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A Member of the Met/HGF-Receptor Family Is Expressed in a BMP-4-like Pattern in the Ectoderm of *Xenopus* Gastrulae

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The importance and involvement of growth factors and their corresponding receptors in embryonic induction has been more and more recognized during the past decade, in particular by loss-of-function experiments using dominant negative receptors. Here, we report the isolation of XHR, a *Xenopus* receptor-type tyrosine kinase, with homology to members of the Met/hepatocyte growth factor (HGF)-receptor family. Sequence comparison of XHR with other members of the Met/HGF-receptor family as well as in situ expression analyses suggest that XHR represents a novel member of this family of receptor-type tyrosine kinases. As could be shown by whole-mount in situ analysis, XHR transcripts are first expressed in the entire ectoderm at the onset of gastrulation. As gastrulation proceeds, XHR-transcription is turned off in cells induced by dorsal mesoderm to form neural tissue and thus, becomes predominantly confined to prospective epidermis. The strikingly similar expression patterns of XHR and Bone Morphogenetic Protein-4 (BMP-4), an inducer of epidermis and inhibitor of neural development, suggest an involvement of XHR signalling in the early cell-fate decision of ectodermal cells to form either neural derivatives or epidermis.

The establishment of the embryonic nervous system is one of the first steps in tissue differentiation of early vertebrate embryos.

More than 70 years ago Spemann and Mangold (1) have discovered that the region around the dorsal blastopore lip, termed the organizer, is capable of inducing ectodermal cells to differentiate into neural tissue instead of epidermis. Although this discovery has led to enormous efforts on the identification of a neural inducer, only recently a few candidates for a natural neural inducer have been isolated.

For instance, noggin protein, which is expressed in the organizer, is able to neuralize ectodermal cells. Likewise, overexpression of follistatin, an antagonist of activin expressed in the organizer, activates neural markers in animal cap explants (2). Chordin, an organizer-specific *Xenopus* homolog of the Drosophila short gastrulation (sog) gene product, is also capable of inducing neural development of ectodermal cells (3). Finally, *cerberus*, a secreted protein expressed in the endomesodermal cells of the organizer, is able to induce anterior neural structures (4).

Recently, it has been shown that both noggin and chordin protein bind and inhibit BMP-4 protein (5, 6) However, only anterior neural markers are induced by chordin and noggin, indicating that additional signals are required for induction of a fully patterned and organized nervous system.

In order to gain more insight into the signalling events that determine the fate of ectodermal cells, we screened for receptor-type tyrosine kinases expressed during the early steps of neural induction in *Xenopus*.

In this paper we report the isolation of a new *Xenopus* member of the Met/HGF-receptor family, termed XHR. By virtue of our results obtained from sequence analysis and studies on XHR expression in both early embryos and adult frogs, we propose that XHR represents a novel *Xenopus* member of the Met/HGF-receptor family. Interestingly, the distribution of XHR transcripts resembles that of BMP-4, an inducer of epidermis (7), in the ectoderm of gastrulae (8), suggesting an involvement of XHR signalling in induction of epidermis and inhibition of neural tissue formation, respectively.

MATERIALS AND METHODS

Embryos and embryology. Superovulation, fertilization and production of exogastrulae were carried out as described previously (9).

Disaggregation and delayed reaggregation of ectodermal explants were performed as described by Grunz and Tacke (10). Reaggregated

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cells and ectoderm explants (isolated at stage 9) were either cultured for 30h or frozen immediately after isolation at −70°C.

RNA isolation and RT-PCR. For RT-PCR amplification of receptor tyrosine kinases, 5μg of total RNA from gastrula stage embryos were used for cDNA synthesis. The nucleotide sequences of the PCR primers were as follows: 5′AGTCTAGACAYMGNGAYYTNGCNG-CNMGNAA3′ and 5′GAGCTCG-AGGGNGCCATCCAYTTNAC-NGG3′. The following program was run: 5min at 95°C; 1min at 94°C, 1min at 46°C and 1min at 72°C for 35 cycles. To increase the specificity of the reaction, a second round of amplification was performed using a sense primer located more 3′ than the first sense primer. This primer had the nucleotide sequence 5′GGGTCTAGAGAYT-TYGGNYTNGCNMG3′. The conditions for the second round of amplification were as follows: 5min at 95°C; 1min at 94°C, 1min 30sec at 43°C, 1min at 72°C for 2 cycles; 1min at 94°C, 1min at 60°C, 1min at 72°C for 30 cycles. All amplifications were performed with Dynazyme thermostable DNA polymerase (Finnzyme) in a Cetus 2.0 thermocycler (Perkin Elmer).

Amplified fragments with an expected length of approximately 100 bp were digested with XhoI and XbaI (Boehringer), subcloned into pBluescript II SK+ and sequenced with the T7-sequencing kit (Pharmacia). One fragment showed highest homology to members of the MET/HGF-receptor family.

Cloning of a Xenopus member of the Met/HGF-receptor family. In order to clone a larger fragment, we designed a sense primer for PCR based on the Met/HGF-receptor family-specific sequence HFGSVY-YHG and an antisense primer specific for the isolated *Xenopus* homologue of the Met/HGF-receptor family. As template, 1μg double stranded cDNA from stage 12 embryos was used. The nucleotide sequences of the primers and the conditions for PCR amplification were as follows: 5′GAGTCTAGACAYTTY-GGNWSNGTNTAY-CAYGG3′ for the sense primer and 5′GAGCTCGAG-GTTCTTGTG-GCGTCTAACACTG3′ for the antisense primer. PCR was run for 5min at 95°C; 1min at 94°C, 1min at 43°C, 1min at 72°C for 1 cycle and 1min at 94°C, 1min at 55°C, 1min at 72°C for 35 cycles.

The resulting fragment was further used to screen a *Xenopus* stage 12 plasmid cDNA library. We isolated a 1.3kb partial cDNA clone termed Xsea, which was further used to screen a lung lambda-ZAP cDNA library, according to the manufacturer's instructions (Stratagene). This led to the isolation of an overlapping 3.3kb cDNA clone, termed XHRex, which showed a 100% match in the overlapping region with Xsea. Both cDNA clones together encode a putative protein of 1369 amino acids.

Whole-mount in situ hybridization. Whole-mount *in situ* hybridizations were performed according to Harland (11) with the following modifications: BM blocking reagent (Boehringer) was used instead of 20% sheep serum and BM-purple (Boehringer) was used as substrate for alkaline phosphatase instead of x-phosphate/NBT. Digoxigenin-labelled antisense RNA of XHRex was synthesized with T7 RNA polymerase (Boehringer).

RESULTS AND DISCUSSION

Sequence of XHR. Using a RT-PCR approach we were able to isolate a novel *Xenopus* receptor-type tyrosine kinase, termed XHR (Genbank Accession-No. U77681), expressed only during the phase of neural induction. The deduced amino acid sequence of XHR shows features characteristic of members of the Met/hepatocyte growth factor (HGF)-receptor family. Known members of this family include *c-met* encoding the receptor for hepatocyte growth factor (HGF) (12, 13), *Ron* (14) coding for the receptor for HGF-like protein (15, 16) and *c-sea* (17) of which the ligand has not yet been identified. Simultaneously, *Xron* cDNA that is more than 99.9% identical to XHR was isolated in an independent experiment by Nakamura et al. (18). The only difference between the deduced protein sequences of *Xron* and XHR is that valine at position 552 and threonine at position 1194 in *Xron* are alanine and serine, respectively, in XHR.

In order to address the question of whether XHR is either the *Xenopus* homolog of *c-met, Ron* or *c-sea* or whether XHR encodes a novel member of the Met/HGF-receptor family, we aligned XHR with all known members and their homologs of the Met/HGF-receptor family. In addition, we aligned each member of the family with each other. The result of this alignment is shown in figure 2C. At the amino acid level XHR shows highest homology to chicken *c-sea* (17) (47.2% overall identity), whereas XHR is only 42.8% identical to the putative mouse homolog of *Ron* (19) and 37.8% identical to the mouse homolog of *c-met* gene product (20). In contrast, human, mouse and chick (21) *c-met* share more than 72% sequence identity at the amino acid level among each other. Similarly, human and mouse *Ron* are more than 72% identical, whereas XHR shows only 42.8% homology with mouse Ron and less than 38% identity with mouse *c-met*. By virtue of the relatively low extent of homology of XHR to members of the three Met/HGF-receptor subfamilies (*c-met, Ron* and *c-sea*) we speculate that XHR encodes a new member of the Met/HGF-receptor family, closest related to *c-sea*.

In situ expression of XHR. By whole-mount in situ hybridization, we find that XHR is first weakly expressed in the entire ectoderm at stage 10 of development (data not shown) but as dorsal mesoderm involutes during gastrulation, XHR-transcription is turned off in ectodermal cells that will give rise to neural tissue (Fig. 1A, 1B). Simultaneously, XHR mRNA accumulates predominantly in prospective epidermis (Fig. 1A-E). Histological analysis reveals that XHR is expressed in both layers of presumptive epidermis during gastrulation (Fig. 1H). In addition, we detect XHR-positive cells in the very lateral and anterior part of the epithelial layer of the future neural plate (Fig. 1H-J). By the end of gastrulation, transcription of XHR is also activated in deeper cells at the anterior border of the prospective neural plate (Fig. 1C). As neurulation proceeds, this stripe of XHR expression extends posteriorly, labelling the most lateral cells of the sensorial layer of the neural plate as well as the most dorsal cells of prospective epidermis (Fig. 1D, 1E, 1I, 1J and 1K). From stage 16 onward, XHR transcripts rapidly decrease. At stage 20 trace amounts of XHR transcripts are localized in the eye anlage and anteriormost part of the brain (18).

In order to address the issue of a possible receptor-ligand relationship between XHR and the *Xenopus* ho-

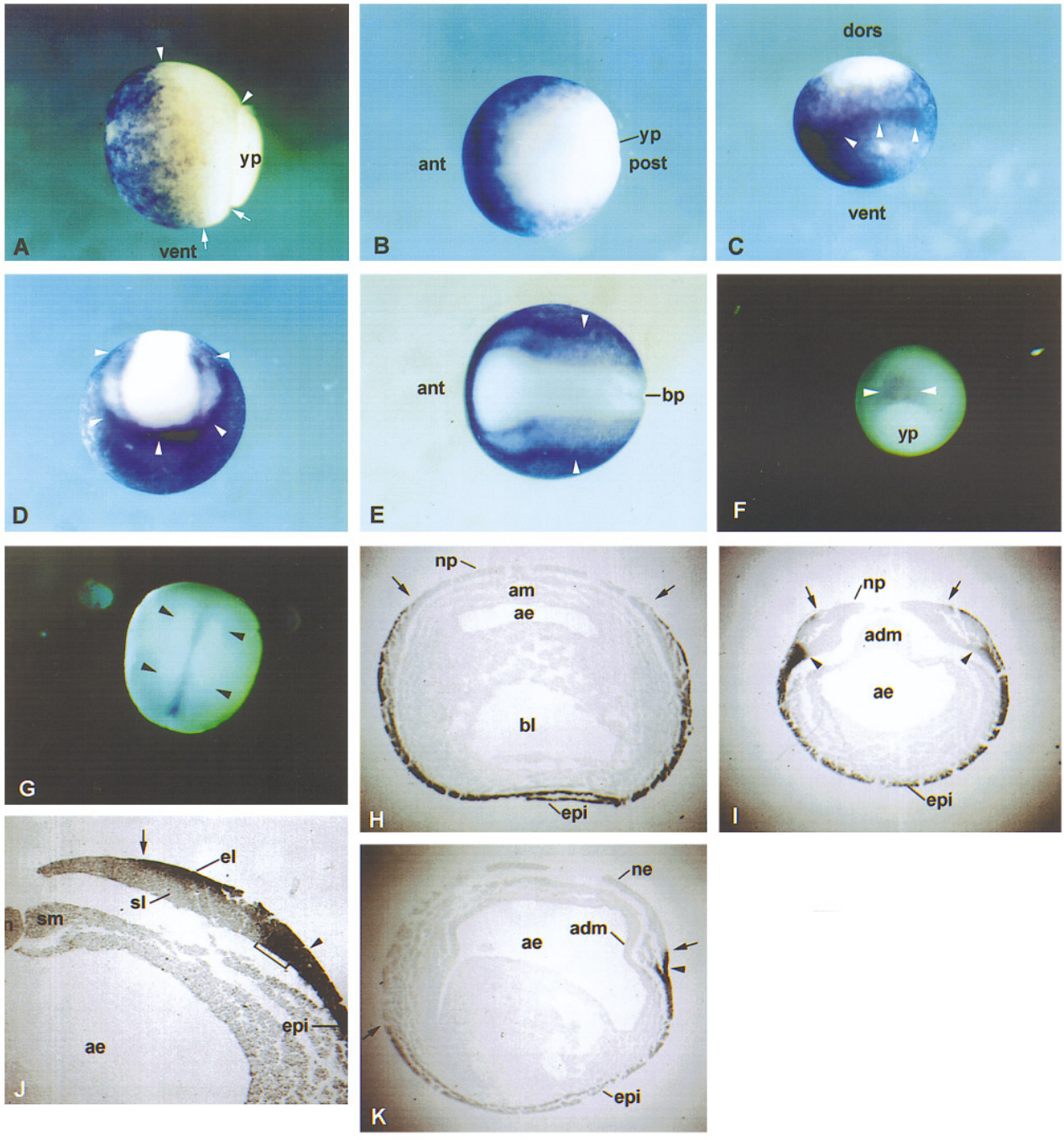


FIG. 1. A-E: In situ distribution of XHR transcripts during gastrula and early neurula stages. A: Lateral view of a mid-gastrula stage embryo. Arrowheads mark the anterior–posterior extension of XHR-negative cells in dorsal ectoderm. Arrows enclose ventral marginal zone. B: dorsal view of a late gastrula. C: anterior view of the same embryo as shown in B. Arrowheads point out a stripe of strong XHR expression at the anteriormost border of the presumptive neural plate. D: anterior view of an embryo at stage 15. Arrowheads mark the anterior and lateral border of the neural plate, also labelled by cells with strong XHR expression. E: dorsal view of the same embryo as shown in D. Arrowheads point out the posterior border of the stripe of cells with strong XHR expression. F-G: Localization of XHL transcripts during gastrulation and early neurulation. F: dorso-vegetal view of a mid-gastrula stage embryo. G: dorsal view of a stage 13 embryo stained for XHL mRNA. The dorsal border of XHR expression is marked by arrowheads. H: Cross-sections of a late gastrula embryo prior subjected to whole-mount in situ hybridization. Arrows mark the dorsal border of XHR expression in ectoderm. I: Cross-section of an early neurula at the level of the eye anlage. Arrows point out the dorsal expression border of XHR in the epithelial layer of the neuroectoderm. Arrowheads mark the lateral border of XHR-positiv cells in the sensorial layer of the neuroectoderm. J: Close-up of a cross-section through an embryo at stage 15 at the level of the prospective trunk region. Arrow points out the dorsal border of XHR expression in the epithelial layer of neuroectoderm. Bracket encloses XHR-expressing cells in the sensorial layer of the neural plate. Arrowhead marks the border between neural plate and epidermis. K: sagittal section of a neurula embryo. Arrows point out the anterior and posterior border of XHR expression. Arrowhead marks the anterior border of the neural plate. adm: anterior dorsal mesoderm; ae: archenteron; am: axial mesoderm; ant: anterior; bl: blastocoel; bp: blastopore; dors: dorsal; el: epithelial layer of ne; epi: epidermis; n: notochord; ne: neuroectoderm; np: neural plate; sl: sensorial layer of ne; sm: somitic mesoderm; post: posterior; vent: ventral; yp: yolk plug.

molog of hepatocyte growth factor-like protein (XHL) (9), we compared the expression pattern of XHR with that of XHL. As shown in figure 1F, XHL mRNA can first be detected at mid-gastrula stage in a region referred to as the non-involuting marginal zone. At early neurula stage XHL transcripts are confined to a narrow stripe labelling the dorsal midline of the neural plate (Fig. 1G) (see also 9, 18). This comparison demonstrates that XHR and XHL are expressed in very different regions of the embryo. Considering the high molecular weight of biologically active XHL (82 kDa) it is unlikely that XHL protein is able to diffuse from the dorsal midline to the very lateral region of the neural plate in order to interact with XHR. Thus, we suggest that XHL does not bind to XHR in the neuroectoderm of gastrula and early neurula stage embryos. Nevertheless, it is possible that XHL interacts with XHR at later stages in the anteriormost part of the embryonic nervous system, where XHL mRNA can be found from mid-neurula stage onward (18).

We further addressed the problem of XHR-XHL ligand interaction by comparison of XHR and XHL expression in adult frogs. In a previous paper we have reported that XHL is strongest expressed in liver (9). Figure 2D shows that XHR is expressed in adult kidney and lung tissue, whereas , in contrast to XHL (Fig.2E), XHR transcripts cannot be detected in liver.

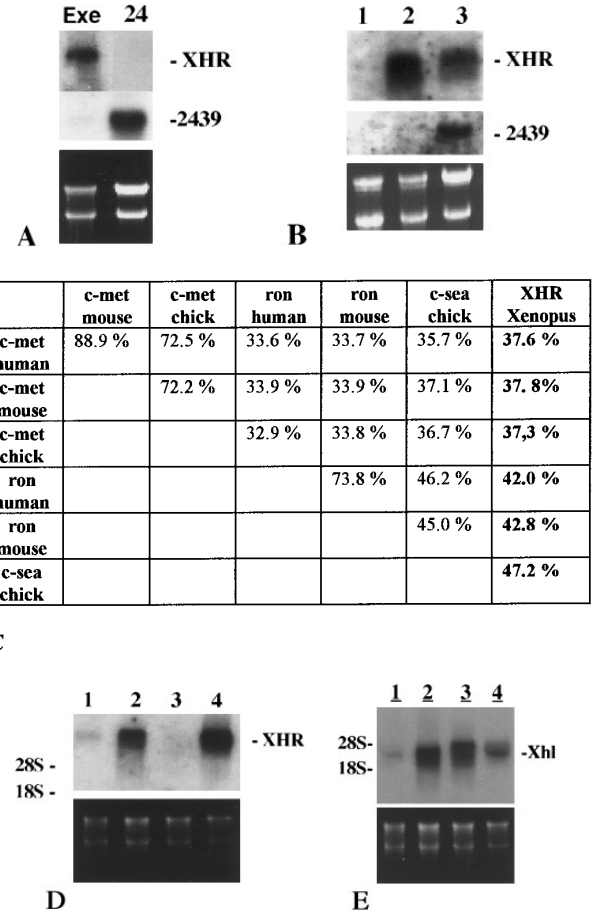
Together with our in situ expression analysis in the early embryo, these findings suggest that first XHR is unlikely the specific receptor forXHL and second XHR encodes a new member of the Met/HGF-receptor family rather than the *Xenopus* homolog of *Ron*, the receptor for HGF-like protein, as proposed by Nakamura et al. (18). Alternatively, XHR could be the *Xenopus* homolog of *c-sea*.

Regulation of XHR expression. In order to investigate the signals that control the spatial and temporal expression pattern of XHR, we performed experiments with exogastrulae, which allow to distinguish among vertical and planar signals emanating from mesoderm, since involution of mesodermal cells is inhibited.

In figure 2A we show that XHR transcripts are present in the ectodermal portion of exogastrulated embryos as expected for a gene expressed in future epidermis. Interestingly, XHR mRNA can still be detected in exogastrulae at stage 24 when XHR expression has already been turned off in wild-type sibling embryos. This result suggests that vertical signals from mesodermal cells are required for correct temporal regulation of XHR expression.

Recently, it has been demonstrated that disaggregation followed by delayed reaggregation of animal cap explants leads to neuralization of ectodermal cells (10), likely by dilution of BMP-4, since the effect can be reversed by addition of BMP-4 to disaggregated cells (7). In order to address the question of whether neuralization caused by delayed reaggregation of animal caps and loss of BMP-4 signalling, respectively, influences XHR expression, we compared XHR transcription in disaggregated-reaggregated ectodermal cells with that in untreated control explants.

We find that XHR expression is essentially unaffected by this process (Fig. 2B) although slightly lower amounts of XHR mRNA are detected in disaggregated/



	c-met mouse	c-met chick	ron human	ron mouse	c-sea chick	XHR Xenopus
c-met human	88.9 %	72.5 %	33.6 %	33.7 %	35.7 %	**37.6 %**
c-met mouse		72.2 %	33.9 %	33.9 %	37.1 %	**37. 8%**
c-met chick			32.9 %	33.8 %	36.7 %	**37,3 %**
ron human				73.8 %	46.2 %	**42.0 %**
ron mouse					45.0 %	**42.8 %**
c-sea chick						**47.2 %**

C

FIG. 2. A: Northern blot analysis showing that XHR mRNA is expressed in the ectoderm of completely exogastrulated embryos (Exe) cultured until sibling embryos reached stage 24 of development. 24: stage 24 sibling embryos. The same blot was reprobed with a neural-specific RNA-binding protein (24-39) (22). B: Northern blot analysis comparing XHR mRNA transcription in disaggregated/reaggregated and untreated animal caps. 1: Ectodermal explants isolated at stage 9 and frozen immediately afterwards. 2: Ectodermal explants isolated at stage 9 but cultured for 30 h. 3: Disaggregated and reaggregated ectodermal explants cultured for 30 h after reaggregation. The same filter was reprobed with a neural-specific RNA-binding protein (24-39) (22). C: Results of alignment analysis of all known members of the Met/HGF-receptor family. Numbers indicate the percentage of identity between the deduced amino acid sequences of human *c-met,* mouse *c-met,* chick *c-met,* human and mouse *Ron,* chick *c-sea* and Xenopus XHR. D-E: Northern blot analysis showing the tissue-specific expression pattern of XHR (D) and XHL (E) mRNA in adult frogs. 1: Brain; 2: Kidney; 3: Liver; 4: Lung; Ethidium bromide-stained gels were photographed for documentation of RNA-loading.

reaggregated animal caps, suggesting that transcriptional regulation of XHR is largly independent of BMP-4 signalling. In contrast, the neural-specific gene 24-39 (22) is exclusively expressed in manipulated ectodermal explants, demonstrating that disaggregation followed by delayed reaggregation has caused neural development.

DISUCUSSION

Our data provide evidence for an involvment of a member of the Met/HGF-receptor family in early *Xenopus* embryogenesis. Based on our sequence alignment and in situ expression studies, we propose that XHR represents a novel member of the Met/HGF-receptor family rather than the *Xenopus* homolog of *Ron,* the receptor for hepatocyte growth factor-like protein as suggested by Nakamura et al. (18). Support for this presumption comes from our observation that neither overlapping nor adjacent expression domains of XHR and XHL exist at least until mid-neurula stages. In addition, we cannot find any correspondence of XHR and XHL expression in adult liver, where XHL but not XHR is expressed. Finally, ectopic expression of XHL during embryogenesis neither alters the behaviour nor changes the fate of cells expressing XHR (9).

We show that XHR transcripts are first expressed in the entire ectoderm at the onset of gastrulation. Interestingly, as mesoderm involutes XHR mRNA disappears in cells that are induced by underlying dorsal mesoderm to differentiate into nervous system such that XHR mRNA becomes restricted predominantly to prospective epidermis. There is a striking similarity between the expression patterns of XHR and BMP-4 (8), of which the latter has been shown to be a natural inducer of epidermis and inhibitor of neural development (7). Of particular interest is that two neural inducers, noggin and chordin, act as antagonists of BMP-4 (5, 6). Based on the spatial distribution of XHR transcripts during neural development, we hypothesize that XHR might be involved in the signalling events that induce embryonic ectoderm to form either epidermis or neural tissue, although we do not provide any experimental evidence for such an activity. In this context it is noteworthy that the *Xenopus* homolog of hepatocyte growth factor (XHGF) is expressed in ventral mesoderm in close proximity to XHR expressing cells (23). By virtue of this observation it is tempting to speculate that XHR could encode a novel receptor for XHGF that differs from the *c-met* gene product. This would be another example for an epithelial-mesenchymal interaction via HGF and its receptor (24).

Our experiments with disaggregated/reaggregated ectodermal explants provide preliminary evidence that activation of XHR expression is not susceptible to inhibition of BMP-4 signalling. However, we cannot conclude from this observation whether XHR acts upstream, downstream or independently of the BMP-4 signal during the early phase of neural induction.

Further experiments using dominant negative forms of XHR in order to block signal transduction are required to gain more insight into the biological function of this novel receptor-type tyrosine kinase during early *Xenopus* development.

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