limb malformations that resemble those of the human patients presenting the Saethre-Chotzen syndrome, which is caused by *H-twist* mutations (El Ghouzzi *et al.*, 1997; Bourgeois *et al.*, 1998). Twist from *Mus* acts *in vitro* as a repressor of some myogenic genes (Hebrok *et al.*, 1994; Spicer *et al.*, 1996), and *in vitro*, *H-twist* expression is altered in cultured young versus senescent human fibroblasts (Wang *et al.*, 1996).

More recently, we showed that in *Mus*, *M-twist* is expressed in unfertilized eggs, and prior to gastrulation, mainly in extra-embryonic presumptive endomesodermal cells; *M-twist*$^{\Delta bHLH}$ homozygous mutant concept displays misorganized extra-embryonic and embryonic germ-layers and dies at the end of gastrulation (Bolcato-Bellemin *et al.*, 1, submitted) .

We also isolated the *G-twist* gene from *Gallus* and showed that it is expressed weakly in unincubated eggs. Then prior to gastrulation its transcripts accumulate in extra-embryonic tissues, and after gastrulation in cells of the mesoderm germ-layer and its derivatives (Bolcato-Bellemin *et al.*, 1, submitted).

TABLE 1

X-TWI AND XSNA RNA DISTRIBUTION IN SEPARATE PARTS OF UNFERTILIZED EGGS

gene	region	relative volume	amount RNA	amount of RNA (%)	corrected % of RNA per unit of volume
	An. cap	1	1107	38	49
X-twi	Eq. reg.	2	1277	44	28
	Ve. cap	1	511	17	23
	An. cap	1	3133	26	32
Xsna	Eq. reg.	2	4400	37	22
	Ve. cap	1	4514	37	46
	An. cap	1	8180	42	55
EF1-α	Eq. reg.	2	9123	47	31
	Ve. cap	1	2078	11	14
	An. cap	1	10377	48	61
18S rRNA	Eq. reg.	2	9279	42	27
	Ve. cap	1	2099	10	12

RT-PCR experiments were performed on RNA extracted from isolated animal caps, equatorial regions, and vegetal caps of unincubated eggs.
Amplifications were done with 20 cycles for *X-twi* and *EF1-α*, 30 cycles for *Xsna*, and 7 cycles for the 18S RNA.
The amount of RNA is given in arbitrary units.

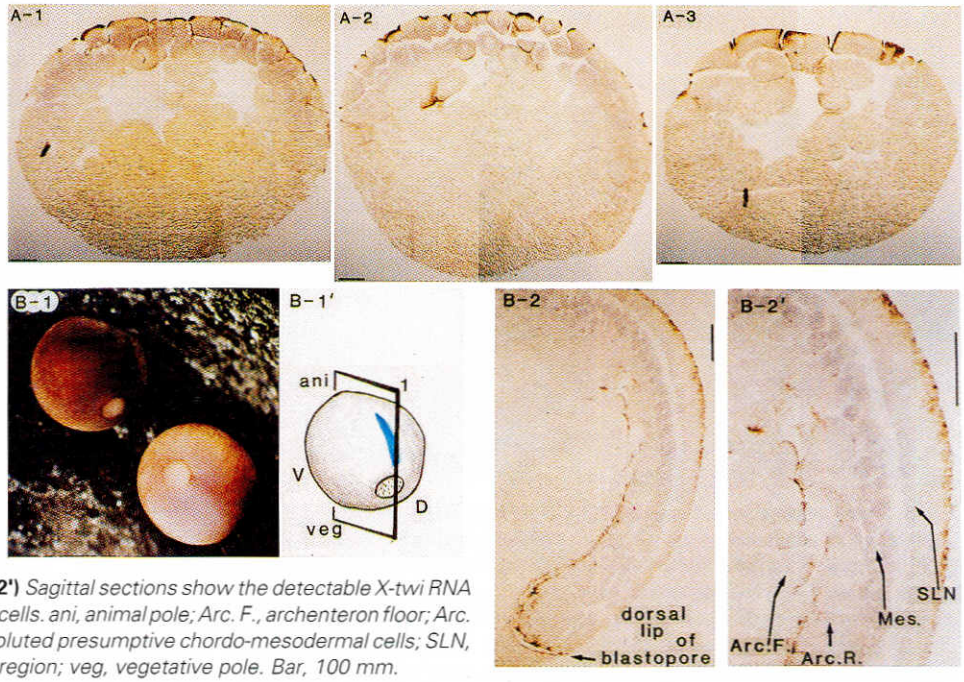

Fig. 2. *X-twi* expression at stages $6^1/_2$-7 and $11^1/_2$. (A-1 and A-2) *Transverse sections of a stage $6^1/_2$ embryo (A-1) and through a stage 7 embryo (A-2) which had been hybridized with the antisense digoxigenin-labeled probe. The X-twi transcripts are detected in blastomeres from animal region. Notice that the transcripts are restricted towards the ventral part of the outermost animal blastomeres, and in the facing cytoplasm of the blastomeres that form the second row of cells.* **(A-3)** *Transverse section of a stage 6/7 embryo which had been hybridized in parallel with the sense digoxigenin-labeled probe. No meaningful staining could be detected within all the sections.* **(B-1)** *On a stage $11^1/_2$ whole embryo, X-twi transcripts are seen in the dorsal/posterior region above the dorsal blastopore (antisense probe: embryo to the left). The control embryo (sense probe) is shown on the right : no signal is detected in it.* **(B-1')** *Schematic drawing of the embryo hybridized with the antisense probe, with the plan of the sections.* **(B-2 and B-2')** *Sagittal sections show the detectable X-twi RNA in the involuted presumptive chordo-mesoderm cells. ani, animal pole; Arc. F., archenteron floor; Arc. R., archenteron roof. D, dorsal region; Mes., involuted presumptive chordo-mesodermal cells; SLN, sensorial layer of the neurectoderm; V, ventral region; veg, vegetative pole. Bar, 100 mm.*

Therefore, we decided to investigate whether in batracians, the initiation of *X-twi* expression could occur earlier than gastrulation, as it is the case for *M-twist* and *G-twist* genes in mice and chicken respectively. We studied the early expression pattern of *X-twi* from unfertilized eggs up to neurula stage using RT-PCR, and illustrated its spatial pattern by whole-mount *in situ* hybridization. These analyses complete the previous study on the expression of *X-twi* described by Hopwood and Gurdon. (1991). The main features of our studies are: *X-twi* transcripts are weakly detected in unfertilized eggs, then during all the early stages before gastrulation, then massively from late gastrula stage onwards. In addition, we show that *X-twi* expression is not modified in dorsalized and in ventralized embryos. We discuss the hypothesis that *X-twi* may have a putative early function in *Xenopus*, as it is the case for its counterparts in *Mus* or in *Drosophila*, and suggest that this gene should be registered as *X-twist*, a true homolog.

Results

X-twi and Xsna transcript distribution in unfertilized eggs

To probe the distribution of the *X-twi* transcripts in unfertilized eggs, we isolated animal caps, equatorial regions and vegetal caps, and assayed their RNA by RT-PCR. The volumes of the respective regions represent $^1/_4$ (animal cap), $^1/_2$ (equatorial part) and $^1/_4$ (vegetative cap) of the total volume of the egg. A band corresponding to the *X-twi* RNA has been visualized in the three isolated parts of the egg, at proportions of 49% (animal cap), 28% (equatorial region) and 23% (vegetative cap) respectively (Table 1). The transcripts for *Xsna* are detected respectively at 32%, 22%, and 46% in the same samples. As controls, *EF1-α* transcripts (Krieg and Melton, 1989) are detected at 55%, 31%, and 14%, and the 18S RNA is detected at 61%, 27% and 12% respectively in the same samples.

X-twi transcript detection up to neurula stage

RNA was extracted from eggs (dejellied or not), from stage 1 to stage $10^{1/2}$ embryos and probed for *X-twi*, *Xsna*, and *EF1-α* transcripts. *EF1-α* transcript detection attests the presence ofroughly equal amounts of RNA in each sample. We show here that *X-twi* and *Xsna* transcripts are faintly detected, at an almost constant rate for *X-sna* up to stage 8, and with some modulations for *X-twi* transcripts; in particular, *X-twi* RNA is still detected up to stage 6 (32 cells), and in stage $10^1/_2$ embryos. Thus, *X-twi* and *Xsna* RNA are detected in samples prior to the stages where the zygotic genome is activated. At stage $10^1/_2$, we estimate that there is about twice as much *Xsna* RNA as *X-twi* RNA (the exposure time was twice as long for *X-twi* as for *Xsna*, to get the same level of signal: Fig. 1A).

More precisely, we observed a boost of *X-twi* expression at stage 8-9, and then from initial gastrula stage (stage $10^1/_4$) onwards, *X-twi* expression increases during the middle and final parts of the gastrulation process (stages 12,13: the medium yolk plug stage, and the slit blastopore stage, respectively) (Fig. 1B).

Localization of X-twi transcripts up to stage 13/14

Whole-mount *in situ* hybridization experiments have been performed at different developmental stages, using as controls the embryos incubated with the sense probe. The embryos were observed, then sectioned, and the localization of the *X-twi* transcripts analyzed.

At about stage $6^{1/2}$-7, whole embryos from controls and assays seem very weakly stained to the same extent. However, a discrete localization of *X-twi* transcripts is observed on serial sections of embryos hybridized with the antisense probe (Fig. 2A-1 for a stage $6^1/_2$ embryo, and 2A-2 for a stage 7 embryo), whereas no definite signal is observed on sections of embryos hybridized with the sense probe (Fig. 2A-3). *X-twi* transcripts are essentially

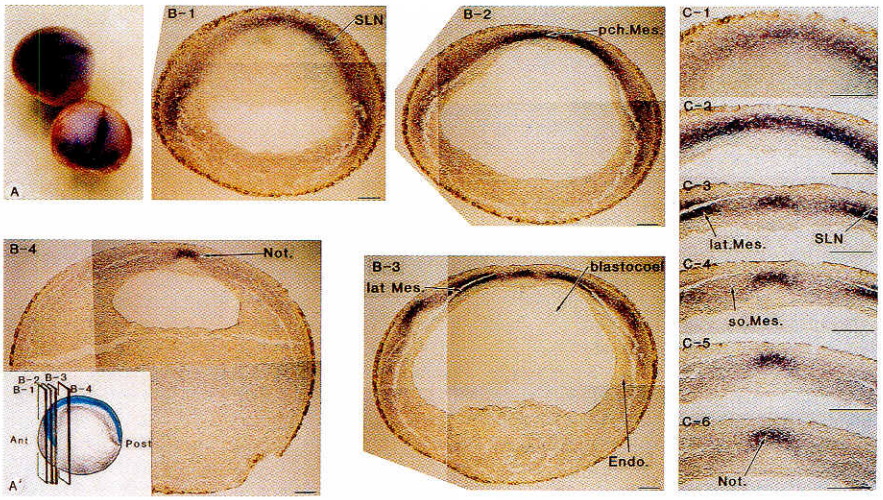

Fig. 3. *X-twi* expression at stage 13/14. (A) *Stage 13/14 embryos exhibit an anchor-shaped distribution of X-twi transcripts in their antero-dorsal region.* **(A')** *Schematic drawing of the embryo with the plan of different sections.* **(B-1 to B-4)** *On serial transverse sections, the most antero-lateral label is located within the SLN and the prechordal mesoderm (B-1 and B-2); more caudally, the X-twi transcripts are also located within the dorsal layer of the lateral mesoderm (B-3) and finally, within the posterior regions, X-twi RNA are mainly restricted to the forming notochord (B-4).* **(C-1 to C-6)** *Higher magnifications of the transverse sections point towards the restricted location of the X-twi transcripts in the prechordal mesoderm and SLN (C-1 and C-2), the forming notochord (C-3 to C-6), the SLN (C-2 and C-3), the lateral plate mesoderm (C-3 to C-6) and its exclusion from the somitogenic mesoderm (C-3 and C-4). Ant., anterior region; Endo., endodermal cells; lat. Mes., lateral plate mesoderm; Not., notochord; pch. Mes., prechordal mesoderm; so. Mes., somitogenic mesoderm; Post., posterior region; SLN, sensorial layer of the neurectoderm. Bar, 100 mm.*

observed at the animal pole, in the two-cell thick layer covering the blastocoel.

In whole embryos at about stage $11^{1/2}$, *X-twi* transcripts are faintly detected on the dorsal midline region as a short streak of labeled cells near the blastopore (Fig. 2B-1 for a posterior dorsal view of two embryos: the left one was hybridized to the antisense probe, the right one is the control hybridized with the sense probe). Sections show that in the embryo that was hybridized with the antisense probe, *X-twi* transcripts are detected in the involuted cell layer forming the presumptive chordo-mesodermal layer, and also in the part of the sensorial layer of the dorsal ectoderm (prospective neurectoderm: SLN) facing the involuted presumptive chordo-mesoderm, but not in the cells forming the archenteron roof (Fig. 2B-2 and 2B-3 for sections). No *X-twi* transcripts are detected in the involuting dorsal mesoderm near the blastopore. We noticed that at that stage, those cells that do express *X-twi* appear to be subsets or groups of cells rather than an homogenous germ-layer.

In stage 13/14 embryos, at initial neural plate stage, *X-twi* transcript location paints an arrow, with the two lateral branches following the anterior/lateral regions of the embryo, and a streak of labeled cells lining the dorsal region (Fig. 3-A for two whole embryos viewed from their dorsal side). The analysis of serial sections shows that the major sites of *X-twi* expression at that stage are the prechordal plate, the axial mesoderm and the forming notochord, the SLN, and the lateral mesoderm ; there is an obvious decreasing gradient of expression from anterior to posterior regions (Fig. 3B-1 to 3B-4 and 3C-3 to 3C-6 for more anterior to more posterior sections). The SLN, which is markedly thicker in the anterior lateral regions, contains less *X-twi* transcripts than the center of the notoplate (Fig. 3B-2 and 3B-3), and in posterior regions the only obvious *X-twi* transcript accumulation is in the notochord (Fig. 3B-4). Higher magnifications of anterior to posterior sections point towards the notochord and axial mesoderm formations (Fig. 3C-1 to 3C-6). It is noteworthy that X-*twi* is expressed both in the thin layer of mesoderm forming the prechordal plate (Fig. 3C-1) and along the entire developing notochord (Fig. 3C-2 to 3C-6), while in the axial mesoderm nearby, its expression is down regulated at that stage (see in particular Fig. 3C-3 and 3C-4).

X-twi expression at the neural groove stage

Two hybridized embryos at about stage 17, the lower being albinos, are presented from a dorsal-anterior view in Figure 4A, with *X-twi* expression mainly in the neural crests. Serial sections from anterior to posterior regions show that *X-twi* expression occurs in the

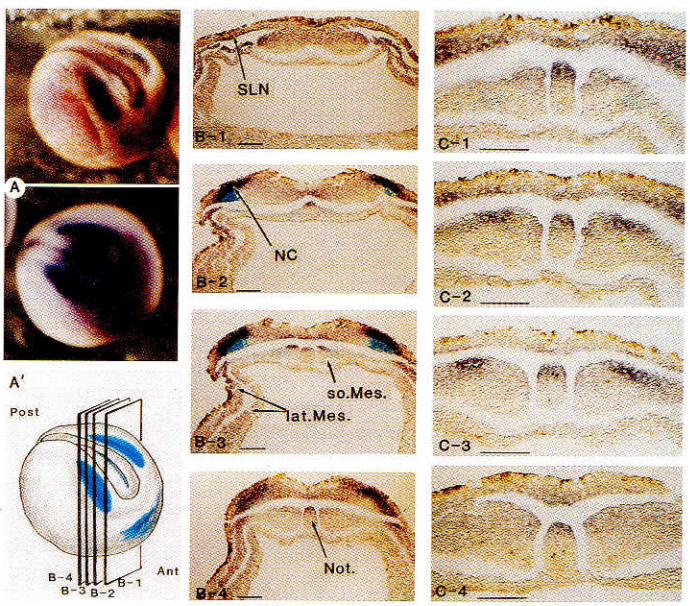

Fig. 4. *X-twi* expression at stage 16/17. (A) *A dorsal-anterior view of two embryos shows massive accumulation of X-twi transcripts within the neural crest cells.* **(A')** *Schematic drawing of the embryo, with the plan of different sections.* **(B-1 to B-4)** *Transverse sections through the embryo (anterior to posterior) show X-twi transcripts in the SLN (B-1), and in the neural crest cells, the inner layer of the neural floor, in the dorsal part of the notochord and of the somites (B-2 to B-4), and more weakly in the lateral mesoderm on more caudal sections (B-3 and B-4).* **(C-1 to C-4)** *Serial sections from anterior to posterior regions of another embryo at the same stage illustrate the restricted location of X-twi in the somitogenic mesoderm. Ant., anterior region; so. Mes., somitogenic mesoderm; lat. Mes., lateral plate mesoderm; NC, neural crest cells; Not., notochord; Post., posterior region; SLN, sensorial layer of the neurectoderm. Bar, 100 mm.*

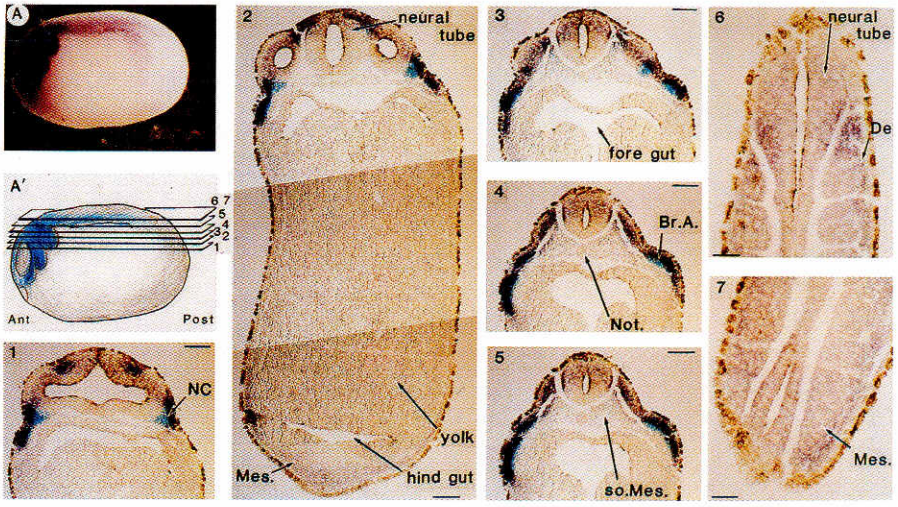

Fig. 5. *X-twi* expression at stage 22/23. (A) *On a whole-mount lateral view of a stage 22/23 embryo, X-twi transcripts are located in the migrating neural crest cells in the cephalic region and along the anterior/posterior dorsal axis of the embryo.* **(A')** *Schematic drawing of the embryo with the plan of different sections.* **(1** *to* **7)** *Frontal sections show that X-twi transcripts are located within the migrating neural crest cells of the head (1 to 5). The somitic mesoderm contains also X-twi transcripts (6 and 7), with a higher level of transcripts within the dermatome region of the anterior differentiated somites (6). Ant., anterior region; Br. A., branchial arch cells. De., dermatome. Mes., mesodermal cells; so. Mes., somitogenic mesoderm; NC, migrating neural crest cells; Not., notochord; Post., posterior region. Bar, 100 mm.*

SLN (Fig. 4B-1), massively within cells of the neural crest (Fig. 4B-2), at lower levels in the floor plate, in the dorsal part of the notochord and of the somites and in the lateral plate mesoderm (Fig. 4B-3 and 4B-4). At a higher magnification are shown serial sections from anterior to posterior regions of another embryo at about the same stage where an obvious dynamic restriction of *X-twi* expression is observed within the forming and differentiating axial mesoderm: in anterior regions, *which is* at a more "advanced stage" in the differentiation of the axial mesoderm, *X-twi* is weakly expressed in the somitogenic mesoderm, as well as in the dorsal part of the notochord and in the SLN (Fig. 4C-1 and 4C-2). As sections progress towards posterior regions, the SLN contains less *X-twi* transcripts, and the latter are progressively excluded from the somitogenic mesoderm near the notochord (Figs. 4C-2 and 4C-3), while there is a low but almost uniform expression in the dorsal moiety of the caudal mesodermal layer (Fig. 4C-4).

X-twi expression at early tail bud stage

At early tail bud stage (stages 22/23), *X-twi* transcripts are abundant at the anterior side of the embryo, in the three first visceral pouches (future mandibular, hyoid and branchial arches respectively) and more diffusely located along the dorsal part of the embryo (Fig. 5A).

On frontal sections of the anterior region, *X-twi* RNA is abundant in cells of the branchial pouches and at lower levels in the dorsalmost and lateral parts of the diencephalon (Fig. 5-1). On Figure 5-2, *X-twi* transcripts are visualized in the head mesenchyme and in the branchial pouches, in the dorsal part of the diencephalon and of the eye vesicles, and weakly in the lateral and caudal mesodermal layers. On slightly more posterior/dorsal sections, *X-twi* transcripts are observed within the dorsalmost part of the rhombencephalon, in the branchial pouches, and in the somitic mesoderm (Fig. 5-3 to 5-5 for more anterior to more posterior sections of the embryo respectively), but no *X-twi* transcripts are detected in the notochord. In the most posterior/dorsal sections, *X-twi* transcripts are in the dorsal part of the neural tube and the lateral region of the anterior somites (Fig. 5-1), while in the posterior region they are less abundant (Fig. 5-2).

At stage 29/30, *X-twi* transcripts are observed in the head region, within the mandibular, hyoid and branchial arches, and caudally in the dorsal/lateral regions of the embryo (Fig. 6A). On frontal sections, *X-twi* transcripts are observed in the mandibular arch cells lying near to the cement gland cells derived from the epithelial layer of the ectoderm (Fig. 6B-1), in the hyoid and massively in the branchial arch cells (Fig. 6B-1 to 6B-3). On median sections *X-twi* transcripts are also observed within the dorsalmost part of the somites and of the neural tube, and the caudal somitogenic mesoderm contains more *X-twi* transcripts than the anterior somites which are still differentiating (Fig. 6B-2 and 6B-5). Higher magnification shows *X-twi* transcripts within the mandibular arch cells (Fig. 6C-1), very faintly in part of the heart anlage (Fig. 6C-2), in the anterior somites (Fig. 6C-3) and in the posterior mesoderm (Fig. 6C-4).

At stage 35/36, frontal sections from more anterior to more posterior regions show that *X-twi* transcripts are detected in the whole mesenchyme and in the heart (Fig. 7A-1 and 7A-2), in the aligned mesodermal cells of the hyoid and branchial arches but not within the epidermis cells, (Fig. 7B-1 and 7B-2), and in parts of the otic and optic vesicles, the head mesenchyme, the dorsal parts of the neural tube and of the somites (Fig. 7C-1 and 7D-1). There are less *X-twi* transcripts in the anterior somites than in the posterior ones (compare Fig. 7C-2 and 7C-3). The optic cup and forming lens (Fig. 7D-2), the forming otic vesicles (Fig. 7D-3) and the somites (Fig. 7D-4) are also shown at high magnification.

X-twi expression in "dorsalized" and "ventralized" embryos

To study the relation between *X-twi* expression and the regional organization of the mesodermal layer, early embryos were submitted, independently, to either UV-irradiation, and to LiCl treatment, incubated, and then labeled by whole-mount *in situ* hybridization, using the *X-twi* antisense digoxigenin probe.

A UV-irradiation of the eggs blocks the cortical rotation, and allows the formation of mesoderm with purely ventral character. If *X-twi* transcripts are not detected, dorsal-character specific proteins which are lacking in these ventralized embryos would be required to induce *X-twi*. If *X-twi* RNA are visualized, *X-twi* activation occurs even in the absence of dorsal-specific mesodermal inducers. In UV-irradiated embryos ("acephalic" appearance), the level of *X-twi* transcripts is moderate at equivalent stage 23/24 and higher at equivalent stage 32/33. In all cases, *X-twi* transcripts are

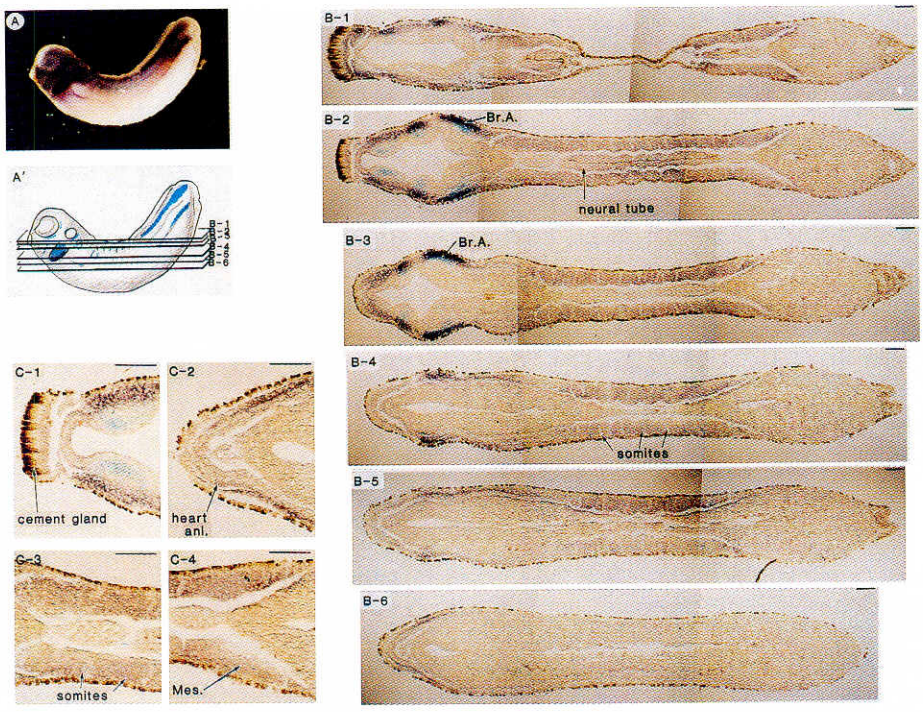

Fig. 6. *X-twi* expression at stage 29/30. (A) *On a whole-mount lateral view of a stage 29/30 embryo, a high X-twi transcript accumulation is seen within the branchial arches and to a lesser extent along the entire dorsal region.* **(A')** *Schematic drawing of the embryo with the plan of different sections.* **(B-1** *to* **B-6)** *Frontal sections show that X-twi transcripts are located within the branchial arch cells in the head region (B-1 to B-4), in the dorsal part of the neural tube (B-1 and B-2), and in the somites (B-1 to B-5).* **(C-1** *to* **C-4)** *Higher magnifications of some sections point towards X-twi accumulation in the first visceral arches (C-1), the anterior somites (C-3) and the posterior mesoderm (C-4). There are very weak amounts of X-twi transcripts in part of the heart anlage (C-2). Br. A., branchial arch cells; Mes., mesodermal cells. Bar, 100 mm.*

restricted to the anterior pole (Fig. 8A for the whole-mount at equivalent stage 23/24, Fig. 8B for the equivalent stage 32/33). Sections of those embryos show that they have developed into a three-layered cylinder, and *X-twi* transcripts are located in the cell layer that lies beneath the ectodermal outer layer (SLN of untreated embryos), and in the periphery of the inner cell mass that might correspond to the mesodermal layer of untreated embryos (Fig. 8A-1 to 8A-3 for equivalent stage 23/24, and Fig. 8B1-8B3 for equivalent stage 32/33). Thus X-*twi* is activated in the absence of dorsal-specific proteins.

LiCl treatment of the embryos is done at stage 6, after cortical rotation, and drives the development of embryos which lack ventral structures, but contain mainly dorsal and anterior structures. If X-*twi* transcripts are seen in such embryos at higher levels than in UV-ventralized ones at about the same developmental stage, dorsal-specific proteins enhance *X-twi* basal expression. If no or weak amounts of *X-twi* RNA are detected, ventral-specific proteins regulate the expression of the gene. LiCl treated embryos at equivalent stage 29/30 ("no axis/radial proboscis" appearance), contain high amounts of X-*twi* RNA at their anterior region (they co-localize with the cement gland region: Fig. 8C). On sections it seems that *X-twi* transcripts are localized in those cells that could be the neural crest cells or the SLN of untreated embryos, and also the head mesenchyme lying near the cement gland (Fig. 8C-1 to 8C-3 for sections). Thus dorsal-specific proteins enhance *X-twi* basal expression.

Discussion

We detected low levels of *X-twi* transcripts in RNA extracted from unfertilized eggs and from early *Xenopus* embryos; these molecules are probably due to maternal genome expression, as the expression of genes under zygotic genome control is only thought to occur from stage $7^1/_2$-8 onwards. Initial gastrulation occurs at stage $10^1/_4$, and *X-twi* transcripts are detected in stage $7^1/_2$ embryos, thus *X-twi* early expression occurs prior to gastrulation stage. The amount of *X-twi* RNA detected prior to stage 11 is so low that it is probably undetectable by Northern blot analyses.

In *Mus* and in *Gallus*, maternal and zygotic compounds were observed respectively for the expressions of *M-twist* and *G-twist* (Bolcato-Bellemin *et al.*, 1 and 2, submitted); this early expression prior to gastrulation is possibly relevant to some early function. However, the interesting discussion about the functional significance of the low early *XMyoD* expression reported by Rupp and Weintraub (1991) can also be applied to *X-twi*: a low early expression might not have any functional importance; but it is also possible that an early burst of transcription would lead later to the establishment of a positive feedback loop that stabilizes gene expression.

In unfertilized eggs, we observed that the *X-twi* transcripts present a decreasing distribution, from animal to vegetative pole, that is similar to the gradients observed for *EF1-α* and for the distribution of the 18S rRNA, but contrasts with the distribution of *Xsna* transcripts, which are more abundant in the vegetative pole. And in stage $6^1/_2$ blastulae, we showed that *X-twi* transcripts are located within the animal region of the embryos, whereas when Hopwood *et al.* (1989) isolated pieces of $8^1/_2$ embryos and cultured them up at equivalent stage $12^1/_2$, they did detect *X-twi* transcripts almost exclusively in cells deriving from equatorial pieces. This might suggest that the locations of the *X-twi* maternal and zygotic transcripts are different. Similar observations have been reported by Rupp and Weintraub (1991), as they detected a low ubiquitously distributed level of *MyoD* throughout the embryo shortly before MBT, and only later a restriction of *MyoD* transcripts towards the marginal zone. It is noteworthy also that these studies allowed us to detect a maternal and a zygotic early expression of *Xsna*, whose expression was until now only reported from stage 9 onwards, in the lower tier of the marginal zone (Essex *et al.*, 1993).

Hopwood *et al.* (1989) noticed that *X-twi* is expressed both in ventralmost and in dorsalmost mesoderm. We showed here that *X-twi* transcripts are detected in cells of the entire presumptive mesoderm cell sheet, although sequentially and at different levels. In addition, late "anteriorized/dorsalized" and "posteriorized/ventralized" *Xenopus* embryos express *X-twi* in accordance with the "dorsalized" and "ventralized" specification of their respective tissues. One hypothesis would be that this expression might be only the simple consequence of the fact that *X-twi* is expressed in

those regions of the late embryos. In which case, minimally these experiments show that the hyper-regionalization of these embryos does not seem to enhance nor inhibit the expression of *X-twi* in the presumptive mesoderm cell layer nor in the SLN. The second hypothesis is that the process of mesoderm regionalization might be independent from – and superimposed on– the induction of *X-twi* expression which would occur early during development. As the UV-treatment is done early enough to block the cortical rotation of the embryos, prior to any mesodermal induction, and as we observed that in these ventralized embryos *X-twi* expression occurs in the absence of dorsalizing factors, these experiments would argue in favor of this second hypothesis, and would suggest that expression of *Xtwi* at least is independent from the regionalization of the mesodermal layer by the dorsalizing factors. Of course, *in situ* hybridization of earlier treated embryos, at gastrula stage, would be useful to get more experimental arguments, but at that stage, as there is no obvious modified phenotype, it is not possible to decide by simple examination of the entire population of treated embryos at which levels some would develop as hyper regionalized.

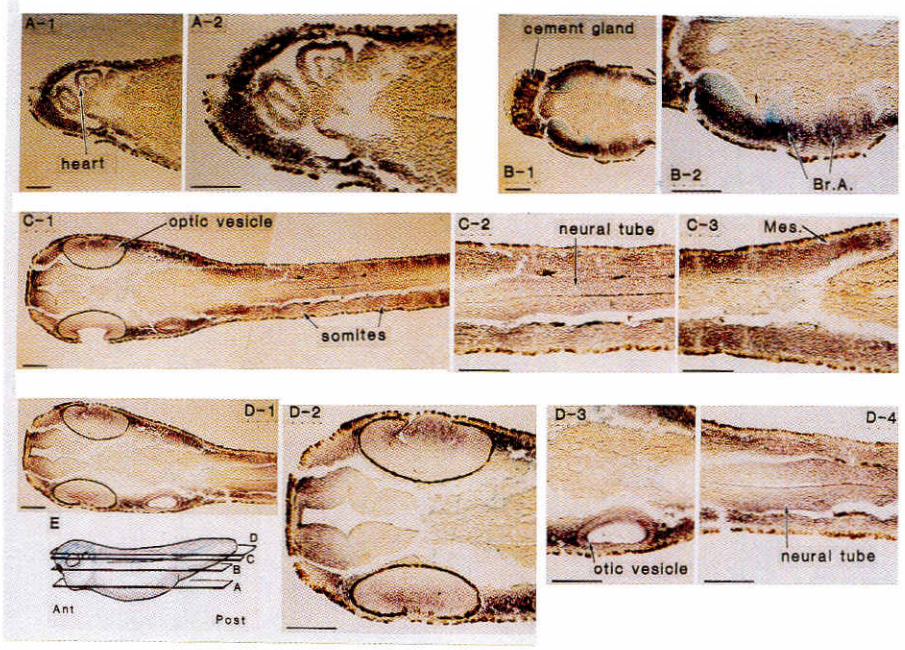

Fig. 7. *X-twi* expression at stage 35/36. *Frontal serial sections show that X-twi transcripts are abundant within the ventral part of the first visceral arches and the forming heart* **(A-1** *to* **B-2)**; *they are less abundant in the anterior somites than in the posterior ones* **(C-1** *to* **C-3)**; *X-twi transcripts are also detected in parts of the otic and optic vesicles* **(D-1** *to* **D-3)**, *and in parts of the neural tube* ***(D-4)***. *E, schematic drawing of the embryo with the plan of different sections; Ant., anterior region; Br. A., cells of visceral arches II and III; Mes., mesodermal cells; Post., posterior region. Bar, 100 mm.*

X-twi was cloned and sequenced by Hopwood *et al.* (1989), and these authors restricted the terminology used in their paper, as no functional analyses had yet been done and as they stated their results could not prove that *X-twi* was homologous to the *Drosophila twist* gene. Our results, that complement their analyses, point towards some common features of the temporal and spatial expressions of the *M-twist*, *G-twist* and *X-twi* genes: these three vertebrate genes are expressed first under maternal genome control and then their transcripts are detected mainly in the entire mesoderm layer of the embryos after gastrulation. Furthermore, gene structure and sequence similarities among them are very high and their temporal and spatial expression patterns are comparable, although some species-specific structures such as the extra-embryonic tissues do not form in *Xenopus*, which is an anamniotic species.

The area of major expression of *X-twi* at neurula stages was later observed using whole-mount *in situ* hybridization. Our observations illustrate a temporal sequence for the accumulation of *X-twi* transcripts, as we show that they are detected earlier in the more presumptive anterior/dorsal structures than in the presumptive ventral/posterior ones, but also that they disappear from the already differentiated structures while still accumulating in the undifferentiated mesoderm cells. Such gradients have also been observed and described for the spatial expression pattern of *M-twist* in murine embryos (Stoetzel *et al.*, 1995). The results that are here presented show also, in particular, that *X-twi* transcripts are transiently detected in the notochord or the neural tube: this fact was already observed for the expression of *M-twist* (Perrin-Schmitt, unpublished).

In *Mus*, and *Homo* genomes respectively, *twist* appears to be a unique gene: Southern blot analyses and chromosomal locations were published for *M-twist* (Mattei *et al.*, 1993) and *H-twist* (Bourgeois *et al.*, 1996; Perrin-Schmitt *et al.*, 1997). For *G-twist* and *X-twist*, Southern blot analyses suggest that this might also be the case (Bolcato-Bellemin *et al.*, 2, Hopwood *et al.*, 1989 and Perrin-Schmitt, unpublished). In any case, all these genes can be considered to be homologous genes, as discussed by Bolker and Raff (1996). Among other sequences related to *twist*, the *Dermo-1* protein bHLH sequence (Li *et al.*, 1995) is highly similar to that of all *twist* proteins. However, the careful alignment of these sequences show that the similarities lie only in that part of their sequences; moreover, the untranslated 5' and 3' sequences of *Dermo-1* are definately divergent from corresponding sequences in all the vertebrate *twist* genes (Perrin-Schmitt *et al.*, 1997, and unpublished). These observations on similarities of the untranslated sequences among the *M-twist*, *G-twist*, *X-twist* and *H-twist* genes, also confirm the hypothesis that these genes might really be considered as homologous genes derived from a common ancestor. Thus, we propose that *X-twi* is a real homolog to the other vertebrate "*twist*" genes and should be called *X-twist*.

Conservation of molecular mechanisms between arthropods and chordates are currently being investigated and demonstrated (Padget *et al.* 1993; Holley *et al.*, 1996; Holley and Ferguson, 1997 for a review).

In *Mus*, we showed that *M-twist* is expressed prior to gastrulation, and the *M-twist*$^{\Delta bHLH}$ mutation, that deletes the bHLH encoding region of the gene, acts as a transient dominant negative allele that synthesizes an abnormal product resulting in an obvious mutant homozygous phenotype from pregastrulation stage onwards, and homozygous lethality at the end of gastrulation (Bolcato-Bellemin *et al.*, 2, submitted). This genetical analysis shows that *M-twist*

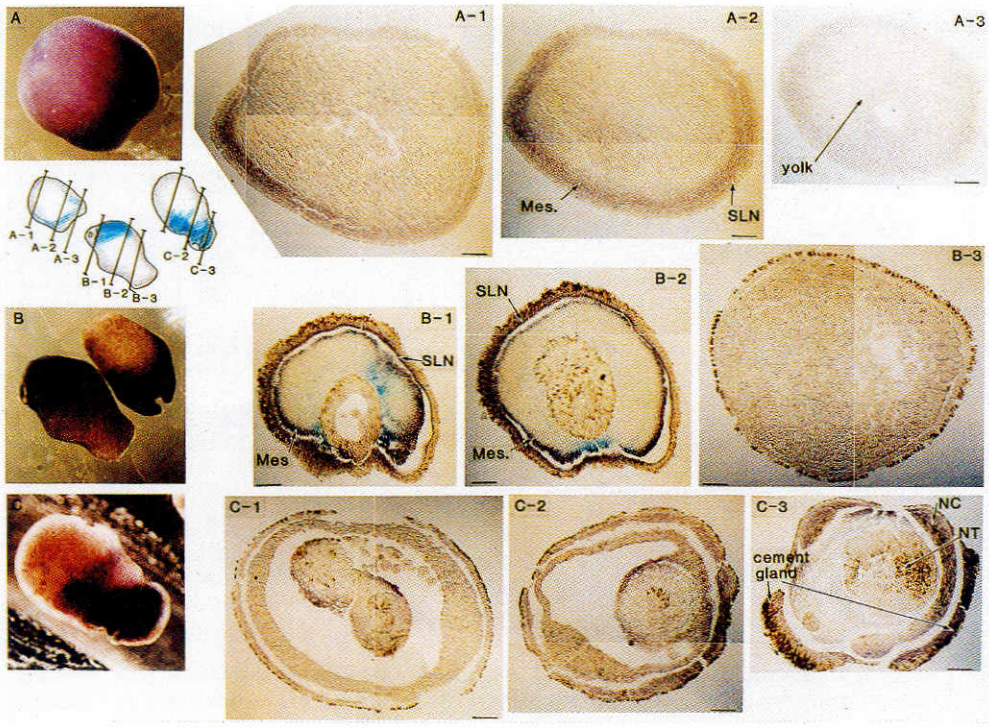

Fig. 8. *X-twi* expression in dorsalized and ventralized embryos. (A and B) *Whole-mounts of UV ventralized embryos at equivalent stages 23/24 (A) and 32/33 (B) respectively.* **(C)** *Whole-mount of a LiCl dorsalized embryo at equivalent stage 29/30. In all cases, X-twi transcripts are restricted to one pole of the embryo.* **(A-1 to C-3)** *Transverse sections through ventralized and dorsalized embryos show that X-twi transcripts are in the cell layers that could be equivalent to the SLN and the facing mesodermal layer of normally developing embryos. Sections A-1 to A-3 correspond to embryo A, sections B-1 to B-3 to embryo B, and sections C-1 to C-3 to embryo C. On drawings, the plan of different sections are presented. Mes., mesodermal cells; NC, neural crest cells; NT, neural tube; SLN, sensorial layer of the neurectoderm. Bar, 100 mm.*

disfunction prior to gastrulation creates major disturbances in embryonic development.

In *Drosophila*, *twist* is the first zygotic gene that is activated in response to *dorsal* (Jiang *et al.*, 1991; Thisse *et al.*, 1991). Twist is required for the proper activation of mesoderm-specific genes such as *tinman* (Bodmer, 1993) and *Dmef2* (Lilly *et al.*, 1995; Taylor *et al.*, 1995) and of *DFR1* (Shishido *et al.*, 1993). Both genetic and molecular studies suggest that differentiation of the mesoderm could be achieved both through the activities of genes such as *even-skipped (eve)* and *fushi-taratzu (ftz)* and through the influence of signals such as *wingless (wg)* and *decapentaplegic (dpp)* (Bate and Baylies, 1996). Thus, both the genes that allow the anterior/posterior axis establishment (such as *eve* and *ftz*), and those that imply germ-layer interactions (such as *wg* and *dpp*) control the mesoderm patterning.

If a parallel might be suggested in *Xenopus*, it is also possible that *X-twi* expression would have two types of functions: first prior to gastrulation, to determine the mesodermal layer, then, as regionalization of the mesoderm occurs, to allow the patterning of its derivatives. These hypotheses need to be verified, but the results that we show here might help to direct future experiments aimed at an understanding of gastrulation in *Xenopus*.

Materials and Methods

Embryological methods

Xenopus embryos were obtained, left to develop and staged; histology was performed as previously described (Nieuwkoop and Faber, 1967; Moon and Christian, 1989; Wolff *et al.*, 1991; Meyer *et al.*, 1995). The different egg regions were obtained by cutting eggs perpendicularly to their animal/vegetative axis into three equal parts; the animal region represents thus $^1/_4$ of the volume of the whole egg, the equatorial region $^1/_2$, and the vegetative region $^1/_4$. The vegetative poles of the embryos were exposed to UV light (254 nm - 0.2 Joules/cm^2) in a UV crosslinker RPN 2500/2501 Amersham Life Science; often all the embryos presented disturbed morphologies, with phenotypes ranging from mild (DAI 2, microcephalic appearance) to strong (DAI 0, bauchstücke appearance). For LiCl treatment, embryos were incubated for 6 min in 0.3 M LiCl at stage 6, and currently they were all with disturbed morphologies ranging from short axis (DAI 7) to radial proboscis (DAI 10). Unincubated eggs were either dejellied with 2% cysteine or directly collected.

Molecular biology techniques

RNA was extracted according to Auffray and Rougeon (1980), and basic molecular biology techniques were as in Sambrook *et al.* (1989).

RT-PCR experiments were performed according to Stoetzel *et al.* (1995). Briefly: the RNAs extracted from 10 embryos of each stage were collected in 20 µl of DEPC-treated H2O. An aliquot of 2 µl of each sample (equivalent to one embryo) was used for reverse transcription, and $^1/_{10}$ of the reaction mixture was then used for PCR reactions with 30 cycle amplifications.

Primers were the following:

X-twi (sense): 5'-CAGGTCTTACAGAGCGACGAGCTGGACTCC-3' *X-twi* (antisense): 5'-GGCTCCCTGCTGTATAGACTGAGTGTCCCA-3' *EF1-α* (sense): 5'-CAGATTGGTGCTGGATATGC-3' *EF1-α* (antisense): 5'-ACTGCCTTGATGACTCCTAG-3' *Xsna* (sense): 5'-AGACCTCACGTCCTTCTCCAGCGAA-3' *Xsna* (antisense): 5'-CGTGCTTGTGAAGGAGGGACATTCT-3'

The *X-twi* cDNA probe was kindly provided to us by J. Gurdon. The *EF1-α* 275bp probe was derived from Krieg and Melton (1989).

The 30-mer 5'-TGCACATCCGGAGCCACACGCTGCCCTGCG-3' *Xsna* probe was synthesized, and γ^{32}P-ATP labeled as in Sambrook *et al.* (1989).

In situ hybridization

Whole-mount *in situ* hybridization and subsequent embryo sectioning were carried out basically as described by Harland (1991), and modified as in Meyer *et al.* (1995). We used the BMPurple (Boehringer, Manheim) as substrate for the AP-enzyme. In some embryos, nearby the standard dark-blue precipitate that marks a high RNA level in some tissues, a turquoise color appears. It labels sites with lower specific RNA concentrations, as it does not

appear in any sense-hybridized control experiments, nor in embryos that contain only low RNA levels. The *X-twi* cDNA fragment used as a matrix to synthesize the digoxigenin probe was prepared as follows: reverse transcription was carried out on total RNA extracted from st. 21 and st. 39 embryos, using the 5'-CTGTAAGTGCTC-3' sequence as primer. A PCR amplification was then performed using the sense (5'-ATGATGCAGGAAGAGTCC-3') and the antisense (5'-GTGAGATGCAGACATGGAC-3') primers, generating a 498bp fragment (nt 152 to 649 on *X-twi* sequence). This cDNA fragment was subsequently cloned into the *EcoRV* site of pBluescript KS$^+$ vector. Digoxigenin labeling reagents were from Boehringer Mannheim (Germany). For the experiments on the blastulae, we used 5 µg/ml RNase for the washes, for all the other stages, we used 10 µg/ml.

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