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Early Regionalized Expression of a Novel *Xenopus* Fibroblast Growth Factor Receptor in Neuroepithelium[1]

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A cDNA encoding a novel *Xenopus* fibroblast growth factor receptor, XFGFR4B, has been cloned. XFGFR4B mRNA is detected throughout embryogenesis. However, from the late gastrula stage on, XFGFR4B transcripts are expressed in two defined areas of the anterior neural plate. Interestingly, these two regions are fated to become parts of the retina, midbrain, hindbrain, and otic vesicle, all of which continue to express XFGFR4B mRNA in tailbud stage embryos and early tadpoles. Expression of XFGFR4B mRNA can be maintained at neurula stage in isolated blastula ectoderm in response to mesodermal induction by activin or neural induction by noggin, suggesting that XFGFR4B expression might be regulated by early cell interactions. Distribution of XFGFR4B mRNA suggests that XFGFR4B might serve an important function during patterning of neuroepithelium.

Establishment of body pattern in *Xenopus* embryos is dependent on successive cell interactions taking place during cleavage and gastrulation. In cleaving embryos, mesoderm is first induced as a consequence of interactions between animal and vegetal blastomeres. Dorsally, this process results in the formation of the Spemann organizer. During gastrulation, interaction of dorsal ectoderm with organizer cells lead to the induction of neural tissue.

A growing body of evidence indicates that several members of the transforming growth factor-β superfamily and of the fibroblast growth factor (FGF) family are playing a crucial role during cell interactions in the early *Xenopus* embryo. Activin, BMP-4 and FGFs with the exception of KGF are able to induce mesoderm in isolated blastula ectoderm (1). FGFs are probably involved in different steps of the inductive processes. For example, it has been recently shown that induction of mesoderm by activin requires functional FGF signalling pathways (2). In addition, basic FGF (bFGF) is able to induce neuron and melanophore differentiation in dissociated gastrula ectodermal cells (3).

Understanding of the exact role of FGFs in early cell interactions of the *Xenopus* embryo implies that the molecular bases of embryonic cell competence to respond to FGF signals have been elucidated. A first step to achieve this goal is the identification of FGF receptors (FGFR) expressed in the different tissues of the embryo. FGFRs are tyrosine kinase transmembrane proteins. They are defined by comparison to members of the human FGFR family, FGFR1 (*flg*), FGFR2 (*bek*), FGFR3 and FGFR4 (4). There is increasing evidence that all FGFRs are not functionally equivalent. Functional specificity of FGFRs arises from the differential affinity for their various ligands (4) and from the substrates they can phosphorylate (5). To date, four FGFRs expressed in *Xenopus* embryos have been identified. They include XFGFR1 and XFGFRA1 encoded by two different genes and homologous to FGFR1 (6, 7), XFGFR2 (8) and recently XFGFR4 (9). XFGFR-1, A1 and 4 are expressed throughout development. XFGFR2 expression begins during gastrulation.

In this paper, we report the cloning of a cDNA encoding XFGFR4B, a novel *Xenopus* FGFR

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related to FGFR4. The restricted expression of XFGFR4B mRNA in neuroectoderm suggests that XFGFR4B plays a role in the patterning of neural tissue.

MATERIALS AND METHODS

Cloning of XFGFR4B 1cDNA

PCR amplification of *Xenopus* FGFR tyrosine kinase sequences was carried out from tailbud stage embryo cDNA using degenerate primers encoding amino acid sequences EGCFGQV (upstream) and KWMAPE (downstream). These sequences are derived from tyrosine kinase domain of FGFRs and are conserved among FGFRs. Primer sequences were: GARG-GiTGYTTYGGiCARGT (sense) and TCWGGiGCCATCCAYTT (antisense). First strand cDNA was obtained using the Superscript kit (BRL, Gaitherburg, MD) according to manufacturer instructions, from total embryo RNA extracted as described (10). cDNA was subjected to 40 cycles of amplification at 94°C for 30 sec, 45°C for 1 min and 72°C for 1 min with a final elongation step of 15 min.

A gastrula stage λZAP cDNA library kindly provided by Dr D.W. DeSimone was screened using one amplified cDNA encoding XFGFR4B as probe according to standard procedures (10).

The 5′ end of XFGFR4B coding sequence was amplified by anchored PCR using the Marathon kit (Clontech, Palo Alto, CA) according to manufacturer instructions.

Analysis of XFGFR4B mRNA Expression

RNase protection was carried out as previously described (11). XFGFR4B antisense probe was transcribed with T3 RNA polymerase from a KpnI-Xhol fragment of XFGFR4B cDNA (nt 230-795 of the coding sequence) cloned into Bluescript SKII-plasmid, after linearization with PvuII. XFGFR1 probe was transcribed with T3 RNA polymerase from a HincII-HindIII fragment of XFGFR1 cDNA (6) cloned in Bluescript SKII-plasmid, after linearization with XhoI. The EF-1αprobe (12) used as internal control was obtained as described (11).

In situ hybridization in toto was performed according to Harland (13). The 5′ EcoRI fragment of the 2AD1 clone (nt 283-873 of XFGFR4B coding sequence) was subcloned into Bluescript SKII-plasmid in both orientations to generate templates for sense and antisense riboprobe transcription. In both cases, digoxigenin-labelled probes were transcribed using T3 RNA polymerase after linearization with HindIII.

Mesodermal and Neural Inductions in Vitro

For mesodermal inductions, ectoderm was dissected at the midblastula stage and cultured in the presence of 20U/ml of *Xenopus* activin A or 20 ng/ml of bovine b-FGF as described (14).

Neural induction with noggin was performed by microinjecting 50 pg of pCSKA-noggin (15) into both of the blastomeres at the 2 cell-stage. Injected embryos were cultured until the midblastula stage and animal caps were dissected.

Induced animal caps were cultured until midgastrula or late neurula stage and subjected to RNase protection analysis.

RESULTS AND DISCUSSION

Structure of XFGFR4B cDNA

Sequence analysis of 11 amplified FGFR tyrosine kinase cDNA showed that 9 were identical to previously identified FGFRs. Sequences of the two other clones were identical but differed from previously published sequences. One of these cDNA was used to screen a gastrula stage cDNA library. Eight positive clones were isolated. The largest clone 2AD1 was sequenced and found to contain most of the sequence encoding an FGFR along with the 3′UTR. The missing 5′ portion of the coding sequence was obtained by anchored PCR. Sequences of the overlapping region of the amplified fragment and of 2AD1 (about 100 bp) were totally identical. They contain a BstBI site used to generate a full-length cDNA. Analysis of the sequence revealed an ORF of 2487 nt encoding 829 amino acid residues. The amino acid sequence was compared to that of other vertebrate FGFRs. It was highly homologous to the urodele PFR4 (10) (75% identity) and avian FREK (16) (77% identity) and to a lesser extent to human FGFR4 (17) (65% identity). Comparison with *Xenopus* FGFRs showed low homologies with XFGFR1 and 2 (59%) but a high homology (95% identity) with the recently identified XFGFR4 (9) (Fig. 1). Nucleotide sequences of both cDNAs were 93% identical. It is very likely that the cloned cDNA corresponds to the product of a second XFGFR4 gene resulting from the genome duplication of *Xenopus laevis* (18). It is

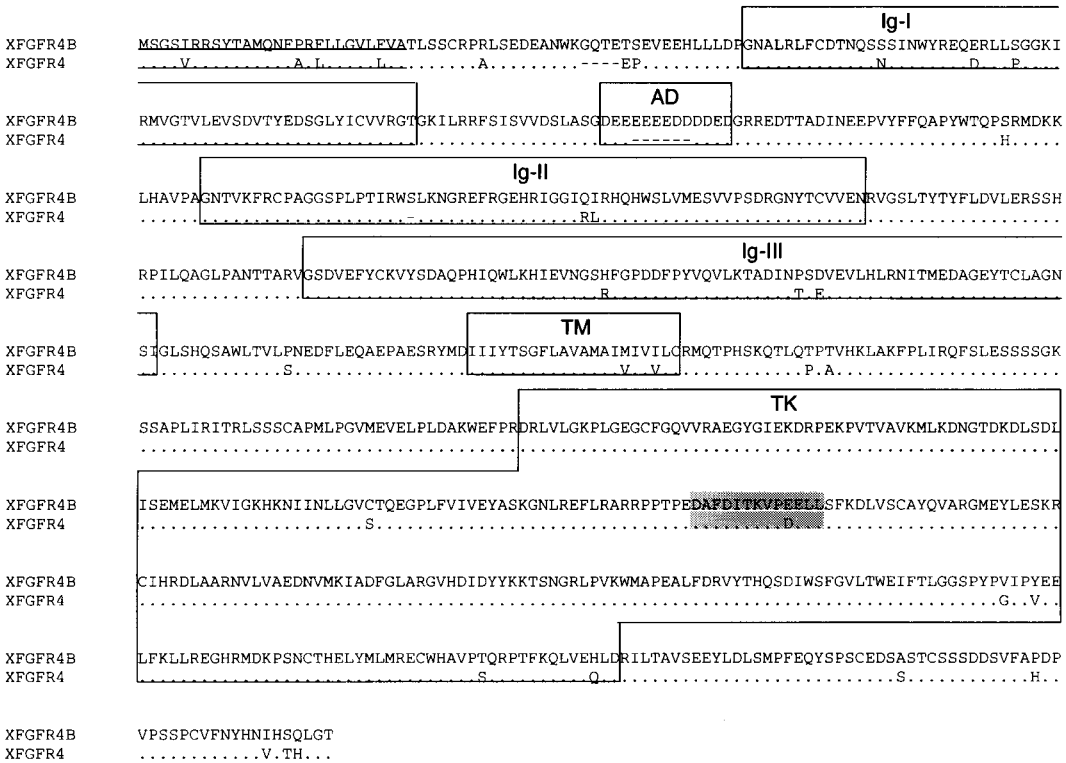

FIG. 1. Comparison of predicted amino acid sequences from XFGFR4B and XFGFR4. Dashes represent gaps in the sequences. Underlined sequences correspond to putative signal peptides. Ig-I, II and III are immunoglobulin-like domains I, II and III, respectively. AD, acidic domain; TM, transmembrane domain; TK, tyrosine kinase domain. Kinase inserts sequences are shadowed.

identical to a recently published 160 bp tyrosine kinase sequence of a FGFR expressed in the brain of late tailbud stage *Xenopus* embryos (19). We propose to name it XFGFR4B.

Developmental Expression of XFGFR4B mRNA

Riboprobes used for RNase protection and in situ hybridization were generated from the 5′ end of the coding region of XFGFR4B cDNA which is most divergent from that of XFGFR4 (91% identity). We assume that these probes are specific of XFGFR4B on the basis of results showing different temporal expressions of XeFGF pseudoalleles using riboprobes with a similar homology (90% identity) (20).

Fig. 2 shows that XFGFR4B mRNA was expressed throughout embryogenesis with a significant increase during gastrulation while XFGFR1 expression remained constant. The doublet of bands generated by the XFGFR4B probe most probably resulted from RNase digestion artifacts. XFGFR4 (9) and XFGFR4B therefore have similar temporal expression patterns. It is noteworthy that temporal expressions of several gene pseudoalleles in *Xenopus* embryos are not regulated similarly (20,21).

Analysis of the spatial expression of XFGR4B mRNA was carried out from the onset of gastrulation. In situ hybridization in toto did not reveal any clear regionalization of XFGR4B mRNA before the late gastrula stage. At the early gastrula stage, XFGFR4B transcripts were detected in all tissues and it was impossible to evaluate whether differences in staining intensities resulted from very different thickness of tissues or from different levels of XFGFR4B expression (not shown). This was therefore studied further by RNase protection (Fig. 3). Early gastrula stage

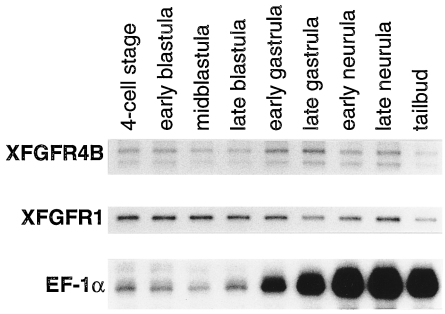

FIG. 2. Temporal expression of XFGFR4B transcripts during embryogenesis. RNase protection analysis. XFGFR4B mRNA expression was compared with that of XFGFR1. EF-1α was used as internal control. XFGF4B mRNA is detected at all stages. Although expression of XFGFR1 transcripts is constant, XFGFR4B mRNA expression diminishes at late blastula stage to reach a maximum during gastrulation.

embryos were dissected into eight pieces including animal ectoderm, supraequatorial or subequatorial dorsal, lateral or ventral marginal zones, and vegetal endoderm (Fig. 3A). XFGFR4B mRNA was detected in all dissected tissues but a much higher level of expression was observed in the endoderm than in the other tissues (Fig. 3B). At the late gastrula stage, neural tissue appeared to be the prominent site of XFGFR4B mRNA expression. Expression of XFGFR4B mRNA was strongly detected in the anterior half of the neural plate. Labelling delineated the border of anterior neural folds and was consistently observed as two lateral patches in the neural plate. The central region of the neural plate was not stained (Fig. 4A, B). At the tailbud stage, neural tissue remained the major site of XFGFR4B mRNA expression. XFGFR4B transcripts were principally detected in retina, midbrain, otic vesicle and hindbrain and at a lower level in the more posterior neural tube (Fig. 4 C-E). In early tadpoles, high expression of XFGFR4B mRNA was also observed in the developing head skeleton (Fig. 4E). XFGFR4B mRNA was also detected in differentiating mesodermal tissues. They were present from the early tailbud stage on in the pronephros, and later in the heart primordium and the myotomes (Fig. 4 C-E). It is striking that as soon as the embryo reaches the late gastrula stage, the expression pattern of XGFR4B transcripts in the neural plate

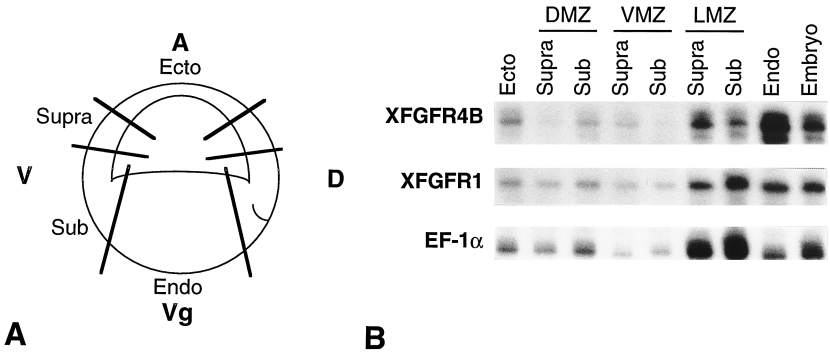

FIG. 3. Spatial expression of XFGFR4B mRNA in early gastrulae. A) Diagram of an early gastrula stage embryo (sagital section) showing the position of the various dissected tissues. Thick lines indicate the position of cuts. Animal ectoderm (Ecto), supraequatorial (Supra), subequatorial (Sub) and vegetal endoderm (Endo) were dissected. Marginal zone explants were subsequently divided into dorsal, lateral and ventral parts. B) RNase protection analysis in dissected tissues. XFGFR4B, XFGFR1 and EF-1α transcripts expressions were simultaneously analyzed as in Fig. 2. XFGFR4B transcripts are expressed in every tissue. A high level of XFGFR4B expression was detected in the endoderm. A, Animal Pole; D, Dorsal; DMZ, Dorsal Marginal Zone; LMZ, Lateral Marginal Zone; V, Ventral; Vg, Vegetal Pole; VMZ, Ventral Marginal Zone.

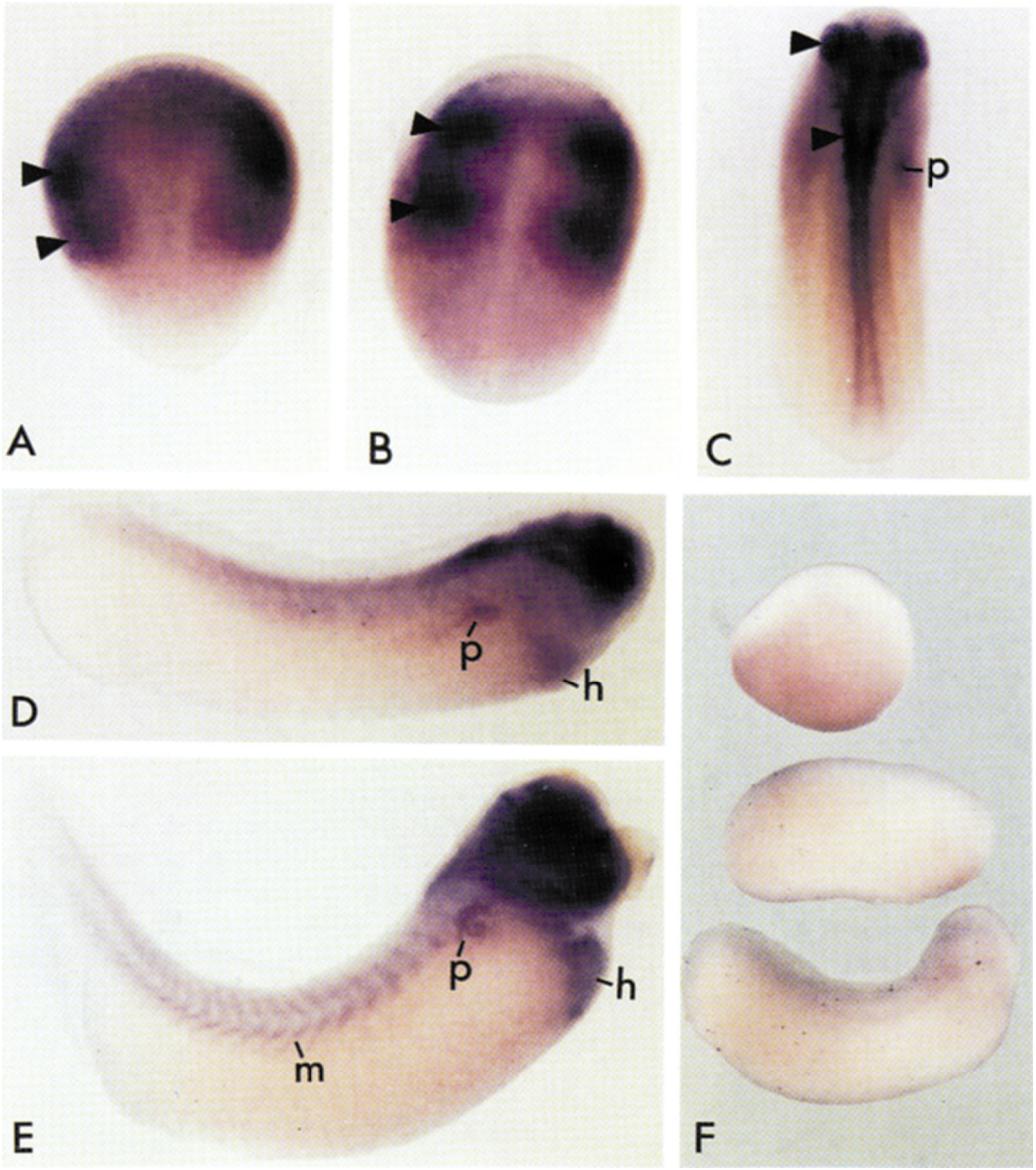

FIG. 4. In situ hybridization analysis of XFGFR4B during embryogenesis. A–C) Dorsal views. A) Late gastrula stage. B) Early neurula stage. C) Early tailbud stage. Arrowheads indicate the patches of XFGFR4B expression in the neural plate (A, B) and the corresponding regions at tailbud stage (C) including retina, midbrain and hindbrain. XFGFR4B transcripts are detected in more posterior regions of the neural tube at a lower level. Note the staining of the developing pronephros (p). D–F) Lateral views. D) Late tailbud stage. E) Early tadpole. F) Controls with a sense probe (lower magnification). Besides neural expression, XFGFR4B mRNA is detected in pronephros (p) heart (h) and myotomes (m) (D,E). Developing head part expresses high levels of XFGFR4B mRNA at the tadpole stage (E).

strictly corresponds to the expression pattern observed in the developing nervous system at the tailbud stage. The fate map of the neural plate indeed shows that the two regions expressing high levels of XFGFR4B mRNA give rise to midbrain and retina on one hand and hindbrain on the other (22). These observations suggest that XFGFR4B is playing an important function during patterning of the neuroepithelium.

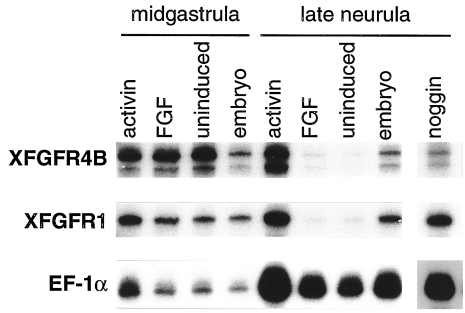

FIG. 5. Expression of XFGFR4B mRNA in response to mesodermal and neural inductions. RNase protection analyses in blastula ectoderm were treated with 20 U/ml of activin, 20 ng/ml of bFGF or expressing noggin and cultured until midgastrula or late neurula stages. XFGFR4B, XFGFR1 and EF-1α transcript expressions were simultaneously analyzed as in Fig. 2. Explants cultured until the midgastrula stage expressed high levels of XFGFR4B and XFGFR1 mRNA whether they were treated with activin, bFGF or were uninduced controls. When cultured until the late neurula stage, only explants treated with activin or expressing noggin continue to express XFGFR4B and XFGFR1 at a similar level while their expressions dropped in bFGF-treated and uninduced explants. Embryo lanes correspond to midgastrula and late neurula stage whole embryo samples used as positive controls.

Effect of Mesodermal and Neural Inducers on XFGFR4B Expression

We tested the effect of activin, bFGF and noggin on the expression of XFGFR4B and XFGFR1 mRNAs in isolated blastula ectoderm cultured until neurula stage. High concentration of activin and low concentration of bFGF were used to obtain dorsal and ventral mesoderm respectively (23). Expression of noggin was performed to induce neural tissue (15). Fig. 5 shows that XFGFR4B and XFGFR1 transcripts were detected in all cases when explants were cultured until midgastrula stage. After culture until late neurula stage, high levels of expression were only detected when explants were treated with activin or expressed noggin. In explants treated with bFGF or uninduced explants, expressions of XFGFR4B and XFGFR1 mRNAs dropped dramatically. Maintenance of high levels of XFGFR4B mRNA expression in response to noggin is consistent with the expression of XFGFR4B mRNA observed in the anterior neuroepithelium. The effect of activin might be more complex. It is possible that maintenance of XFGFR4B mRNA by activin results from indirect neural induction by the induced dorsal mesoderm or from the direct induction of mesodermal tissues like pronephros. Our results slightly differs from those of Friesel and Dawid (7) who observed the maintenance of XFGFR1 expression in response to bFGF at late neurula stage. This probably results from the much lower dose of bFGF used in our experiments (we used 20ng/ml, but they used 50 ng/ml). We observed that explants treated with 20ng/ml of bFGF reexpress high levels of both XFGFR4B and XFGFR1 when cultured until the midtailbud stage (unpublished results). It is possible that such a process occurs earlier in response to a higher dose of bFGF. In conclusion, our observations indicate that regionalization of XFGFR4B expression during neurulation is likely to be regulated by early cell interactions.

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