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Original Article

Expression and functions of FGF-3 in *Xenopus* development

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ABSTRACT We have analyzed the expression pattern of the *Xenopus FGF-3* gene during early development and examined its biological activity in three different bioassays using *Xenopus* embryos. We show that from the early gastrula stage there is a domain of expression around the blastopore which becomes a posterior domain as the blastopore closes. An anterior ectodermal domain becomes detectable from mid-gastrula stages in the prospective hind-brain, and there are several later domains of expression: the midbrain-hindbrain junction, the otocyst, the pharyngeal pouches and the tailbud region. By using double whole-mount *in situ* hybridizations we show that the *XFGF-3* expression in the brain is dynamically regulated both in time and space during development. The anterior domain of early neurula stage embryos corresponds to the prospective rhombomeres 3-5. By the time the neural tube is closed, *XFGF-3* expression is restricted to r4 and later a new domain of expression is established at the midbrain/hindbrain junction. In addition, we show that, despite its difference in receptor specificity, XFGF-3 can induce the formation of mesoderm from animal caps similarly to other FGFs. It also displays a posteriorizing activity on whole embryos similar to other FGFs. Although the absence of maternal expression makes it unlikely that XFGF-3 is involved in mesoderm induction *in vivo*, its posterior domain of expression during gastrulation and its posteriorizing activity suggests that it participates in the maintenance of mesodermal gene expression and in the FGF mediated patterning of the anteroposterior axis during gastrulation.

KEY WORDS: *Xenopus, FGF-3, FGF receptor, mesoderm induction, posteriorization, rhombomeres, mid-brain-hindbrain junction (isthmus)*

Introduction

In recent years it has become clear that members of the fibroblast growth factor (FGF) family play several important roles in pattern formation during vertebrate development.

During the very early stages of development, FGFs have been shown to be required in *Xenopus* for the establishment and patterning of the mesoderm (Kimelman and Kirschner 1987; Slack *et al.*, 1987; Amaya *et al.*, 1991,1993) and for the anteroposterior patterning of trunk and tail (Isaacs *et al.*, 1994; Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995; Pownall *et al.*, 1996). At later stages, work in chicken and mouse has shown that FGFs are involved in a wide range of developmental processes such as formation of the midbrain (Crossley *et al.*, 1996a), inner ear development (Mansour *et al.*, 1993), the induction and outgrowth of the limbs (Cohn *et al.*, 1995; Crossley *et al.*, 1996b; Vogel *et al.*, 1996; Ohuchi *et al.*, 1997), apoptosis in the tooth (Vaahtokari *et al.*, 1996), lung growth (Peters *et al.*, 1994), lens development (Robinson *et al.*, 1995) and the regulation of hair growth (Hebert *et al.*, 1994).

Signaling by the members of the FGF family depends upon complex interactions with high and low affinity cell surface receptors that eventually lead to the activation of the MAP kinase signal transduction pathway (Dionne *et al.*, 1991; Johnson and Williams, 1993). Four high affinity receptor genes (FGFR-1 to FGFR-4), members of the tyrosine kinase receptor family, are known to exist. Alternative splicing of the four genes generate multiple variants of the receptors characterized by distinct ligand binding specificities (Johnson and Williams, 1993). Of these the most significant is the distinction between the c type isoforms that bind tightly to FGF-2 and the b type isoforms that bind tightly to FGF-7.

One member of the FGF family, *FGF-3* (previously known as *int-2*), was first identified as a proto-oncogene because of its transcriptional activation in tumors induced by mouse mammary

Abbreviations used in this paper: FGF, fibroblast growth factor; eFGF, embryonic fibroblast growth factor; FGFR, fibroblast growth factor receptor; r, rhombomere; En-2, Engrailed-2; st, stage.

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tumor virus (Peters *et al.*, 1983; Dickson *et al.*, 1984). Although hardly detected in adult tissues, both mouse and chicken *FGF-3* transcripts exhibit a complex pattern of expression in the developing embryo suggesting multiple functions for this growth factor during embryogenesis (Wilkinson *et al.*, 1988,1989; Mahmood *et al.*, 1995). The knockout of murine *FGF-3* gene function (Mansour *et al.*, 1993) causes abnormalities in the tail and defects in the development of the inner ear. *Xenopus* FGF-3 binds avidly to the FGFR2b isoform (Mathieu *et al.*, 1995) and in this regard differs from FGF-2, the factor on which most work has so far been done. Because of this different receptor specificity we felt that FGF-3 might show biological activities distinct from those of FGF-2. In this paper we present a detailed expression pattern of *Xenopus FGF-3* during early development and examine its biological activities in three different bioassays, animal cap autoinduction, posteriorization, and bead implantation.

Results

XFGF-3 expression in the Xenopus embryo

XFGF-3 is not expressed maternally but only zygotically, being first detected at the early gastrula stages. At stage 10.5 faint staining is detected in a complete ring around the blastopore with a higher level of expression dorsally (Fig. 1A). As gastrulation proceeds, *XFGF*-3 expression becomes downregulated on the dorsal side relative to the lateral and ventral aspect of the blastopore (Fig. 1B). At this stage its expression is complementary to that of *eFGF*, which becomes concentrated on the dorsal side (Isaacs *et al.*, 1995).

In the middle gastrula stage, a prominent stripe of *XFGF*-3 expression becomes established on each side of the neural plate about half way along the embryo and is maintained throughout neurulation (Fig. 1B-F). As the blastopore closes, the periblastoporal ring becomes concentrated in the posterior (Fig. 1C-E). The level of expression remains reduced in the dorsal midline of the posterior although some faint staining extending up the midline to the anterior stripe is detectable (arrowhead in C and D). By the end of neurulation (stage 20) the anterior stripe has become much narrower and due to the closure of the neural tube has converged towards the dorsal midline (Fig. 1F). Posteriorly, there is still a strong expression in the regions lateral to the closed blastopore that appears to extend for some distance along the developing axis (Fig. 1G).

By the tailbud stages of development *XFGF-3* expression has become quite complex. Figure 1H shows that high levels of expression are detectable in the lining of the pharyngeal pouches and in the otocyst. By this stage the expression in the hindbrain has disappeared while a new domain has appeared at the level of the isthmus (midbrain/hindbrain junction). In the posterior, *XFGF-3* is expressed in the developing tailbud both in the chordoneural hinge and in the posterior wall of the neuroenteric canal (Fig. 1I).

XFGF-3 expression in the developing Xenopus hindbrain and midbrain

In an attempt to define in more detail to what region of the neuroectoderm the anterior stripe of *XFGF-3* expression corresponds, we carried out double whole-mount *in situ* hybridizations comparing *XFGF-3* to three other genes known to be expressed in specific regions of the brain during development: *En-2* which is expressed at the midbrain/hindbrain junction (isthmus) (Hemmati-Brivanlou and Harland, 1989), *Krox-20* whose two medial stripes of expression correspond to rhombomere 3 and 5 (r3 and r5) of the developing hindbrain (Bradley *et al.*, 1992) and *HoxB1*, expressed in rhombomere 4 (Godsave *et al.*, 1994).

Throughout neurulation the band of *XFGF-3* expression (blue staining) lies posterior to that of *En-2* (orange staining) showing that *XFGF-3* expression at this stage is restricted to the future hindbrain rather than the isthmus (Fig. 1N). *Krox 20* starts to be detectable in the hindbrain, in early neurula stage embryos (st. 13-14), when its expression is initially restricted to the prospective rhombomere 3 (Fig. 1J, orange staining). At this stage the *XFGF-3* domain shares the same anterior boundary but extends about 2 rhombomere widths further to the posterior (Fig. 1J). In slightly older embryos (st. 15), in which *Krox 20* is expressed as two bands in rhombomere 3 and 5, it becomes apparent that *XFGF-3* is expressed in r3, r4 and r5 (Fig. 1K). A comparison with Hox B1, which identifies the prospective rhombomere 4 (Fig. 1M), shows that during the neural fold stages (st. 16–st. 17) *XFGF-3* is downregulated in the prospective rhombomere 3 but it is still expressed at high levels in the prospective r4 and r5. By neural tube closure *XFGF-3* domain has narrowed further and its level of expression has been reduced: at this stage it is absent from r3 and r5 but still present in r4 (Fig. 1L).

By tailbud stages, *XFGF-3* expression in the hindbrain has dropped below detection. But a new domain of *XFGF-3* expression has arisen at the midbrain/hindbrain junction, shown by the fact that *En-2* and *XFGF-3* domains of expression are now found to overlap (Fig. 1O).

Effects of FGF-3 overexpression

Mesoderm induction

Direct binding and binding competition analysis have shown that XFGF-3 has a different receptor specificity from the other FGFs in that it binds with high affinity to both b and c splice variants of FGFR-2 while it has only a weak affinity for FGFR-1 (Mathieu *et al.*, 1995). This difference in receptor preference raises the possibility that XFGF-3 might show different biological activities to other members of the FGF family.

In order to test this possibility we have examined the biological properties of *XFGF-3* in early amphibian development by injecting various amounts of its mRNA into two cell stage embryos. To assay for mesoderm induction, animal caps were taken from blastula stage embryos and cultured for three days and then scored for the formation of fluid-filled vesicles as an indication of mesoderm induction (Godsave and Slack, 1988; Isaacs *et al.*, 1994) (Table I and Fig. 2).

The histological analysis of the induced animal cap explants shows the presence of muscle tissue, mesenchyme, and mesothelial cells, demonstrating that, in common with other members of the FGF family, XFGF-3 can elicit the formation of ventrolateral type of mesoderm (Fig. 2D). In addition, occasionally (6/76), proctodeum-like structures were seen to be formed in animal caps induced with variable doses (14-56 pg) of *FGF-3* mRNA (Fig. 2 E-F). The dose-response data presented in Table I show that *XFGF-3* has a comparable specific activity as a mesoderm-inducing agent to *XFGF-2, XFGF-8* and *XFGF-9*, but a lower activity by about 10x, than eFGF (Isaacs *et al.*, 1992; Song and Slack, 1996; Christen and Slack, 1997).

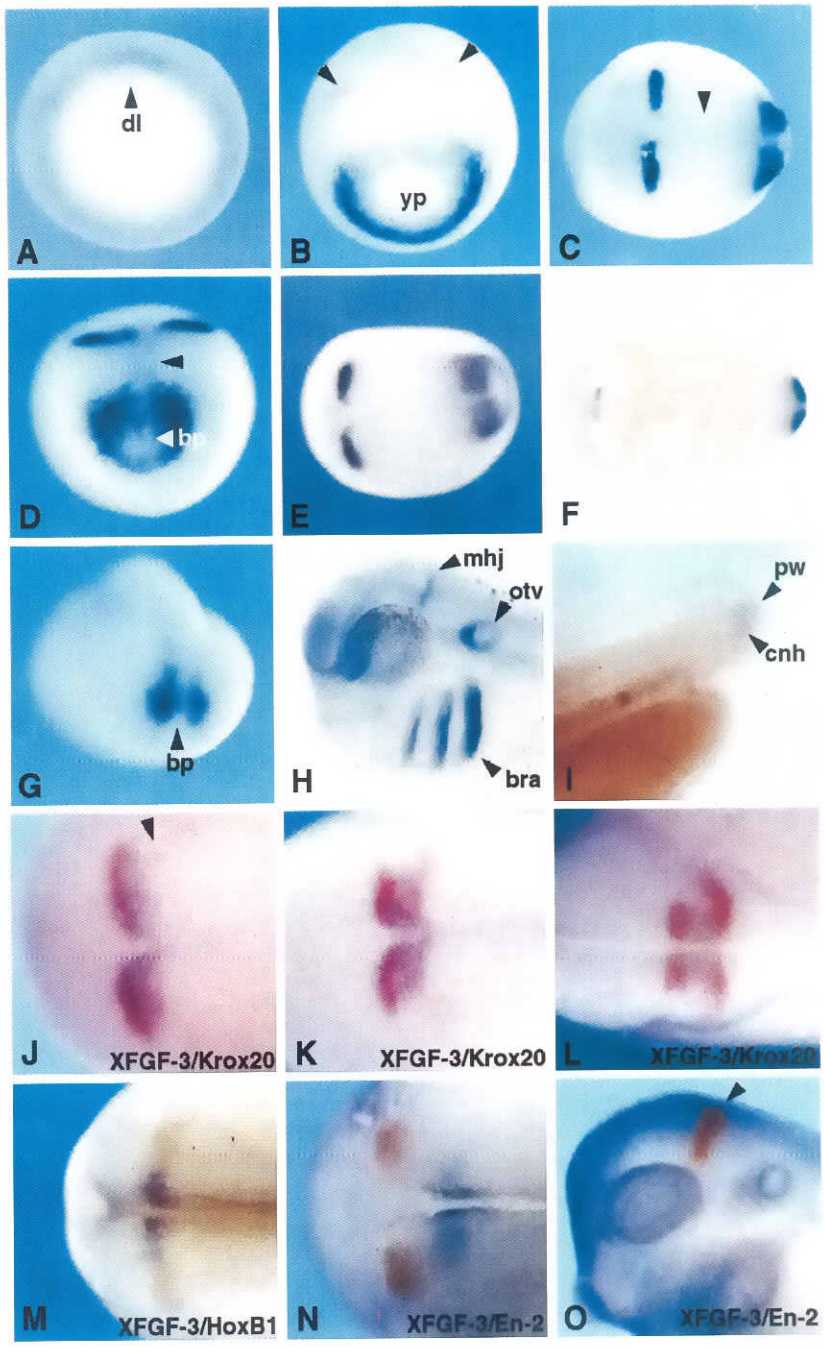

Fig. 1. XFGF3 expression in the developing *Xenopus* embryo. (A) *Vegetal view of an early gastrula stage embryo (st.10.5); dorsal to the top. A faint staining is detected in a complete ring around the blastopore with higher levels of expression visible dorsally. dl, dorsal blastopore lip.* **(B)** *Dorsovegetal view of a late gastrula stage embryo (st. 12.5) showing the periblastoporal expression of XFGF-3. Higher levels of XFGF-3 mRNA are present in the lateral and ventral aspects of the blastopore. Arrowheads point to the anterior stripe of expression that begins to be visible at this stage on each side of the neural plate. yp, yolk plug.* **(C-E)** *Early neurula stage embryos.* **(C,E)** *Dorsal views of a st13 and st 14 embryos, respectively (anterior to the left). Expression in the neural plate has become more prominent by this stage. A faint staining is detected either side of the midline extending up to the anterior stripe (black arrowhead in C and D).* **(D)** *Posterior view of a st. 13 embryo showing XFGF-3 expression around the closed blastopore (bp).* **(F)** *Dorsal view of a cleared embryo at the end of the neurula stage (st. 20). The anterior stripe of XFGF-3 expression has become much narrower and has converged towards the midline.* **(G)** *Posterior view of a stage 20 embryo showing a strong expression in region lateral to the closed blastopore (bp) extending for some distance along the developing axis.* **(H,I)** *Tailbud stage embryo (st. 32).* **(H)** *Side view of the head region of a cleared embryo showing expression in the branchial arches (bra), in the otic vesicle (otv) and at the level of the midbrain/hindbrain junction (mhj).* **(I)** *Side view of the posterior region of a cleared embryo showing XFGF-3 expression in the tailbud in the chordoneural hinge (cnh) and in the posterior wall (pw) of the neurenteric canal.* **(J-L)** *Double whole-mount in situ hybridizations showing XFGF-3 and Krox 20 expression (blue and orange staining, respectively).* **(J)** *Early neurula stage embryo (st. 14). Krox 20 and XFGF-3 expression are found to overlap at the level of the prospective rhombomere 3 of the hindbrain . At this stage Krox 20 expression in the prospective rhombomere 5 is only barely detectable (arrowhead).* **(K)** *Stage 15 embryo. XFGF-3 at this stage is expressed in rhombomeres 3-5.* **(L)** *Stage 20 embryo. By the end of neurulation the domain of XFGF-3 expression has narrowed, being restricted to the prospective rhombomere 4.* **(M)** *Double whole-mount in situ hybridization showing XFGF-3 and Hox B1 (orange staining) expression in a stage 17 embryo. Hox B1 band of expression identify the prospective rhombomere 4.* **(N,O)** *Double whole-mount in situ hybridization showing En-2 (orange staining) and XFGF-3 expression.* **(N)** *Neurula stage embryo (st. 16). Throughout neurulation the band of XFGF-3 expression lies posterior to that of En-2.* **(O)** *Side view of a tailbud stage embryo (st. 30). By tailbud stages XFGF-3 expression in the hindbrain has disappeared while a new domain of expression has arisen at the isthmus (arrowhead) that overlaps with that of En-2.*

In order to test whether *XFGF-3* function is subject to the inhibitory effect of the dominant negative FGFR-1 receptor [XFD: (Amaya *et al.*, 1991)], we coinjected *XFGF-3* mRNA with an excess of XFD mRNA. Table 1 shows that XFD can effectively block XFGF-3 inducing activity indicating that the receptors stimulated by XFGF-3 can be inhibited by this reagent.

Posteriorization

In whole embryos, overexpression of *eFGF* during gastrula stage causes a reduction of anterior structures (Isaacs *et al.*, 1994; Pownall *et al.*, 1996). This effect can also be observed following injection of mRNA into fertilized eggs. We found that *XFGF-3* mRNA would cause anterior truncation in a dose-dependent way. Low doses (up to 35 pg RNA) cause mild to moderate anterior truncations characterized by a partial or complete loss of the eyes and the cement gland (Fig. 2G). Higher doses (70 to 280 pg) lead to a severely posteriorized phenotype characterized by a loss of most of the head (Fig. 2H). The trunk is abnormally bent ventrally and an enlarged proctodeum region is found at the posterior of some embryos whereas the tail seems only lightly affected. Although these results differ somewhat from those seen with *eFGF* mRNA (Isaacs *et al.*, 1994) in that *XFGF-*

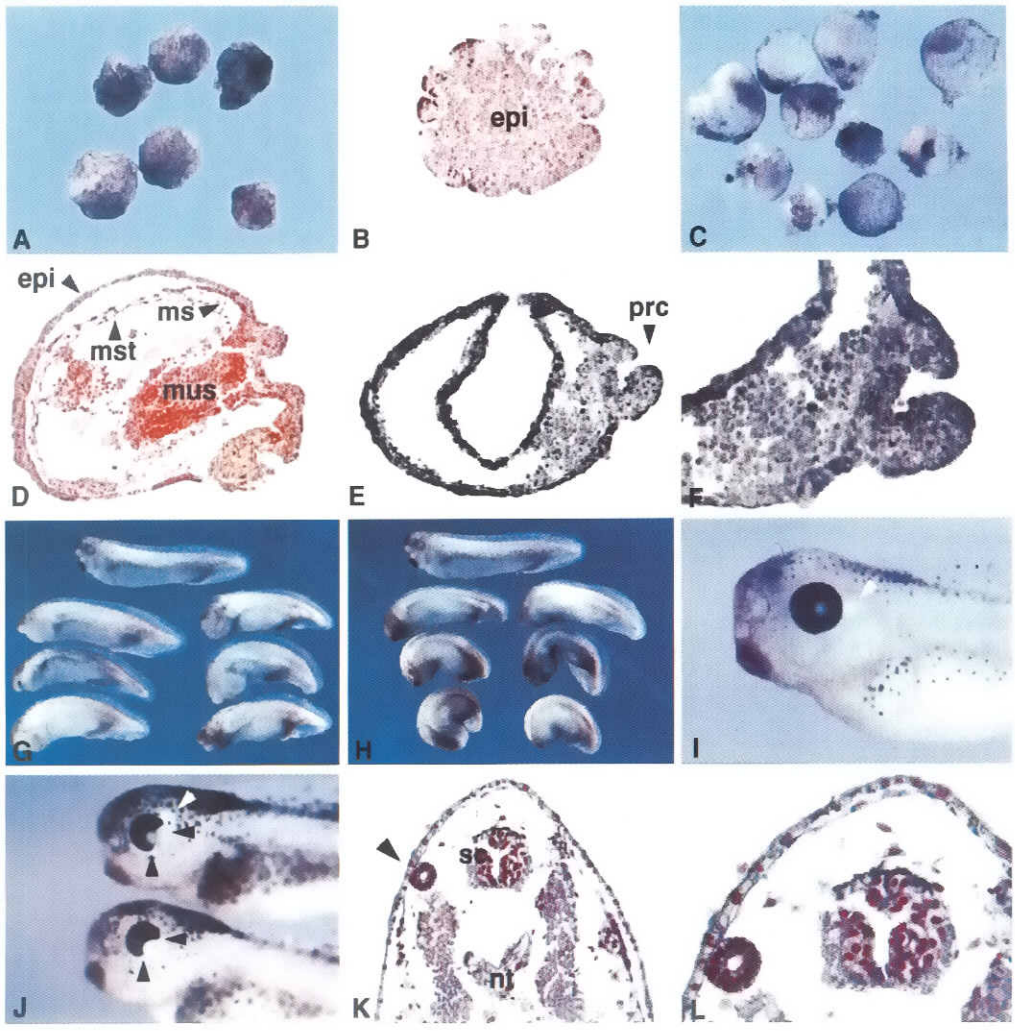

Fig. 2. Effects of XFGF-3 overexpression. **(A,C)** *Whole animal caps after three days in culture.* **(B,D)** *Sections of such animal caps.* **(A,B)** *Controls;* **(B,C)** *Induced animal caps from embryos that had been injected with 28 pg of XFGF-3 mRNA. epi, epidermis; ms, mesenchyme; mst mesothelium; mus, muscle.* **(E)** *Induced animal cap from an embryo injected with 14 pg of XFGF-3 mRNA showing a proctodeum like structure (prc).* **(F)** *High magnification of the same animal cap.* **(G)** *Top embryo is a control while the bottom six embryos have been injected with 35pg of XFGF-3 mRNA at the 2-cell stage.* **(H)** *Top embryo is a control while the bottom six embryos have been injected with 70pg mRNA at the 2-cell stage.* **(I,J)** *PBS or XFGF-3 soaked beads were implanted close to the left optic vesicle of late neurula stage embryos.* **(I)** *The presence of the PBS bead (arrowhead) did not have any effect on the development of the eye of control embryos.* **(J)** *XFGF-3 beads affected the differentiation of the pigmented epithelium of the eye. Black arrowheads point to the wide region of the pigmented epithelium where pigmentation is absent. White arrowhead points to the position of the implanted XFGF-3 bead.* **(K)** *Transverse section at the level of the trunk of a stage 40 embryo which had an XFGF-3 bead implanted posteriorly at the early neural plate stage. The presence of the XFGF-3 bead has induced the formation of an ectopic otic vesicle (arrowhead). Sc spinal cord; nt, notochord.* **(L)** *Higher magnification view of the same specimen.*

3 overexpression does not significantly affect normal gastrulation, they are comparable to those seen with *FGF-2* (Thompson and Slack, 1992), *FGF-8* (Christen and Slack, 1997) and *FGF-9* (Song and Slack, 1996). Again, the results of our analysis have shown that although XFGF-3 has a different receptor preference from other members of the family, it nonetheless appears to share their biological activities.

Later effects

Because of the multiple late expression domains we considered it possible that XFGF-3 would show novel biological activities associated with these domains. To investigate this we grafted heparin beads soaked in XFGF-3 protein (370 μg/ml) into various sites in the neurula.

XFGF-3 beads implanted anteriorly between early neural fold and early tailbud stages (st. 15 to st. 22) were found to affect the differentiation of the pigmented epithelium of the eye and to cause abnormalities in the layer structure of the retina in 15 out of 20 embryos (Fig. 2J), while posterior bead implantations at early neural plate stage (st. 13-14) were found to induce the formation of ectopic otic vesicles in 2 out of 4 embryos (Fig. 2K,L). However, the effects found with XFGF-3 were very similar to those elicited by the implantation of FGF-2 beads in similar stage embryos (Lombardo and Slack, 1998) and no specific effects that are not shown by FGF-2 were found in our study. Moreover the indications are that the results with similar doses of FGF-2 are stronger than with XFGF-3.

Discussion

In this study we have extended our characterisation of the *Xenopus* FGF-3 expression pattern published in an earlier report (Tannahill *et al.*, 1992), and also examined its biological activity in various assays using *Xenopus* embryos.

By using double whole-mount *in situ* hybridizations we have been able to show that *XFGF-3* expression in the brain is complex, being dynamically regulated both in time and in space during development. These results are comparable to those reported for both the chicken and mouse homologs (Mahmood *et al.*, 1995). However, the pattern revealed by our study does differ slightly from what other investigators have observed. Expression was reported in r5 and r6 in both the chicken and the mouse hindbrain (Wilkinson *et al.*, 1988; Mahmood *et al.*, 1995), whereas we have not seen expression in r6 in *Xenopus*. In addition, although r4

appears to be a common site of *FGF-3* expression in mouse, chicken and *Xenopus*, neither in mouse nor in chicken has expression been detected in r3 (Wilkinson *et al.*, 1988; Mahmood *et al.*, 1995). Mahmood *et al.* (1995) do detect *FGF-3* expression in the unsegmented hindbrain of the chick, although they do not comment on what prospective rhombomere/s this region may correspond to. Thus, it remains possible that the early domain of expression that Mahmood *et al.* (1995) describe in the unsegmented chicken hindbrain does encompasses the prospective r3 and that, due to the highly dynamic pattern of expression of this gene, *FGF-3* expression at this level is lost by the onset of rhombomere formation.

As far as biological activity is concerned, we have shown that, despite its difference in receptor specificity, XFGF-3 can induce the formation of mesoderm from animal caps similarly to other FGFs. It also displays a posteriorizing activity on whole embryos similar to other FGFs. The absence of maternal expression makes it unlikely that XFGF-3 is involved in mesoderm induction *in vivo*, but its posterior domain of expression during gastrulation and its posteriorizing activity indicate that it may participate along with eFGF and FGF-8 in the maintenance of mesodermal gene expression and in the FGF mediated patterning of the anteroposterior axis during gastrulation. The biological activities shown in the bead implantation experiments, together with the expression data, also make it likely that FGF-3 contributes to the inductive activity of the isthmus region in the brain, as well as being necessary for the correct morphogenesis of the ears.

The inducing activity of XFGF-3 is completely inhibited by the dominant negative receptor XFD (Amaya *et al.*, 1991). This is a truncated form of FGFR1, but its ability to inhibit XFGF-3 suggests that it can block other receptors in addition to R1 itself. A detailed study of FGF receptor expression in *Xenopus* has yet to be carried out, but in the Spanish salamander, *Pleurodeles waltl*, the presence of several types and isoforms of FGF receptors was reported (Shi *et al.*, 1992,1994a,b; Launay *et al.*, 1994; Riou *et al.*, 1996). Our results show that the *Xenopus* blastula must contain receptors that are stimulated by FGF-3, and also that such receptors can be blocked by XFD. Therefore some of the function of endogenous FGFs, deduced from the phenotype of the XFD embryos (Amaya *et al.*, 1991,1993; Isaacs *et al.*, 1994) is likely to be attributable to *XFGF-3* signaling. In summary, our results suggest that *XFGF-3* contributes to the total endogenous FGF activity, but that it does not have any unique activity of its own.

TABLE 1

MESODERM INDUCTION FOLLOWING XFGF-3 mRNA INJECTION

XFGF-3 mRNA	Uninduced	Induced (%)		*n*
1.75 pg	19	2	(10)	21
3.75 pg	16	2	(11)	18
7 pg	23	8	(26)	31
14 pg	10	14	(58)	24
28 pg	4	34	(89)	38
56 pg	-	16	(100)	16
28pg +2ng XFD	22	-	(0)	22
Water injected	30	-	(0)	30

Various amounts of XFGF3 mRNA were injected into two cell stage embryos. Animal caps were taken from blastula stage embryos, cultured for three days and then scored for the formation of fluid-filled vesicles. The results from two separate experiments were pooled.

Materials and Methods

Embryological methods and RNA injections

Embryos were cultured according to the methods of Godsave and Slack, 1988 and staged according to Nieuwkoop and Faber, 1967.

To create the CS2$^+$FGF-3 plasmid a 5' primer, GAGAGAGATCGATACATATGGTTATAATTTGGATCCT, containing a Cla I restriction site (underlined), and a 3' primer, CGTATCCTGCAGACTAGTTCAATGTCCTCGTCTTTGTCT containing a Pst I restriction site (underlined), were used to amplify a 736bp fragment containing the full *Xenopus FGF-3* coding region from the plasmid XFGF3.1 (Kiefer *et al.*, 1993). The amplified PCR product was then cut with Cla I and PstI restriction enzymes and ligated between the corresponding sites in the CS2$^+$ vector (Turner and Weintraub, 1994).

FGF-3 synthetic mRNA was produced by *in vitro* transcription using the MEGAscript SP6 kit (Ambion), according to the manufacturer's instructions, using as template CS2$^+$FGF-3 plasmid linearized with Not I.

Injections were performed as described in Isaacs *et al.*, 1994. Different amount of synthetic mRNA dissolved in 10 nl of water were injected into each blastomere of two cell stage embryos in NAM+5% Ficoll. At stage 6 the embryos were transferred to NAM/10+5% Ficoll and allowed to develop until the appropriate developmental stage. Animal cap explants were taken at stages 8-9 and cultured in NAM/2 until the appropriate control stage was reached, at which time they were fixed for histological analysis.

Histological methods were as described in Godsave and Slack, 1988.

Preparation of XFGF-3 protein and bead implants

XFGF-3 protein was purified from the conditioned medium from COS-1 cells transfected with the plasmid XFGF3.1 as described in Mathieu *et al.* (1995) and bead implantations were performed as described in Lombardo and Slack (1998). The concentration of FGF-3 protein used in this study was 370 µg/ml. For control experiments, beads were incubated in PBS instead of XFGF-3.

In situ hybridization

Albino embryos were cultured to various developmental stages and then fixed in MEMFA (0.1 M MOPS, 2 mMEDTA, 1 mM MgSO4, 3.7% formaldehyde) for an hour at room temperature and stored in 100% ethanol at -20°C until further processing.

Whole-mount *in situ* hybridizations were carried out as in Harland (1991), with modifications as in Pownall *et al.*, 1996.

For double *in situ* hybridizations the probes were transcribed from linearized plasmids using either 10X DIG RNA labeling mix *(FGF-3)* or 10X Fluorescein RNA labeling mix (Boehringer Mannheim) *(En-2, Krox20 and HoxB1)*.

FGF3 was linearized with Bam HI and transcribed with T3 polymerase. *En-2* and *Krox 20* were detected as in (Doniach *et al.*, 1992). *HoxB1* (Godsave *et al.*, 1994) was linearized with NdeI and transcribed with SP6 polymerase.

Hybridization of probes to endogenous RNAs was detected by anti-DIG *(XFGF-3)* or anti-fluorescein *(En-2; Krox-20; HoxB1)* alkaline phosphatase conjugated antibodies. *XFGF-3* expression was visualized by a color reaction using BM-purple (Boehringer Mannheim) (blue staining) while to visualize that of *En-2*, *Krox-20* and *HoxB1* INT/BCIP (Boehringer Mannheim) was used (red/orange staining).

Acknowledgments

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