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Original Article

Comparative analysis of *Engrailed*-1 and *Wnt*-1 expression in the developing central nervous system of *Xenopus laevis*

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ABSTRACT **Expression of the *Engrailed*-1 (*XEn*-1) gene was studied in *Xenopus* embryogenesis by Northern blot analysis and whole-mount *in situ* hybridization. One transcript of 2.2 kb was detected from stage 17 (midneurula) onwards, until stage 47 (swimming tadpole). The expression pattern of the *XEn*-1 gene as revealed by *in situ* hybridization can be divided in three regions. The first domain of transient expression appears at the midneurula stage (st. 17) in the anterior part of the neural fold, forming a complete ring of positive cells at the mid/hindbrain border after neural tube closure. A second region of transient expression is detected as groups of ventro-lateral cells in the spinal cord and the hindbrain from late-neurula till tadpole stages. A third area of transient expression of *XEn*-1 is formed by the anterior part of the developing pronephros. Comparison of *XEn*-1 expression at the mid/hindbrain border with that of the *Xenopus wnt*-1 and *engrailed*-2 genes reveals that *XEn*-1 and *Xwnt*-1, in contrast to *XEn*-2, are both detected in a narrow stripe of positive cells in this region. Analysis in exogastrulated embryos reveals that expression of *XEn*-1 and *Xwnt*-1, but not *XEn*-2, is induced by planar signaling in the presumptive midbrain. Of the three genes only *XEn*-1 is expressed in the floorplate at the mid/hindbrain border, while *Xwnt*-1 is expressed in adjacent cells in the neural ectoderm. The results suggest that in vertebrates at the interface between cells in the floorplate and in the paraxial neuroectoderm, at the limited region of the mid/hindbrain border, *En*-1 interacts with *wnt*-1 in a signaling pathway analogous to the *engrailed/wingless* signaling in the parasegments of the *Drosophila* embryo.**

KEY WORDS: *engrailed, wnt-1, central nervous system, development, Xenopus laevis*

Introduction

Homeodomain proteins act as sequence specific transcription factors that regulate cell fate during development (Levine and Hoey, 1988). The *engrailed* genes define a subset of homeobox genes that have been highly conserved during evolution (Patel *et al.*, 1989). In *Drosophila*, mutational analysis has demonstrated that *engrailed* plays multiple roles during development (Morata and Lawrence, 1975; Nüsslein-Volhard and Wieschaus, 1980; Kornberg, 1981a,b). *Engrailed* functions in segmentation of the blastoderm (Kornberg *et al.*, 1981b; Coleman *et al.*, 1987), and is expressed during neurogenesis in a subset of neurons and neuroblasts (Brower, 1986; DiNardo *et al.*, 1988; Patel *et al.*, 1989). In all species examined, expression of *engrailed* is found in the central nervous system in line with the suggestion that one of the functions of *engrailed* is to control cell fate during neurogenesis (Patel *et al.*, 1989).

In most vertebrates, two different *engrailed*-like genes have been identified, and have been designated originally as *En*-1 and *En*-2 (Joyner and Martin, 1987). Expression of both *engrailed* genes during mouse development is observed in the early neural plate at the mid/hindbrain junction (Joyner *et al.*, 1985; Joyner and Martin, 1987; Davidson *et al.*, 1988; Davis and Joyner, 1988; Davis *et al.*, 1988). Detailed analysis of the expression patterns of both *En* genes showed that *En*-1 RNA can be detected first in the central nervous system (CNS) at the one somite stage, extending to the anterior margins of the midbrain rostrally, and into the metencephalon caudally (McMahon *et al.*, 1992). The expression of *En*-1 is transient and decreases at 15 days of embryonic development. *En*-2 expression is not detected until the 5-somite stage and is consistently weaker until the midsomite stage (9.5 dpc), and still detectable in the adult brain (Bally-Cuif *et al.*, 1992; McMahon *et al.*, 1992).

In the zebrafish, three *engrailed* genes have been identified, two of which are closely related to the *En*-2 gene (*eng*2 and *eng*3) (Fjose *et al.*, 1992), one resembling most the *En*-1 gene (*eng*1)

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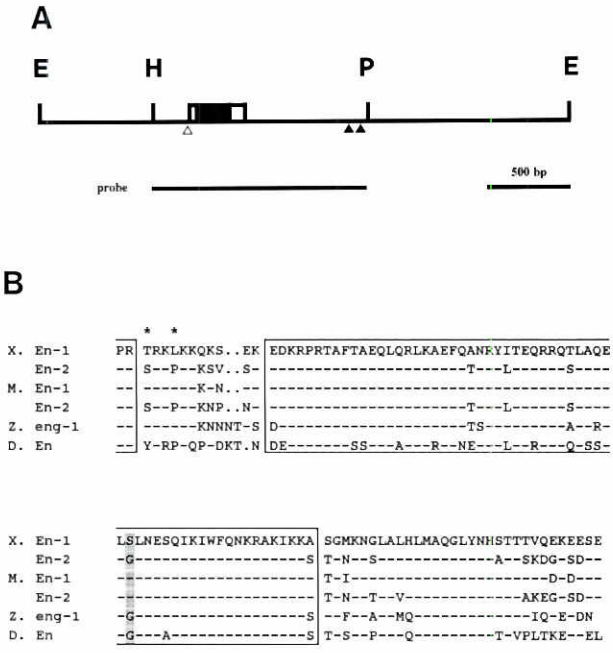

Fig. 1. Analysis of *Xenopus engrailed*-1 genomic DNA. (A) *Schematic representation of the isolated 3.0 kb genomic clone. The open box represents the open reading frame which starts at a splice acceptor site indicated by an open triangle. The filled box represents the homeodomain. Filled triangles represent putative polyadenylation signals. The probe used for Northern blotting experiments and in situ hybridization is indicated. Abbreviations: E, EcoRI; H, HincII; P, PstI.* **(B)** *Alignment of the predicted amino acid sequences of Xenopus engrailed-1 and engrailed-2 (Hemmati-Brivanlou et al., 1991), mouse En-1 and En-2 (Joyner and Martin, 1987), zebrafish eng1 (Ekker et al., 1992) and Drosophila engrailed (Poole et al., 1985). Amino acids identical to XEn-1 are replaced by dashes and gaps were inserted to improve alignment. Boxed areas represent homology regions 2 and 4 (homeodomain) (Logan et al., 1992). The Serine/Glycine residue important for recognition by the antibody 4D9 is indicated. Asterisks represent specific amino acids for engrailed-1 genes.*

(Ekker *et al.*, 1992). Expression of all three genes is detected at 12 h (6-somite stage) at the border of the mid- and hindbrain. The expression patterns of the three *engrailed* genes overlap, the *eng*1 expression being the most restricted in antero-posterior direction (Ekker *et al.*, 1992).

In *Xenopus*, only an *En*-2 gene and its expression have so far been studied in detail (Hemmati-Brivanlou and Harland, 1989; Davis *et al.*, 1991; Hemmati-Brivanlou *et al.*, 1991). Northern analysis showed two transcripts of 1.7 and 3.0 kb that were first detected at early neurula stage (stage 14, stages according to Nieuwkoop and Faber, 1967), peak around late neurula stage (stage 20) and persist in tadpoles (stage 36) (Hemmati-Brivanlou *et al.*, 1991). Immunohistochemical studies using the monoclonal antibody 4D9 (Patel *et al.*, 1989) showed the presence of En-2 protein in the CNS at the mid/hindbrain border (Hemmati-Brivanlou and Harland, 1989; Davis *et al.*, 1991; Hemmati-Brivanlou *et al.*, 1991).

We isolated a *Xenopus engrailed*-1 gene and determined its expression pattern. Comparison of the spatio-temporal expression pattern of *XEn*-1 with those of *Xwnt*-1 and *XEn*-2, show colocalization of *XEn*-1 and *Xwnt*-1 RNA at the mid/hindbrain border of the developing CNS. The *XEn*-1 expression in the floorplate, suggests that at least in *Xenopus En*-1 functionally interacts with *wnt*-1 at the interface between the floorplate and paraxial neural plate ectoderm at the mid/hindbrain border.

Results

Isolation of genomic XEn-1 sequences

A *Xenopus* genomic library (Kintner and Melton, 1987) was screened with a mouse *En*-1 probe (Martin and Frohman, 1989). A hybridizing 3.0 kb *Eco*RI fragment (Fig. 1A) was subcloned and partially sequenced. The nucleotide sequence reveals an open reading frame starting at a position corresponding to a splice acceptor site as identified in *engrailed* genes from other species (Logan *et al.*, 1992). Comparison of *engrailed* genes of different species has identified 5 highly conserved regions, the homeobox

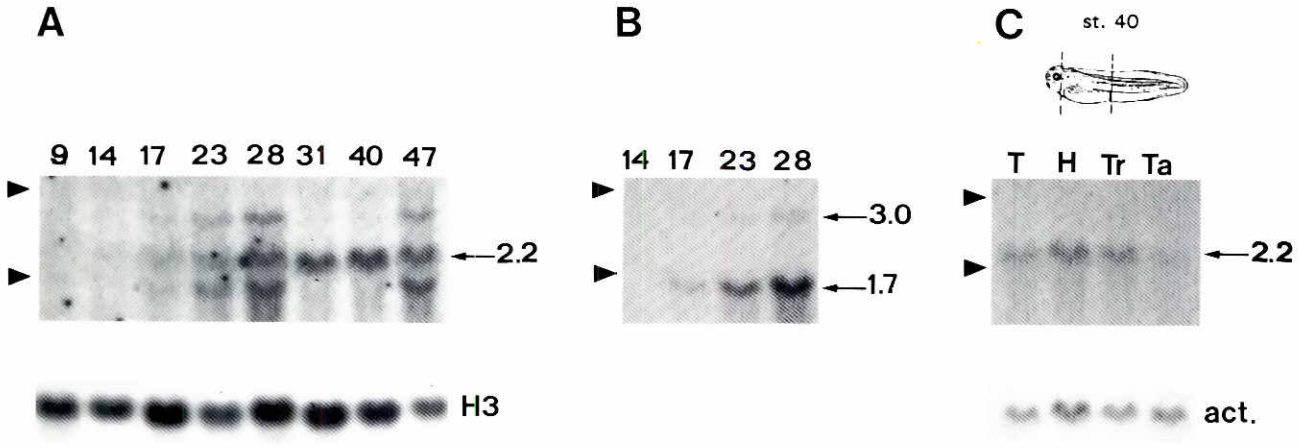

Fig. 2. Northern blot analysis of *Engrailed* expression in different developmental stages. (A) *Expression pattern of the XEn-1 RNA from blastula to swimming tadpole, the bottom panel reflects reprobing the same filter with a Histone H3 probe.* **(B)** *Expression of the XEn-2 RNA detected by reprobing the same filter as used for panel A.* **(C)** *Spatial distribution of the XEn-1 transcripts in a stage 40 tadpole. The top panel represents the dissection. The middle panel shows the XEn-1 transcripts are present in all three regions. The bottom panel shows a reprobing of the same filter with an actin probe. The 28S and 18S ribosomal RNA markers are indicated by arrowheads. H, Head; Tr, Trunk; Ta, Tail.*

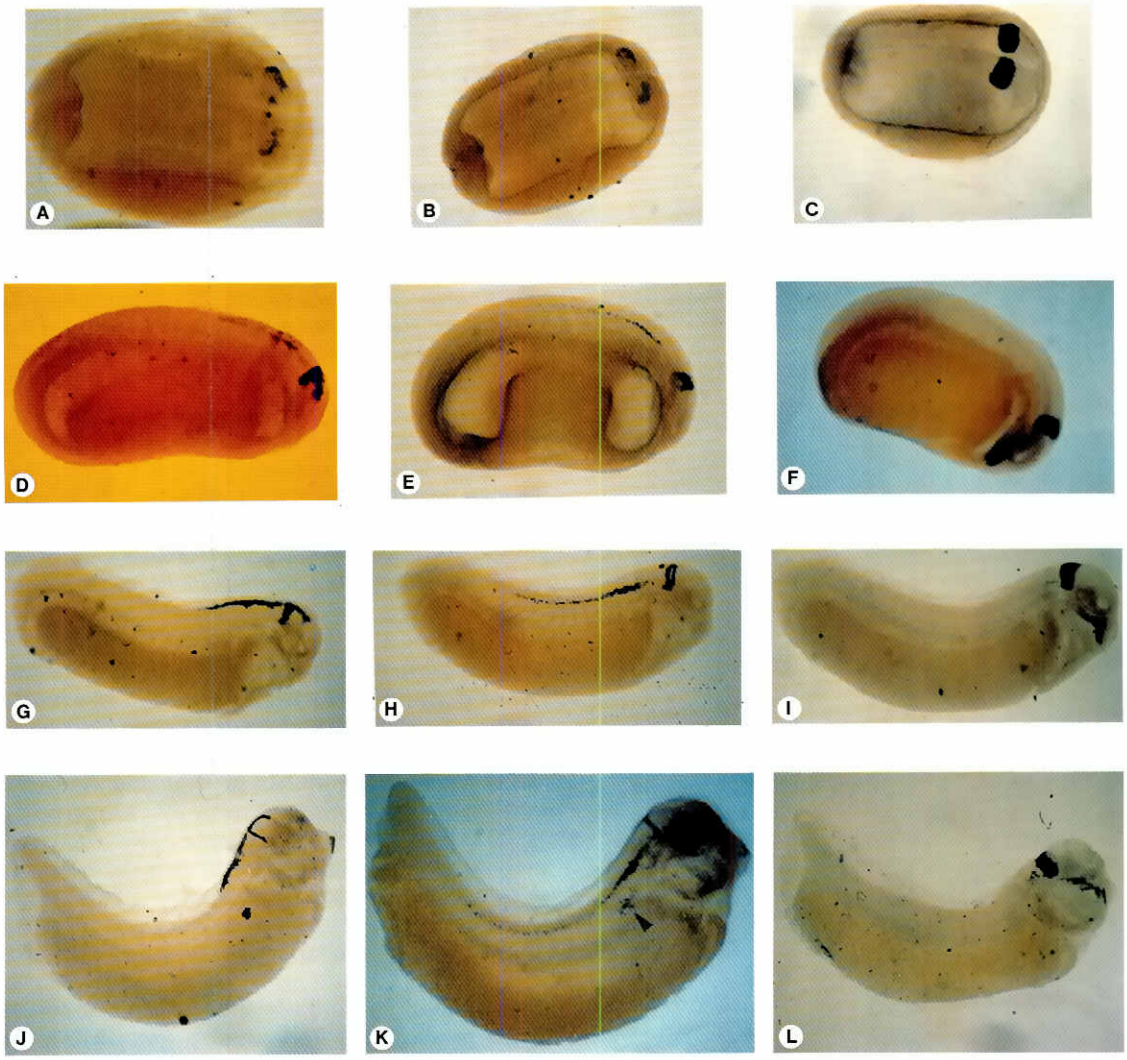

Fig. 3. Comparison of the expression pattern of *XEn*-1 with those of *XEn*-2 and *Xwnt*-1. *Expression of XEn-1* **(B,E,H,K)**, *XEn-2* **(C,F,I,L)** *and Xwnt-1* **(A,D,G,J)** *during development. Whole-mount in situ hybridization of stage 17* **(A,B,C)**, *stage 22* **(D,E,F)**, *stage 26* **(G,H,I)** *and stage 35* **(J,K,L)** *Xenopus embryos. XEn-1 transcripts are localized at the presumptive midbrain early in development* **(B)**, *forming a ring at the mid/hindbrain border later in development* **(E,H,K)**. *XEn-1 transcripts are also detected in groups of ventro-lateral cells in the spinal cord at late neurula stages* **(E,H,K)**. *This expression pattern extends anteriorly during development up to the mid/hindbrain border* **(K)**. *The XEn-1 gene is transiently expressed in part of the developing pronephros starting at stage 35 (arrow in* **K**). *Xwnt-1 and XEn-2 transcripts are also detected at the presumptive midbrain early in development (***A** *and* **C** *respectively) forming a ring at the mid/hindbrain border later in development (***D,G,J** *and* **F,I,L** *respectively) however XEn-2 transcripts are detected over a broader region in antero-posterior direction compared to both XEn-1 and Xwnt-1. Xwnt-1 transcripts are also detected at the dorsal midline of the mes- and rhombencephalon* **(D,G,J)**. *XEn-2 transcripts are also detected in the mandibular arch* **(F,I,L)** *and optic tectum and pituitary* **(L)**. *Embryos are oriented with their anterior part to the right.*

forming the fourth region (Logan *et al.*, 1992). The putative coding region of the present *Xenopus engrailed* gene contains the last 2 amino acids of region 2 and the complete regions 3-5.

Fig. 1B shows the amino acid sequence of *engrailed* proteins from *Drosophila*, zebrafish, mouse, *Xenopus* and the deduced sequence of the present *XEn*-1 protein (see box Fig. 1A). The homeodomain of the predicted protein from the *XEn*-1 clone is homologous to that of the murine *En*-1 and shares similarity with murine *En*-2 (95%), *Xenopus En*-2 (92%), zebrafish *eng*-1 (90%) and with *Drosophila engrailed* (75%). We conclude that we have isolated genomic sequences of the *Xenopus engrailed*-1 gene. Comparison of the predicted amino acid sequences of *XEn*-1 outside the homeodomain (similarity regions 3 and 5) with those of other *engrailed* genes supports this conclusion (Fig. 1B, asterisks).

Previously, the monoclonal antibody 4D9 has been used for immunohistochemical studies. This antibody has been shown to recognize a stretch of 14 amino acids in the engrailed homeodomain. Especially the Glycine residue present at position 5 (as indicated by the hatched box in Fig. 1B) of this stretch is important. For both murine *engrailed* genes, substitution of a Serine for this Glycine abolishes recognition by the 4D9 antibody (Patel *et al.*, 1989). Since the *XEn*-1 gene codes for a Serine at this position (Fig. 1B), the protein product of the *XEn*-1 gene can not be recognized by the 4D9 antibody (K. Eizema, unpublished results).

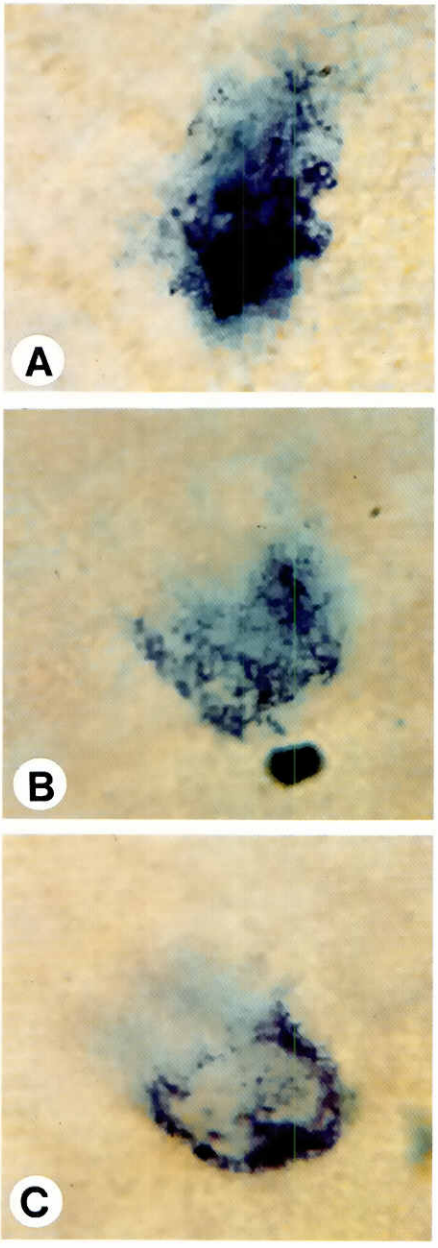

Fig. 4. Transient expression of the *XEn*-1 gene in the developing pronephros. *Enlargement of the expression pattern of the XEn-1 gene in the pronephros at stage 37* **(A)**, *stage 40* **(B)** *and stage 41* **(C)**.

The 3' part of the *XEn*-1 genomic fragment contains two possible adenylation sites and represents the complete trailer region (cf. Watanabe *et al.*, 1993). Interestingly, the 78 nucleotides preceding the first possible adenylation site shows 70% homology with the corresponding regions in the mouse and human *En*-1 genes. This region is contained within a larger region of homology between mouse and human *En*-1 genes suggesting a functional significance and a possible role in gene expression regulation (Logan *et al.*, 1992).

Sequence analysis of the 5' part of the fragment showed no similarity to known coding sequences. Further analysis of the *XEn*-1 gene is performed to identify more 5' coding, leader and promoter sequences (Eizema *et al.*, in preparation).

Expression of Xenopus engrailed-1 mRNA during early development

Northern blot analysis of RNA isolated from embryos at different stages of development with a probe containing the coding region of *XEn*-1 (indicated by the black bar in Fig. 1A) revealed three transcripts, 1.7, 2.2 and 3.0 kb in length (Fig. 2A). Two transcripts, 1.7 and 3.0 kb in length, were detected by Hemmati-Brivanlou *et al.* (1991) in similar developmental stages using an *engrailed*-2 probe. After rehybridization of the filter with the *Ava*II-*Eco*RI fragment of the 1.4 kb cDNA containing the coding region of *XEn*-2 (Hemmati-Brivanlou *et al.*, 1991) indeed two *XEn*-2 transcripts (i.e., 1.7 and 3.0 kb in length) were detected (Fig. 2B), suggesting that the 2.2 kb messenger is derived from the *XEn*-1 gene (cf. Fig. 2A and B). The 2.2 kb *XEn*-1 messenger is present from stage 17 onwards until at least stage 47 (Fig. 2A). The relative abundance of the *XEn*-1 transcript increased until stage 28 (tailbud), whereafter it remained constant until stage 47 (swimming tadpole).

To determine the regional distribution of *Xenopus engrailed*-1 RNA we dissected stage 40 embryos into head, trunk and tail. Figure 2C shows that the messenger of *XEn*-1 is present in all three parts of the embryo but predominantly in the head and trunk regions.

Spatio-temporal expression of engrailed-1 in Xenopus embryos as determined by whole-mount in situ hybridization

To determine the spatio-temporal expression pattern of *XEn*-1 whole-mount *in situ* hybridization was performed using the same probe as for the Northern blot experiments. An overview of the expression of the *XEn*-1 gene in embryos ranging from stage 17 to stage 35 is presented in Figure 3 (B,E,H,K).

In short, expression of *XEn*-1 was detected in three areas of the developing embryo; (i) in groups of ventro-lateral cells in the spinal cord and hindbrain, (ii) in the pronephros and (iii) at the midbrain/hindbrain border, the latter region being the first to show expression.

The staining of cells in the ventro-lateral part of the CNS was first detected at stage 20. This staining has an anterior limit just posterior to the otic vesicle — where an accumulation of positive cells is observed — and extends posteriorly along the spinal cord (Fig. 3E). The cells showing expression of *XEn*-1 in this ventro-lateral domain are scattered. During development (until stage 40) this expression extends anteriorly all the way to the ring of *XEn*-1 expressing cells at the mid/hindbrain border (Fig. 3H,K).

Starting around stage 35, the expression of the *XEn*-1 gene in the developing pronephros is detected. This expression is transient and was no longer present by stage 42. Expression was detected only in the most anterior part of the pronephros which develops rapidly at these stages (Nieuwkoop and Faber, 1967) (Fig. 4).

At stage 17, before neural tube closure, *XEn*-1 transcripts were detected in two patches on either side of the midline in an area of the neural plate which corresponds to the presumptive midbrain (Eagleson and Harris, 1990) (Fig. 3B). After neural tube closure (stage 22) expression was confined to the presumptive mid/hindbrain border forming a complete ring — including the floorplate — around the cavity of the neural tube (Fig. 3E). Starting at stage 26, the expression at the mid/hindbrain border becomes very restricted in antero-posterior direction resulting in a narrow stripe of only a couple of cells wide expressing *XEn*-1 just posterior of the constriction that separates the mesencephalic and metencephalic vesicles (mid/hindbrain border) (Figs. 3H,K and 5A). Around stage 40 expression of the *XEn*-1 gene is decreasing in the lateral parts

of the ring and becomes restricted to the dorsal and ventral regions of the ring (results not shown).

Comparison of the expression pattern of XEn-1 with those of XEn-2 and Xwnt-1

During development, the *Xenopus engrailed*-2 gene is expressed at a much higher level and over a broader antero-posterior region at the mid/hindbrain junction than is *XEn*-1 (Fig. 3C,F,I,L resp. B,E,H,K). Transcripts of the *XEn*-2 gene are never found in the floorplate at the mid/hindbrain border (data not shown). Consistent with data obtained from northern blots of dissected embryos, *XEn*-2 transcripts are not detected in trunk and tail areas whereas transcripts of the *XEn*-1 gene are found in head, trunk and tail regions (Fig. 2C compared to Hemmati-Brivanlou *et al.*, 1991).

Expression of the *Xwnt*-1 gene can be detected in two areas of the developing CNS; the mid/hindbrain border and the dorsal midline of both the midbrain and the hindbrain. The first expression of *Xwnt*-1 at the mid/hindbrain border shows resemblance to that of the *XEn*-1 gene in this area: Expression as detected around stage 17 is in two patches on either side of the midline and during development this expression becomes restricted in antero-posterior direction resulting in a narrow stripe of *Xwnt*-1 expressing cells just posterior of the mid/hindbrain border (Fig. 3A,D,G,J). Analysis of frontal sections showed colocalization of *XEn*-1 and *Xwnt*-1 transcripts at the ventral side of the border whereas at the dorsal side *Xwnt*-1 is expressed just anterior of *XEn*-1 (data not shown). However, the *Xwnt*-1 gene was never found to be expressed in the floorplate at the mid/hindbrain border (Fig. 5B). Expression of *Xwnt*-1 is also seen along the dorsal midline of the mesencephalon already in stage 20 embryos (Fig. 3D), whereas expression along the dorsal midline of the rhombencephalon is first observed at stage 22 (Fig. 3D) which is consistent with the *wnt*-1 expression pattern as described for the mouse (McMahon *et al.*, 1992) and *Xenopus* tailbud stages (Wolda *et al.*, 1993).

Expression of XEn-1 but not of Xwnt-1 and XEn-2 in the floorplate at the mid/hindbrain border

Fig. 5 shows a comparison of the expression patterns of *XEn*-1 and *Xwnt*-1 at the mid/hindbrain border of stage 32 embryos. Transcripts of *Xwnt*-1 were never detected in the floorplate in contrast to *XEn*-1 which is abundantly expressed in this region at the mid/hindbrain border. Note that the *Xwnt*-1 negative area in the floorplate coincides with the highly positive *XEn*-1 area (Fig. 5). *XEn*-2 expression was never detected in the floorplate of the developing CNS (data not shown). Expression of the *XEn*-1 gene in the floorplate was detected from stage 20 till stage 42 while expression in the lateral part of the neural tube at the mid/hindbrain border was by then hardly detectable (results not shown).

Comparison of the expression pattern of XEn-1 with those of Xwnt-1 and XEn-2 in exogastrulae

Exogastrulae are embryos of which the normally involuting mesodermal cells are forced to evolute out of the embryo thereby forming a meso/endodermal sack which is normally found in the interior of the embryos (Holtfreter, 1933a). The presence of mesoderm is essential for the proper determination of the neuroectoderm (Holtfreter, 1933b). In exogastrulae the development of the neuroectoderm depends on signaling via the adjacent ectoderm and is not influenced by the mesoderm (Holtfreter, 1933a; Ruiz i Altaba, 1992, 1993). Therefore, we used exogastrulae to test whether our genes of interest, *XEn*-1, *Xwnt*-1 and *XEn*-2, are

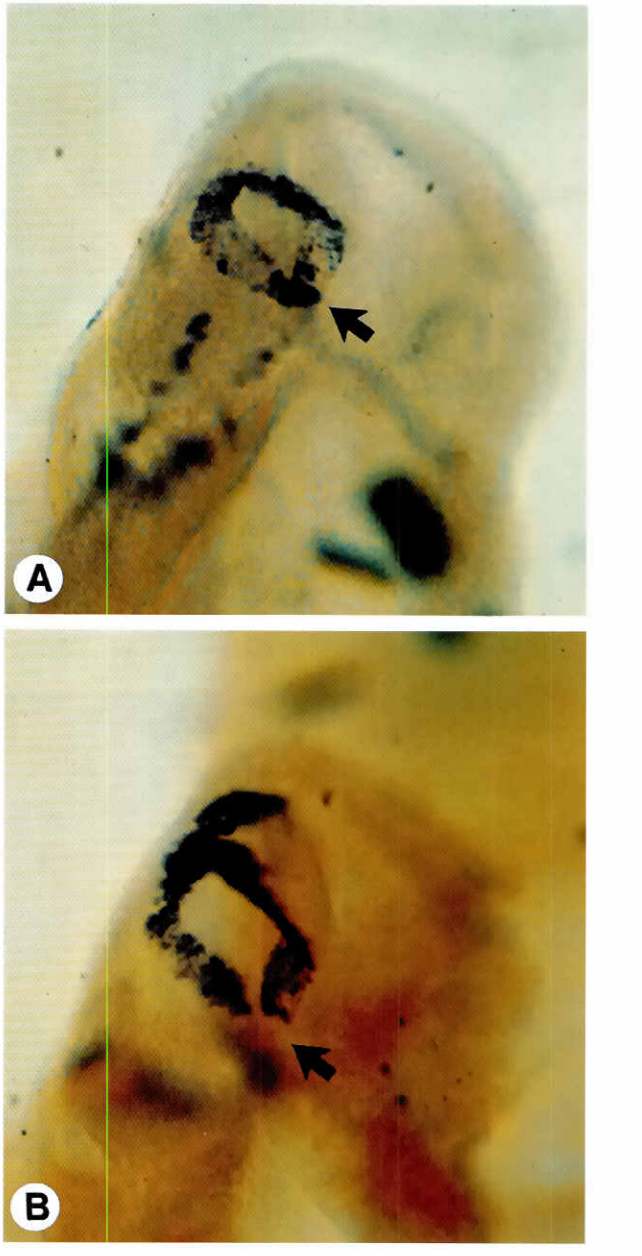

Fig. 5. *XEn*-1 and *Xwnt*-1 expression in the floorplate of the neural tube at the mid/hindbrain border. *Whole-mount in situ hybridization of stage 32 Xenopus embryos with XEn-1* **(A)** *and Xwnt-1* **(B)** *probes viewed from the dorsal side. Both genes display a very restricted area of expression in antero-posterior direction (cf Fig. 3). In dorso-ventral direction the XEn-1 and Xwnt-1 expression patterns differ. In the floorplate XEn-1 transcripts are present* **(A)** *while no Xwnt-1* **(B)** *and/or XEn-2 (data not shown) transcripts can be detected.*

dependent on signals from the mesoderm for their expression in the neuroectoderm (Ruiz i Altaba, 1990). Fig. 6 shows that transcripts of both the *XEn*-1 and the *Xwnt*-1 gene can be detected in the ectodermal part of exogastrulae at stage 25. The expression of the *XEn*-1 and the *Xwnt*-1 genes starts later in time in exogastrulating embryos compared to normally developing siblings (results not shown). The expression pattern of *XEn*-1 in exogastrulae is only partial compared to that of normally developed embryos. In

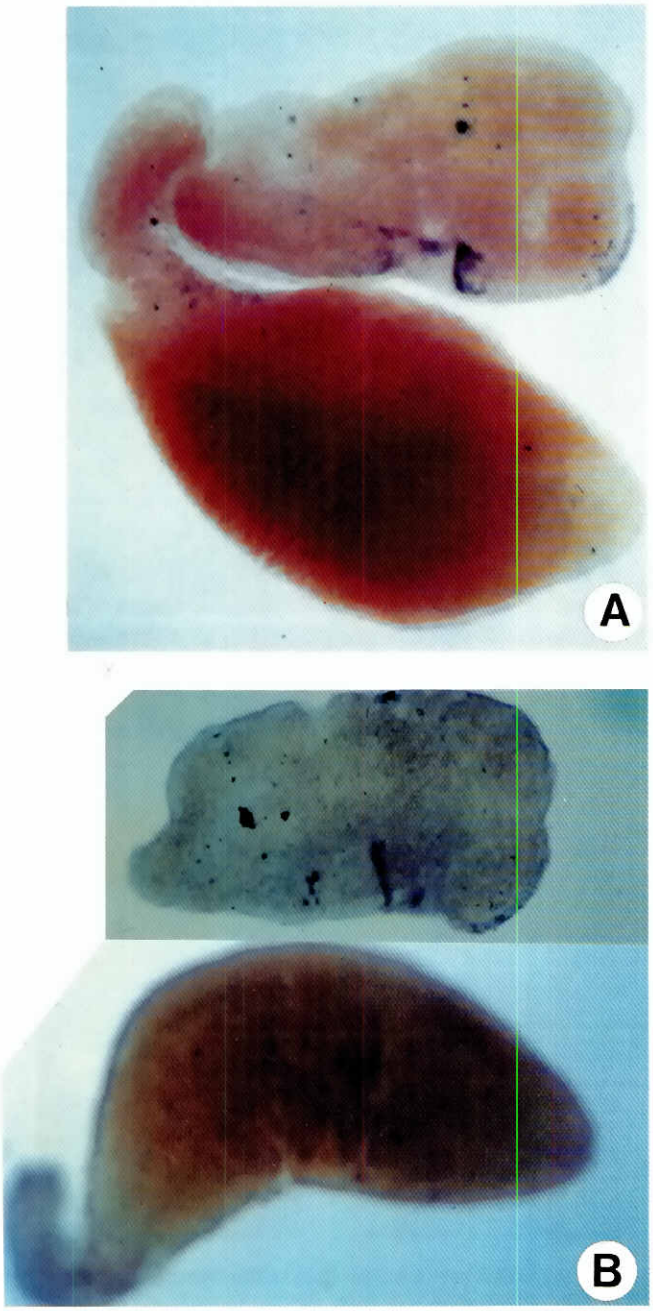

Fig. 6. Expression of *XEn*-1 and *Xwnt*-1 in exogastrulae. *Whole-mount in situ hybridization of exogastrulae corresponding to stage 25 control embryos. Embryos are oriented with anterior to the right and the ectodermal part is at the top of the embryo.* **(A)** *Transcripts of the Xwnt-1 gene are detected only in the ectodermal part of the exogastrulae.* **(B)** *Transcripts of the XEn-1 are only detected in the ectodermal part of the exogastrulae.*

exogastrulae *XEn*-1 expression is restricted to an area of the neuroectoderm presumably corresponding to a region of the mid/hindbrain border (Ruiz i Altaba, 1994). In addition a couple of *XEn*-1 positive cells, which may represent the ventro-lateral expression in the spinal cord and hindbrain, can be detected (Fig. 6B). The pattern of *Xwnt*-1 expression is also partial in exogastrulae: The most anterior neural ectoderm forms a circle equivalent to the expression at the mid/hindbrain border in control embryos. Behind this follows a broad domain of expression equivalent to the dorsal hindbrain expression in normal embryos (Fig. 6A). Transcripts of the *XEn*-2 gene cannot be detected in the ectodermal part of exogastrulae (data not shown) in line with the results of Hemmati-Brivanlou and Harland (1989) and confirming the lack of mesodermal contamination (Ruiz i Altaba, 1994).

Discussion

The amino acid sequence deduced from the open reading frame of the *Xenopus engrailed*-1 (*XEn*-1) studied in this paper, is identical to a partial sequence of the *Xenopus En*-1b homeodomain (Holland and Williams, 1990) and shows one amino acid difference with that of a recently published *XEn*-1 cDNA sequence (Watanabe *et al.*, 1993). Holland and Williams (1990) isolated two *Xenopus engrailed* genes which they designated as *XEn*-1a and *XEn*-1b. At the amino acid level the partial sequences of these genes differ by one amino acid, whereas at the nucleotide level they differ by 9 nucleotides (of a total of 181 nucleotides). On the basis of this sequence information, we cannot exclude the possibility of also detecting *XEn*-1a transcripts using the genomic *XEn*-1 DNA that we isolated as a probe and we therefore designate the gene as *XEn*-1.

The coding region of our genomic *XEn*-1 DNA shows 97% similarity to the published cDNA at the nucleotide level (Watanabe *et al.*, 1993). The differences may be due to variations in the bred of *Xenopus* used to create the DNA libraries.

En-1 expression during Xenopus embryogenesis

Expression of *XEn*-1 was detected in three areas of the developing embryo: in the pronephros, in groups of ventro-lateral cells in the spinal cord and hindbrain, and at the mid/hindbrain border.

The transient expression observed in the pronephros is the only mesodermal *En*-1 expression detected so far in *Xenopus*. *En*-1 expression has been detected in other mesodermally derived tissues in the mouse (cf., dermamyotome and sclerotome *En*-1 expression in the mouse (Davis *et al.*, 1991)). The pronephros and glomus grow considerably at stage 33/34 (Nieuwkoop and Faber, 1967). Starting at stage 37 the pronephros becomes much shorter relative to the growing axial system. Its complexity increases gradually by a further coiling of its tubuli and collecting tube. From stage 53 onwards degeneration phenomena start in the pronephros. Regression of the posterior portion of the glomus, however, starts at stage 41 and terminates at stage 51 (Nieuwkoop and Faber, 1967). It is during the growth and differentiation of the pronephros and the glomus that *XEn*-1 gene expression is observed. *XEn*-1 expression is lost at the time when degeneration of this organ starts (i.e., at stage 41), suggesting a function of En-1 in the transient existence of this embryonic organ.

A second area of *XEn*-1 expression was observed in ventro-lateral stripes in the hindbrain and spinal cord after neural tube closure. Using an antibody recognizing both En-1 and En-2 protein (αEnhb-1), Davis *et al.* (1991) detected staining in two ventro-lateral stripes extending from the rostral hindbrain to the end of the spinal cord in mouse, chicken and frog embryos. Because an En-2 specific antibody did not yield the same staining pattern, Davis *et al.* (1991) suggested that the ventro-lateral staining reflected En-1 rather than En-2 expression. Here we present evidence supporting the view that it is *XEn*-1 and not *XEn*-2 which is expressed in the ventro-lateral stripes. Moreover, our *in situ* data

reveal that at tailbud stages in the developing *Xenopus* embryo, the *XEn*-1 positive cells forming the ventro-lateral groups extend rostrally all the way to the band of *XEn*-1 expressing cells at the mid/hindbrain border. This ventro-lateral *XEn*-1 expression is not segmented as observed in the zebrafish using the αEnhb-1 antibody (Hatta *et al.*, 1991), but appears scattered nonsymmetrically along both sides of the neural tube.

The latter expression pattern of *XEn*-1 may be functional in pattern formation of specific axonal tracts in the central nervous system. In vertebrates, each region of the developing neural tube has its own program of cell division and birthdates of neurons of a given type are generally found to lie within a strictly limited period of development (Jacobson, 1978). As a result, the ventricular zone becomes a mosaic of specialized progenitor cells (Yu *et al.*, 1994). The scattered nonsymmetrical pattern of *XEn*-1 expression along the spinal cord and hindbrain might therefore mark the involvement of XEn-1 in the development of a set of temporally and spatially defined neurons, possibly interneurons (Davis *et al.*, 1991). Whether the *XEn*-1 expression pattern is the consequence or the cause of the processes involved in axon determination needs further research.

Different expression patterns for the XEn-1 gene and the XEn-2 gene at the mid/hindbrain border

The first domain to show expression of the *Xenopus engrailed*-1 gene lies within the CNS, i.e. the presumptive midbrain (Eagleson and Harris, 1990), including the cells at the border of the midbrain and the hindbrain. During neurula stages, *XEn*-1 expression in this area of the developing brain is relatively broad in antero-posterior direction, when compared to the very sharp ring of *XEn*-1 positive cells at tailbud stages. In relation to the fate map of ancestral cell groups in the *Xenopus* neural plate (Eagleson and Harris, 1990), this suggests that after the initial induction of expression of the *XEn*-1 gene at midneurula stage *XEn*-1 expression is maintained in a lineage dependent way.

In the zebrafish, *eng*-1 is expressed in a narrow stripe about half a dozen cells wide at the mid/hindbrain border (Ekker *et al.*, 1992), comparable to the expression of *XEn*-1.

In contrast, expression of the *engrailed*-2 gene is detected over a broad antero-posterior region at the mid/hindbrain border in mouse (McMahon *et al.*, 1992), chicken (Gardner and Barald, 1992), *Xenopus* (Hemmati-Brivanlou *et al.*, 1991, this study) and zebrafish (Ekker *et al.*, 1992; Fjose *et al.*, 1992). On the basis of these distinct expression patterns Ekker *et al.* (1992) proposed that *eng*1 functions in establishing the mid/hindbrain border whereas *eng*2 and *eng*3 may specify other aspects of development in this region of the CNS. The functional distinction between the *En*-1 and *En*-2 genes might be conserved between all vertebrate species with respect to the development in this region of the CNS. Direct evidence for this distinction comes from monitoring the effects of the lack of either the *engrailed*-1 or the *engrailed*-2 gene on the development of mouse embryos. Mice lacking the *engrailed*-2 gene are viable but exhibit abnormal cerebellar foliation, which suggested functional redundancy of *En*-1 and *En*-2 (Joyner *et al.*, 1991; Millen *et al.*, 1994). In contrast, mice lacking the *engrailed*-1 gene die shortly after birth and show multiple developmental defects. Mutant *En*-1 mice have a deletion of mid/hindbrain tissue that includes part of the cerebellum and colliculi (Wurst *et al.*, 1994), suggesting an essential role of En-1 in the formation of parts of both the mid- and hindbrain.

The initial induction of expression of XEn-1 and Xwnt-1 at the mid/hindbrain border is independent of vertical signaling from the axial mesoderm

Comparison of the expression patterns of the *wnt*-1 gene to that of the *engrailed* genes in *Xenopus* embryos, revealed that at the mid/hindbrain border both the *Xwnt*-1 and the *XEn*-1 expression, in contrast to the *XEn*-2 expression, is restricted to a very narrow stripe of cells surrounding the cavity of the neural tube. Furthermore, the onset of expression of both the *XEn*-1 and the *Xwnt*-1 genes is in the presumptive midbrain in two patches at either side of the neural tube at midneurula stages. This suggests that *XEn*-1, rather than *XEn*-2, functionally interacts with *Xwnt*-1 in this area of the developing CNS. This suggestion is supported by our results obtained with exogastrulated embryos. In embryos which have been forced to exogastrulate, both the *XEn*-1 and the *Xwnt*-1 genes are expressed — although at a lower level and later in development compared to control embryos — in adjacent or overlapping areas in the ectodermal part. In contrast, the *XEn*-2 gene is not expressed in the ectoderm of exogastrulae. This indicates that the antero-posterior pattern of expression of *XEn*-1 and *Xwnt*-1 can develop in the absence of underlying axial mesoderm, i.e., in the absence of vertical signals, most likely as a result of induction by planar signals (Ruiz i Altaba, 1994). The *XEn*-2 expression, however, may depend on vertical signaling. For both the *XEn*-1 and the *Xwnt*-1 gene the pattern of expression in the exogastrulae is partial and likely to be equivalent to the region of the mid/hindbrain border in control embryos. This suggests that in vertebrates the expression of *wnt*-1 and *En*-1, but not *En*-2, at the mid/hindbrain border is initiated by common regulators acting in a planar direction, reminiscent of the activation of segment polarity genes (e.g., *wingless* and *engrailed*) by pair rule genes in *Drosophila* embryos (Lawrence, 1992).

En-1/wnt-1 interaction at the interface between the floorplate and the paraxial neural plate ectoderm at the mid/hindbrain border

In *Drosophila*, *wingless* and *engrailed* are expressed in adjacent rows of cells in the posterior half of each segmental primordium and are mutually dependent on each other for the maintenance of their expression (Kornberg *et al.*, 1985; Martinez-Arias *et al.*, 1988; Van den Heuvel *et al.*, 1989; Vincent and Lawrence, 1994). This is achieved through a complex genetic pathway involving several segment polarity genes (DiNardo *et al.*, 1988; Martinez-Arias *et al.*, 1988; Heemskerk *et al.*, 1991; Sampedro *et al.*, 1993; Noordermeer *et al.*, 1994; Siegfried *et al.*, 1994). *wingless* and its vertebrate homologue *wnt*-1 are members of a growing family of related genes that participate in distinct aspects of development (Nusse and Varmus, 1992; Moon, 1993). Grafting part of the mes-metencephalic region of mouse embryos into prosencephalic regions of chicken embryos revealed that the area of *wnt*-1 expression at the mid/hindbrain border was most capable in inducing *engrailed* expression in 'new' sites in the posterior prosencephalon (Bally-Cuif *et al.*, 1992). Requirement of the wnt-1 protein for En-1 expression was confirmed by mice lacking *wnt*-1, showing loss of the complete domain of normally expressing *En*-1 cells (McMahon *et al.*, 1992).

The segment polarity gene *hedgehog* functions in the *wingless/engrailed* signaling cascade where it is required to maintain *wingless* as indicated by genetic analyses (Ingham and Hidalgo, 1993). Recently, vertebrate homologues of the *hedgehog* gene have been cloned and expression of this gene is found in the notochord and floorplate, both of which are important signaling centers in the

regulation of polarity of the developing CNS (Echelard *et al.*, 1993; Krauss *et al.*, 1993; Riddle *et al.*, 1993). Since in *Drosophila hedgehog* and *engrailed* are expressed in the same cells (Lee *et al.*, 1992), a conservation of function of these gene products in vertebrates implies a colocalization of the transcripts of these genes. In *Xenopus laevis* embryos this would be the case in the floorplate of the mid/hindbrain border where the *Xenopus engrailed*-1 gene is highly expressed.

The *Xwnt*-1 gene, however, is not expressed in the floorplate (cf. Fig. 5). Therefore, at the interface between the floorplate and the paraxial neural plate ectoderm at the mid/hindbrain border, *En*-1 might function via the *hh* gene product as a signal, in the maintenance of *wnt*-1 expression and vice versa. Although another *wnt* gene, *Xwnt*-4 (McGrew *et al.*, 1992), is expressed in the floorplate, this gene has a different effect in the biological *Xenopus* embryo assay (Moon, 1993), suggesting that the latter gene product functions in a different signaling cascade.

The following model for the function of En-1 together with wnt-1 and hh can be proposed in the formation of the mid/hindbrain region. At first, before neural tube closure, both *En*-1 and *wnt*-1 are induced by common regulators as part of an antero-posterior patterning system. After neural tube closure *wnt*-1 expression is maintained at the mid/hindbrain border via a signaling center acting in a medio-lateral direction, present in the floorplate, involving En-1 and hh.

Materials and Methods

Isolation of genomic XEn-1 sequences

A genomic library from *Xenopus laevis* stage 33 embryos in EMBL4 (kindly provided by Dr. D.A. Melton) was screened using a homeobox containing 750bp *Bam*HI-*Eco*RI fragment from the mouse *En*-1 genomic clone (Martin and Frohman, 1989). Hybridization with random primed DNA probes was carried out at 65°C in hybridization mixture (6xSSC (1xSSC: 150mM NaCl, 15 mM Na-citrate pH 7.2), 10x Denhardts (1xDenhardt: 0.02% each of BSA, ficoll and polyvinylpyrollidone), 0.1% SDS, 0.1% sodiumpyrophosphate, 0.05 mg/ml salmon sperm DNA). Filters were washed in 2xSSC, 0.1% SDS at 50°C. Positive plaques were isolated and rescreened twice before further analysis.

DNA sequencing and alignment

Subclones of insert DNA were sequenced on both strands by the dideoxy chain termination method (Sanger *et al.*, 1977) using a T7 DNA polymerase sequencing system (Pharmacia). Alignment of sequences to published *engrailed* sequences was performed using the software of MULTALIN (Corpet, 1988).

Northern blotting

Xenopus laevis embryos were obtained by *in vitro* fertilization and developmental stages were determined according to Nieuwkoop and Faber (1967). Total RNA was extracted from intact or microdissected embryos as described by Auffray and Rougeon (1980). Total RNA (23 µg) was fractionated by electrophoresis through 1% agarose gels containing formaldehyde (6% V/V) and transferred to nylon membranes (Qiabrane, Qiagen (USA)/Diagen (Germany). Markers were run in separate lanes and stained with ethidium bromide. After UV-crosslinking of the RNA, filters were hybridized to ^{32}P-random primed labelled DNA probes in hybridization mix (3xSSC, 5xDenhardts, 0.1% SDS, 10% dextransulphate, 0.05 mg/ml salmon sperm DNA and 0.2 mg/ml yeast RNA) at 65°C for at least 12 h. The final wash was in 0.2xSSC, 0.1%SDS at 65°C. Filters were analyzed by autoradiography and relative amounts of RNA transcripts were determined using a phosphor-imager (Molecular Dynamics). The *Hin*cII-*Pst*I fragment of the *XEn*-1 gene (Fig. 1), the *Ava*II-*Eco*RI fragment of the 1.4 kb *XEn*-2 gene (Hemmati-Brivanlou *et al.*, 1991) and the Histone H3 cDNA (Destrée *et al.*, 1984) were used as probes.

Whole-mount in situ hybridization

The protocol of Harland (1991) was followed with minor modifications. Albino *Xenopus laevis* embryos were hybridized to digoxigenin-labeled antisense RNA (1.7 µg/ml) at 65°C in hybridization mix containing total yeast RNA (1 mg/ml). The *Hin*cII-*Pst*I fragment of the *XEn*-1 gene (Fig. 1), the *Ava*II-*Eco*RI fragment of the 1.4 kb *XEn*-2 gene (Hemmati-Brivanlou *et al.*, 1991) and the 1.5 kb *Eco*RI-*Eco*RI fragment of the *Xwnt*-1 cDNA (Noordermeer *et al.*, 1989) were used as probes. The chromogenic reaction with alkaline phosphatase was allowed to proceed up to 24 h after which the embryos were fixed for 4 h in MEMPFA (0.1M MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$ and 4% paraformaldehyde). Embryos were mounted in Murrays clear (2:1 benzyl benzoate/ benzyl alcohol) for photography.

Embryo manipulation

Embryos were dissected into three parts (head, trunk and tail) in 25% MMR (Newport and Kirschner, 1982) using tungsten needles. Using stage 40 embryos two transverse cuts were made: one posterior to the eye and one anterior to the beginning of the tailbud (see also cartoon in Fig. 3).

Exogastrulae

Albino embryos were induced to exogastrulate by incubation in 1.3xMMR (Newport and Kirschner, 1982). At the end of gastrulation (stage 13), exogastrulae with ectodermal sacs that displayed the greatest degree of elongation and with a typical anterior mesoderm bud at the anterior end of the endomesodermal region (Ruiz i Altaba, 1992), were selected for whole-mount *in situ* hybridization analysis.

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References

AUFFRAY, C. and ROUGEON, F. (1980). Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem. 107:* 303-314.

BALLY-CUIF, L., ALVARADO-MALLART, R.M., DARNELL, D.K. and WASSEF, M. (1992). Relationship between *wnt*-1 and *En*-2 expression domains during early development of normal and ectopic met-mesencephalon. *Development 115:* 999-1009.

BROWER, D.L. (1986). *Engrailed* gene expression in *Drosophila* imaginal discs. *EMBO J. 10:* 2649-56.

COLEMAN, K.G., POOLE, S.J., WEIR, M.P., SOELLER, W.C. and KORNBERG, R. (1987). The *invected* gene of *Drosophila:* sequence analysis and expression studies reveal a close kinship to the *engrailed* gene. *Genes Dev. 1:* 19-28.

CORPET, F. (1988). Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res. 16:* 10881-10890.

DAVIDSON, D., GRAHAN, E., SIME, C. and HILL, R. (1988). A gene with sequence similarity to *Drosophila engrailed* is expressed during the development of the neural tube and vertebrae in the mouse. *Development 104:* 305-316.

DAVIS, C.A. and JOYNER, A.L. (1988). Expression patterns of the homeobox containing genes *En*-1 and *En*-2 and the proto-oncogene *int*-1 diverge during mouse development. *Genes Dev. 2:* 1736-1744.

DAVIS, C.A., HOLMYARD, D.P., MILLEN, K.J. and JOYNER, A.L. (1991). Examining pattern formation in mouse, chicken and frog embryos with an *En*-specific antiserum. *Development 111:* 287-298.

DAVIS, C.A., NOBLE-TOPHAM, S.E., ROSSANT, J. and JOYNER, A.L. (1988). Expression of the homeobox containing gene *En*-2 delineates a specific region of the developing mouse brain. *Genes Dev. 2:* 361-371.

DESTRÉE, O.H.J., BENDIG, M.M., LAAF, de R.T.M. and KOSTER, J.G. (1984). Organization of *Xenopus* histone gene variants within clusters and their transcriptional expression. *Biochim. Biophys. Acta 782:* 132-141.

DINARDO, S., SHER, E., HEEMSKERK-JONGENS, J., KASSIS, J.A. and O'FARRELL, P.H. (1988). Two-tiered regulation of spatially patterned *engrailed* expression during *Drosophila* embryogenesis. *Nature 332:* 604-609.

EAGLESON, G.W. and HARRIS, W.A. (1990). Mapping of the presumptive brain regions in the neural plate of *Xenopus laevis*. *J. Neurobiol. 21:* 427-440.

ECHELARD, Y., EPSTEIN, D.J., St.-JACQUES, B., SHEN, L., MOHLER, J., McMAHON, J.A. and McMAHON, A.P. (1993). *Sonic Hedgehog*, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell 75:* 1417-1430.

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

FJOSE, A., NJOLSTAD, P.R., NORNES, S., MOLVEN, A. and KRAUSS, S. (1992). Structure and early embryonic expression of the zebrafish *engrailed*-2 gene. *Mech. Dev. 39:* 51-62.

GARDNER, C.A. and BARALD, K.F. (1992). Expression patterns of *engrailed*-like proteins in the chick embryo. *Dev. Dynamics 193:* 370-388.

HARLAND, R.M. (1991). *In situ* hybridization: an improved whole-mount method for *Xenopus laevis*. *Methods Enzymol. 36:* 685-697.

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

HEEMSKERK, J., DINARDO, S., KOSTRIKEN, R. and O'FARRELL, P.H. (1991). Multiple modes of *engrailed* regulation in the progression towards cell fate determination. *Nature 352:* 404-410.

HEMMATI-BRIVANLOU, A. and HARLAND, R.M. (1989). Expression of an *engrailed*-related protein is induced in the anterior neural ectoderm of early *Xenopus* embryos. *Development 106:* 611-617.

HEMMATI-BRIVANLOU, A., DE LA TORRE, J.R., HOLT, C. and HARLAND, R.M. (1991). Cephalic expression and molecular characterization of *Xenopus En*-2. *Development 111:* 715-724.

HOLLAND, P.W.H. and WILLIAMS, N.A. (1990). Conservation of *engrailed*-like homeobox sequences during vertebrate evolution. *FEBS Lett. 277:* 250-252.

HOLTFRETER, S. (1933a). Die total Exogastrulation, eine Selbstabloesung des Ektoderms von Entomesoderm. *W. Roux Arch. Entw.Mech. Org. 129:* 669-793.

HOLTFRETER, S. (1933b). Nachweis der Induktionsfahigkeit abgetoteter Kiemteile. Isolations und Transplantation Versuche. *W. Roux Arch. Entw.Mech. Org. 128:* 584-633.

INGHAM, P.W. and HIDALGO, A. (1993). Regulation of *wingless* transcription in the *Drosophila* embryo. *Development 117:* 283-291.

JACOBSON, M. (1978). *Developmental Neurobiology*. Plenum Press, New York.

JOYNER, A.L. and MARTIN, G.R. (1987). *En*-1 and *En*-2, two mouse genes with sequence homology to the *Drosophila engrailed* gene: expression during embryogenesis. [Erratum published in Genes Dev. 1, page 521, 1987.] *Genes Dev. 1:* 29-38.

JOYNER, A.L., HERRUP, K., AUERBACH, B.A., DAVIS, C.A. and ROSSANT, J. (1991). Subtle cerebellar phenotype in mice homozygous for a targeted deletion of the *En*-2 homeobox. *Science 251:* 1239-1242.

JOYNER, A.L., KORNBERG, T., COLEMAN, K.G., COX, D.R. and MARTIN, G.R. (1985). Expression during embryogenesis of a mouse gene with sequence homology to the *Drosophila engrailed* gene. *Cell 43:* 29-37.

KINTNER, C.R. and MELTON, D.A. (1987). Expression of *Xenopus* N-CAM RNA in ectoderm is an early response to neural induction. *Development 99:* 311-325.

KORNBERG, T. (1981a). Compartments in the abdomen of *Drosophila* and the role of the *engrailed* locus. *Dev. Biol. 86:* 363-372.

KORNBERG, T. (1981b). *Engrailed:* a gene controlling compartment and segment formation in *Drosophila*. *Proc. Nat. Acad. Sci. USA 78:* 1095-1099.

KORNBERG, T., SIDEN, I., O'FARRELL, P. and SIMON, M. (1985). The *engrailed* locus of *Drosophila*: *in situ* localization of transcripts reveals compartment-specific expression. *Cell 40:* 45-53.

KRAUSS, S., CONCORDET, J.-P. and INGHAM, P.W. (1993). A functionally conserved homolog of the *Drosophila* segment polarity gene *hh* is expressed in tissues with polarizing activity in zebrafish embryos. *Cell 75:* 1431-1444.

LAWRENCE, P.A. (1992). *The Making of a Fly; the Genetics of Animal Design*. Blackwell, Oxford.

LEE, J.J., VON-KESSLER, D.P., PARKS, S. and BEACHY P.A. (1992). Secretion and localized transcription suggest a role in positional signaling for products of the segmentation gene *hedgehog*. *Cell 71:* 33-50.

LEVINE, M. and HOEY, T. (1988). Homeobox proteins as sequence-specific transcription factors. *Cell 55:* 537-540.

LOGAN, C., HANKS, M.C., NOBLE-TOPHAM, S., NALLAINATHAN, D., PROVART, N.J. and JOYNER, A.L. (1992). Cloning and sequence comparison of the mouse, human, and chicken *engrailed* genes reveal potential functional domains and regulatory regions. *Dev. Genet. 13:* 345-358.

MARTIN, G.R. and FROHMAN, M.A. (1989). The mouse *En*-1 gene encodes a nuclear protein expressed in the nervous system of the developing embryo. In *Cell to Cell Signals in Mammalian Development* (Eds. S.W. de Laat, J.G. Bluemink and C.L. Mummery). Springer-Verlag, Berlin, pp. 43-52.

MARTINEZ-ARIAS, A., BAKER, N., and INGHAM, P.W. (1988). Role of segment polarity genes in the definition and maintenance of cell states in the *Drosophila* embryo. *Development 103:* 157-170.

McGREW, L.L., OTTE, A.P. and MOON, R.T. (1992). Analysis of *Xwnt*-4 in embryos of *Xenopus laevis*: a *Wnt* family member expressed in the brain and floor plate. *Development 115:* 463-473.

McMAHON, A.P., JOYNER, A.L., BRADLEY, A. and McMAHON, J.A. (1992). The midbrain-hindbrain phenotype of *wnt*-1^{-}/*wnt*-1^{-} mice results from stepwise deletion of *engrailed*-expressing cells by 9.5 days postcoitum. *Cell 69:* 581-595.

MILLEN, K.J., WURST, W., HERRUP, K. and JOYNER, A.L. (1994). Abnormal embryonic cerebellar development and patterning of postnatal foliation in two mouse *engrailed*-2 mutants. *Development 120:* 695-706.

MOON, R.T. (1993). In pursuit of the functions of the *wnt* family of developmental regulators: Insights from *Xenopus laevis*. *BioEssays 15:* 91-97.

MORATA, G. and LAWRENCE, P.A. (1975). Control of compartment development by the *engrailed* gene of *Drosophila*. *Nature 255:* 614-617.

NEWPORT, J. and KIRSCHNER, M.W. (1982). A major developmental transition in early *Xenopus* embryos: I characterization and timing of cellular changes at the midblastula stage. *Cell 30:* 675-686.

NIEUWKOOP, P.D. and FABER, J. (1967). *Normal Table of Xenopus laevis*. North-Holland, Amsterdam.

NOORDERMEER, J., KLINGENSMITH, J., PERRIMON, N. and NUSSE, R. (1994). Dishevelled and armadillo act in the wingless signalling pathway in *Drosophila*. *Nature 367:* 80-83.

NOORDERMEER, J., MEIJLINK, F., VERRIJZER, P., RIJSEWIJK, F. and DESTRÉE, O. (1989). Isolation of the *Xenopus* homolog of *int-1*/*wingless* and expression during neurula stages of early development. *Nucleic Acids Res. 17:* 11-18.

NUSSE, R. and VARMUS, H.E. (1992). *Wnt* genes. *Cell 69:* 1073-1087.

NÜSSLEIN-VOLHARD, C. and WIESCHAUS, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. *Nature 287:* 795-801.

PATEL, N.P., 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, Annelids, and Chordates. *Cell 58:* 955-968.

POOLE, S.J., KAUVAR, L.M., DREES, B. and KORNBERG, T. (1985). The *engrailed* locus of *Drosophila:* structural analysis of an embryonic transcript. *Cell 40:* 37-43.

RIDDLE, R.D., JOHNSON, R.L., LAUFER, E. and TABIN, C. (1993). *Sonic hedgehog* mediates the polarizing activity of the ZPA. *Cell 75:* 1401-1416.

RUIZ I ALTABA, A. (1990). Neural expression of the *Xenopus* homeobox Xhox3: evidence for a patterning neural signal that spreads through the ectoderm. *Development 108:* 595-604.

RUIZ I ALTABA, A. (1992). Planar and vertical signals in the induction and patterning of the *Xenopus* nervous system. *Development 115:* 67-80.

RUIZ I ALTABA, A. (1993). Induction and axial patterning of the neural plate: planar and vertical signals. *J. Neurobiol. 24:* 1276-1304.

RUIZ I ALTABA, A. (1994). Pattern formation in the vertebrate neural plate. *Trends Neurosci. 17:* 233-243.

SAMPEDRO, J., JOHNSTON, P. and LAWRENCE, P.A. (1993). A role for *wingless* in the segmental gradient of *Drosophila*. *Development 117:* 677-687.

SANGER, F., NICKLEN, S. and COULSON, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci USA 74:* 5463-5467.

SIEGFRIED, E., WILDER, E.L. and PERRIMON, N. (1994). Components of *wingless* signalling in *Drosophila*. *Nature 367:* 76-79.

VAN DEN HEUVEL, M., NUSSE, R., JOHNSTON, P. and LAWRENCE, P.A. (1989). Distribution of the *wingless* gene product in *Drosophila* embryos a protein involved in cell-cell communication. *Cell 59:* 739-749.

VINCENT, J-P. and LAWRENCE, P.A. (1994). *Drosophila wingless* sustains *engrailed* expression only in adjoining cells: evidence from mosaic embryos. *Cell 77:* 909-915.

WATANABE, M., HAYASHIDA, T., NISHIMOT, T. and KOBAYAHI, H. (1993). Nucleotide sequence of *Xenopus* homeobox gene, *En*-1. *Nucleic Acids Res. 21:* 2513.

WOLDA, S.L., MOODY, C.J. and MOON, R.T. (1993). Overlapping expression of *Xwnt*-3A and *Xwnt*-1 in neural tissue of *Xenopus laevis* embryos. *Dev. Biol. 155:* 46-57.

WURST, W., AUERBACH, A.B. and JOYNER, A.L. (1994). Multiple developmental defects in *engrailed*-1 mutant mice: an early mid-hindbrain deletion and patterning defects in forelimbs and sternum. *Development 120:* 2065-2075.

YU, W-P., COLLARINI, E.J., PRINGLE, N.P. and RICHARDSON, W.D. (1994). Embryonic expression of myelin genes: evidence for a focal source of oligodendrocyte precursors in the ventricular zone of the neural tube. *Neuron 12:* 1353-1362.

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