

Differential expression and regulation of two distinct fibroblast growth factor receptors during early development of the urodele amphibian *Pleurodeles waltl*

DE-LI SHI¹, JEAN-JACQUES FEIGE², JEAN-FRANÇOIS RIOU¹, DOUGLAS W. DeSIMONE³ and JEAN-CLAUDE BOUCAUT^{1*}

¹Laboratoire de Biologie Expérimentale, URA-CNRS 1135, Université Pierre et Marie Curie, 9 quai Saint-Bernard, Bâtiment C-30, 75005 Paris, France, ²Laboratoire de Biochimie des Régulations Cellulaires Endocrines, INSERM Unité 244, DBMS/LBIO, Centre d'Etudes Nucléaires de Grenoble, 85 X, 38041 Grenoble Cedex, France and ³University of Virginia, Health Sciences Center and the Molecular Biology Institute, Department of Anatomy and Cell Biology, Box 439, School of Medicine, Charlottesville, VA 22908, USA

*To whom correspondence should be addressed

Summary

Fibroblast growth factor (FGF) has been shown to be involved in mesoderm induction during amphibian development. Its presence in the embryo suggests that FGF is an endogenous inducer. By polymerase chain reaction (PCR) methods and by screening a *Pleurodeles waltl* tail-bud cDNA library with a cDNA probe for human FGF receptor, we have isolated two full-length cDNA clones, which we designate PFR1 and PFR4 based on their homology to the human FGF receptors FGFR-1 and FGFR-4. Both cDNA clones encode *Pleurodeles* FGF receptors that share characteristics common to members of the FGF receptor superfamily. The deduced amino acid sequence of PFR1 is 85% identical overall with the human *fms*-like-gene (FLG). PFR4 is most closely related to the human FGFR-4 (66% overall identity). The tyrosine kinase catalytic domains of both receptors are remarkably conserved. The two receptors show distinct patterns of regulation during early development. PFR1 first appears as a maternally derived mRNA and mRNA levels remain constant during early developmental stages. However, PFR4 mRNA is first

expressed at the late blastula stage, which suggests that its expression is a result of zygotic transcription. Furthermore, northern blot analysis indicates that PFR1 mRNA is distributed evenly in the early gastrula while PFR4 mRNA is predominantly localized to the presumptive ectoderm. At tail-bud stage, PFR1 transcripts are localized primarily to the neural and mesodermal tissues, PFR4 transcripts are most abundantly expressed in neural tissue, and more transcripts are detected in lateral plate mesoderm than in the somites. When animal cap explants of blastulae are cultured in the presence of mesoderm-inducing factors, PFR1 mRNA levels are maintained by bFGF and activin A. In contrast, PFR4 mRNA levels are significantly down-regulated. These observations suggest a differential expression and regulation of FGF receptors in early amphibian development.

Key words: fibroblast growth factor receptor, mesoderm induction, tyrosine kinase, *Pleurodeles waltl*, PFR1, PFR4.

Introduction

Acidic and basic fibroblast growth factors (aFGF and bFGF) are the prototypes of a large family of structurally related multifunctional peptides that play important roles in several biological processes such as angiogenesis, neuronal differentiation, wound-healing, normal and pathological cell proliferation and embryonic development (Slack et al., 1987; Kimelman and Kirschner, 1987; Rifkin and Moscatelli, 1989; Baird and Böhlen, 1990; Goldfarb, 1990). Besides aFGF and bFGF, the FGF family comprises at present five other members. These include the two oncogene products int-2 (Dickson and Peters, 1987) and hst/K-

FGF (Taira et al., 1987; Delli Bovi et al., 1987) and three other gene products termed FGF-5 (Zhan et al., 1988), FGF-6 (Marics et al., 1989) and keratinocyte growth factor (Finch et al., 1989).

The characterization of FGF receptors has progressed rapidly in the last few years. In humans, there are at least four FGF receptor genes and it has been proposed to name them FGFR-1, FGFR-2, FGFR-3 and FGFR-4 (Keegan et al., 1991; Partanen et al., 1991). All these genes encode transmembrane proteins with tyrosine kinase activity. FGFR-1 was initially purified from chicken embryos (Lee et al., 1989) and partial sequencing of the protein revealed a strong homology with the product of the previously char-

acterized *fms*-like-gene (FLG) (Ruta et al., 1988). The second FGF receptor gene, FGFR-2, was identified by screening a mouse liver cDNA expression library with anti-phosphotyrosine antibodies and was named BEK (standing for bacterially expressed kinase) (Kornbluth et al., 1988). Complete human FGFR-1/FLG, FGFR-2/BEK, FGFR-3 and FGFR-4 cDNA clones were isolated recently (Dionne et al., 1990; Keegan et al., 1991; Partanen et al., 1991). Their deduced amino acid sequences reveal that they are similar yet distinct gene products, with structural features shared by the platelet-derived growth factor (PDGF) receptor/colony stimulating factor 1 (CSF-1) receptor/*c-kit* family of tyrosine kinase receptors. The extracellular domains contain three immunoglobulin (Ig)-like domains of similar location and a remarkable stretch of acidic amino acids located between the first and the second Ig-like domains. The intracellular domains contain highly conserved tyrosine kinase domains that are split by 14 amino acid insertions. The functionality of human FGFR-1, FGFR-2 and FGFR-3 has been demonstrated by FGF-dependent $^{45}\text{Ca}^{2+}$ efflux assays in *Xenopus* oocytes microinjected with the respective mRNAs (Johnson et al., 1990; Keegan et al., 1991) and by binding assays on transfected NIH-3T3 cells (Dionne et al., 1990). All three human receptor types appear to bind both aFGF and bFGF with dissociation constants of $(2-15) \times 10^{-11}$ M and to respond to both aFGF and bFGF in the $^{45}\text{Ca}^{2+}$ efflux assays. Interestingly, FGFR-4 binds aFGF with high affinity but it does not bind bFGF (Partanen et al., 1991).

The development of the amphibian embryo involves a series of inductive events. The first of these is thought to be mesoderm induction, which is believed to occur in response to diffusible signals released from vegetal blastomeres (for review see Gurdon, 1987; Smith, 1989). Recently, significant progress has been made in understanding mechanisms of induction since the demonstration that growth factors are involved in this process. First, FGF was shown to induce isolated ectodermal cells, which differentiate as epidermis when cultured alone, into mesodermal derivatives (Slack et al., 1987). This was followed by the discovery that XTC-cell-conditioned medium has a strong mesoderm-inducing activity (Smith, 1987; Smith et al., 1988). The factor responsible for mesoderm induction in this medium has now been identified as activin A (Smith et al., 1990; Van den Eijnden-Van Raaij et al., 1990). Activin A was shown recently to be present in the *Xenopus* embryo (Asashima et al., 1991). In addition, both FGF protein (Slack and Isaacs, 1989) and mRNA (Kimelman et al., 1988) are present in these embryos. Two FGF receptors (130×10^3 and 140×10^3 M_r respectively) were identified in *Xenopus* blastulae using chemical cross-linking approaches (Gillespie et al., 1989). Recently, a FGF receptor from *Xenopus* has been cloned and characterized (Musci et al., 1990; Friesel and Dawid, 1991). This receptor binds and functionally responds to bFGF (Musci et al., 1990). It represents the *Xenopus* homolog of FGFR-1 (Musci et al., 1990; Friesel and Dawid, 1991).

Because it is now well established that protein-tyrosine-kinases are involved in the control of many developmental processes such as cell proliferation, differentiation and migration (reviewed in Ullrich and Schlessinger, 1990), fur-

ther investigation of these activities should help elucidate the mechanisms involved in mesoderm induction as well as in other FGF-mediated developmental processes. We are interested in mesodermal cell migration and in the role of extracellular matrix (ECM) in the gastrulation process in the urodele amphibian *Pleurodeles*. Recent studies have demonstrated that mesodermal cell migration acts as a driving force during urodele amphibian gastrulation (Boucaut et al., 1984a,b; Shi et al., 1987, 1989). In *Pleurodeles*, the dorsal marginal cells have acquired the capacity for autonomous migration as early as the 32-cell stage (Shi et al., 1989). Recently, Smith et al. (1990) have shown that *Xenopus* ectodermal cells exposed to XTC-cell-conditioned medium are able to spread and migrate on a fibronectin-coated surface. However, information concerning the involvement of growth factors in the control of mesodermal cell migration in the urodele amphibian is lacking. The observation made in *Xenopus* raises the possibility that mesoderm-inducing factors may be indeed involved in various aspects underlying gastrulation. The urodele *Pleurodeles* differs markedly from *Xenopus* in that superficial cells of the gastrula, especially in the dorsal region, make an important contribution to mesodermal structures (Smith and Malacinski, 1983; Delarue et al., 1992). Previous work made in *Pleurodeles* represents some of the evidence available that ECM and integrins are involved in a morphogenetic event in vivo (for review see Boucaut et al., 1990). Clearly, therefore, to understand the molecular events leading up to activation of cell-ECM adhesion, it is critical to understand mechanisms of mesoderm induction. Thus, it is of interest to determine the role of these factors and their receptors in the processes that lead up to and follow mesoderm induction. We report here the isolation of two complete cDNA clones encoding FGF receptors in the urodele amphibian *Pleurodeles*. We analyse their developmental expression and their regulation by mesoderm-inducing factors during early development.

Materials and methods

Library screening

An oligo-(dT) primed *Pleurodeles* tail-bud stage cDNA library was constructed in ZAP II (Stratagene, La Jolla, CA) and screened using a 1,100 bp *Bgl*III fragment from the tyrosine kinase domain of the human FGF receptor (FLG) (a generous gift from Dr A. Baird, The Whittier Institute, La Jolla, CA) as a probe. Filters were hybridized overnight at 42°C in $2 \times$ Pipes ($1 \times$ Pipes = 400 mM NaCl, 10 mM Pipes, pH 6.5), 20% formamide and 1% SDS containing 100 µg/ml sheared herring sperm DNA. They were then washed once with $2 \times$ SSC ($1 \times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% SDS for 15 minutes at room temperature and three times at 55°C for 45 minutes. About 5×10^5 recombinant phage were screened using these conditions. After plaque purification, cDNA inserts were obtained in pBlue-script by in vivo excision using helper phage R408 (Stratagene, La Jolla, CA). Complete (PFR1) and partial (PFR4) cDNA clones were obtained. PCR amplification was then used to obtain the 5' sequence of PFR4 as described below.

To obtain a homologous probe for the control of RNA equivalence, the same filters were reused and screened with a 1,300 bp rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA

probe (a generous gift of Dr J. M. Blanchard, Centre Paul Lamarque, Montpellier, France) under the same conditions as described above. A full-length cDNA clone (1.3 kb) was obtained which served as control in northern analysis and in RNAase protection experiments.

PCR amplification of 5' sequence

The upstream 5' sequence of partial cDNA clone (PFR4) was obtained using the RACE protocol (rapid amplification of cDNA ends) as initially reported by Frohman et al. (1988). First strand cDNA synthesis was carried out with the *SuperScript* Preamplification System (Bethesda Research Laboratories). 5 µg of neurula stage total cellular RNA (prepared as described below) were mixed with 20 pmol PFR4-specific antisense oligonucleotide (5'-GAGTCTCAGACTGAGATC-3'). The mixture was heated at 70°C for 10 minutes and quenched on ice. Reverse transcription was done at 42°C for 1 hour in the presence of 200 units *SuperScript* RNAase H⁻ reverse transcriptase. Excess primers were removed by spin dialysis through Centricon 100 microconcentrators (Amicon). The reverse-transcribed cDNAs were then tailed with dATP in the presence of 15 units terminal nucleotidyl transferase (Bethesda Research Laboratories) as described by Frohman et al. (1988) and diluted to 500 µl with TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA).

10 µl of the tailed cDNA were amplified by PCR using *Ampli-Taq* DNA polymerase (Perkin-Elmer-Cetus). Primers used for amplification are the following: 10 pmol of (dT)17-adaptor, 20 pmol of adaptor containing a *XhoI* site (Frohman et al., 1988), and 20 pmol of PFR4-specific antisense oligonucleotide (5'-GTTGCGCAGGATGTGGCCTG-3'), which is preceded by an in-frame *EcoRI* site. The reaction mixture was denatured at 94°C for 5 minutes, annealed at 55°C for 2 minutes and extended at 72°C for 40 minutes. We carried out 40 cycles of PCR amplification using a DNA thermal cycler (Hybaid) with a step program (94°C, 40 seconds; 55°C, 2 minutes; 72°C, 2 minutes) and a final extension at 72°C for 15 minutes. Following amplification, the PCR products were extracted with phenol/chloroform and recovered by ethanol precipitation. The samples were then digested by *EcoRI* and *XhoI* and ligated into the corresponding sites of pBluescript SK (Stratagene) for sequence analysis.

Sequence analysis

Double-stranded DNA was used for sequence analysis. The ends of cDNA clones were sequenced using subclones derived from convenient restriction sites. Additional sequences were obtained using several 17-mer oligonucleotide primers to cover the regions for which suitable subclones were unavailable. DNA was sequenced using the dideoxynucleotide chain termination method (Sanger et al., 1977) with Sequenase Version 2.0 (United States Biochemicals, Cleveland, OH) according to the manufacturer's recommendations. The sequence homology was analysed by consulting the EMBL/GenBank data base.

Embryos, dissections and induction assays

Pleurodeles embryos were obtained as previously described (Shi et al., 1987). They were kept at room temperature until desired stages. Regional explants from early gastrula were dissected in agar-coated Petri dishes containing 10% normal amphibian medium (NAM) (Slack, 1984). Dissections of early tail-bud embryos were performed in 10% NAM containing 1 mg/ml chymotrypsin (Sigma, type II). For induction assays, groups of 20 animal cap explants were treated with the following growth factors for 24 hours at room temperature: 20 ng/ml bovine bFGF, 20 ng/ml human TGF-1, 20 ng/ml porcine TGF-2 (all from R&D System, Minneapolis, MN) and 10 ng/ml human recombinant activin A (Genentech, San Francisco, CA). At the end of the incu-

bation, explants were processed immediately for RNA extraction or stored frozen at -80°C for late use.

Northern blot and RNAase protection analysis

Total cellular RNA was isolated using a modified method of Chomczynski and Sacchi (1987). Briefly, groups of 5 embryos or 20 explants were first homogenized in 200 µl of 4 M guanidine isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% (w/v) sarcosyl. RNA was then extracted with phenol/chloroform followed by sequential precipitation with ethanol and 4 M LiCl. Purity and quantitation were assessed by measurement of absorbance at 260 nm and 280 nm. For northern blot analysis, RNA was fractionated on a 1% agarose gel containing 1 M formaldehyde buffered with 50 mM Hepes/EDTA. An RNA ladder (Bethesda Research Laboratories) was run in parallel to determine mRNA size. RNA was transferred to a nylon membrane (Hybond-N, Amersham, UK) using the PosiBlot pressure blotter (Stratagene, La Jolla, CA) and then crosslinked using the Stratilinker UV crosslinker (Stratagene) according to the manufacturer's instructions. Probes were radiolabelled with ³²P-dCTP (3,000 Ci/mmol, Amersham, UK) using a random primer labelling Kit (Boehringer, Mannheim, Germany) following the manufacturer's recommendations. Hybridization was carried out under high-stringency conditions at 42°C overnight in 50% formamide, 5 × Denhardt's (1 mg/ml of Ficoll 400, polyvinylpyrrolidone and bovine serum albumin), 5 × SSC, 20 mM Tris-HCl, pH 7.5, 0.1% SDS containing 100 µg/ml sheared herring sperm DNA. Filters were washed once in 1 × SSC, 0.1% SDS at room temperature for 15 minutes, and three times in the same solution at 60°C for 45 minutes. They were then exposed to X-ray film with intensifying screens at -80°C. Autoradiograms were scanned using a laser densitometer (Shimadzu).

RNAase protection analysis was performed as described by Krieg and Melton (1987) with minor modifications. All cDNA fragments were cloned into pBluescript SK plasmid (Stratagene) to direct the in vitro synthesis of antisense transcripts using T7 RNA polymerase (Boehringer, Mannheim, Germany) in the presence of [³²P]-UTP (400-800 Ci/mmol, Amersham, UK). Full-length probes were purified from a 0.4 mm thin polyacrylamide gel by elution at 37°C in 2 M ammonium acetate, 1% SDS containing 25 µg/ml tRNA. Hybridizations were carried out in the presence of 80% formamide, 0.4 M NaCl, 40 mM Pipes and 1 mM EDTA at 50°C for 24 hours. The samples were digested by 20 µg/ml RNAase A and 1.5 µg/ml RNAase T1 at 30°C for 1 hour followed by Proteinase K at 37°C for 15 minutes. Following phenol/chloroform extraction and ethanol precipitation, the protected fragments were resolved by electrophoresis on a 5% polyacrylamide gel, and exposed to X-ray film with intensifying screens at -80°C.

Results

Isolation and sequence analysis of *Pleurodeles* FGF receptors

We have used a 1,100 bp probe from the tyrosine kinase domain of the human FGF receptor (FLG) to screen a *Pleurodeles* tail-bud stage cDNA library that we constructed. Under low-stringency conditions of hybridization, eight positive clones were isolated. Partial sequence analysis reveals that all of these clones encode closely related proteins that contain sequences highly related to the human and chicken FGFR-1 (Lee et al., 1989; Dionne et al., 1990). The largest insert (about 3.8 kb), which we designate PFR1, encodes the complete sequence of *Pleurodeles* FGF recep-

tor. The amino acid sequence was deduced from a 2547-nucleotide open reading frame (Fig. 1). It contains a likely initiation methionine located at nucleotide position 100 followed by a stretch of hydrophobic amino acids that represent the signal sequence. The downstream open reading frame (2448 bp) appears to encode a protein of 816 amino acids corresponding to a putative M_r of 91.5×10^3 . Com-

parison of PFR1 with the chicken FGFR-1 sequence (Lee et al., 1989) reveals an overall identity of 87% at the amino acid level. PFR1 is also highly homologous to human FGFR-1/FLG (Dionne et al., 1990) (85% identity overall), to the mouse FGFR-1 (Reid et al., 1990) (84% identity overall) as well as to the recently identified *Xenopus* FGF receptor (Musci et al., 1990; Friesel and Dawid, 1991).

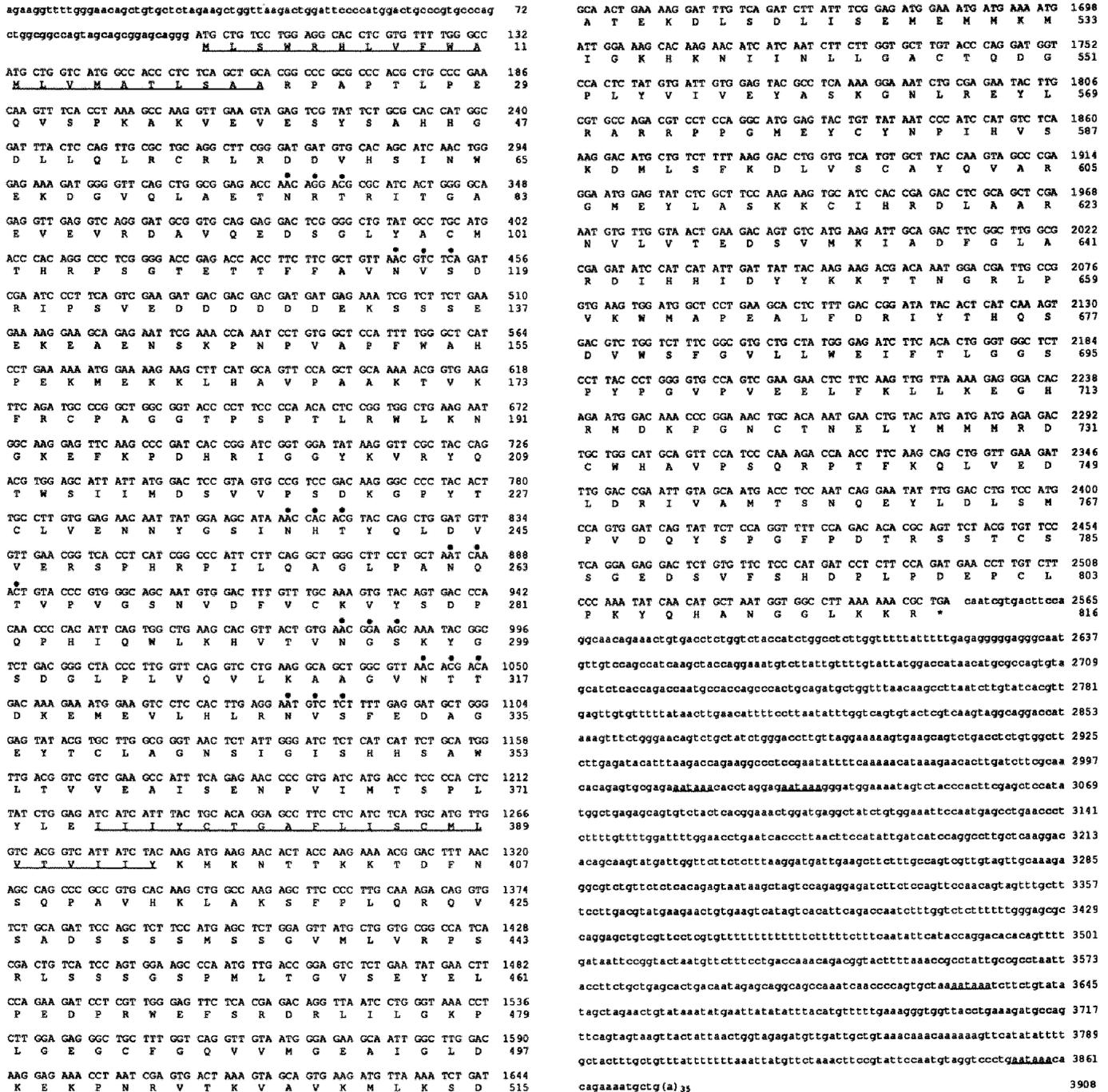


Fig. 1. Nucleotide and deduced amino acid sequences of the PFR1 cDNA clone. The presumed signal-peptide sequence, transmembrane region and the four potential polyadenylation signals (AATAAA) in the 3'-untranslated region are underlined. The possible sites of N-linked glycosylation (N-X-S/T) are marked by superior dots. The nucleotide sequence has been deposited in the EMBL data base with accession number X 59380.

However, only 79% identity overall was found between these two amphibian species. Identities between PFR1 and the human FGFR-2/BEK (Dionne et al., 1990), FGFR-3 (Keegan et al., 1991) or FGFR-4 (Partanen et al., 1991) are less important. Therefore, it is likely that PFR1 is a *Pleurodeles* homolog of FGFR-1/FLG.

Partial amino acid sequence and restriction map analyses of different cDNA clones reveal that an additional cDNA clone (3.0 kb) is different from PFR1. Comparison of the deduced amino acid sequence from the longest open

reading frame of this cDNA clone with other FGF receptors, especially with PFR1, reveals that it partially encodes another distinct but related FGF receptor in *Pleurodeles*, having the same characteristics as PFR1 but lacking the signal sequence and part of the first Ig-like domain. In order to obtain the complete 5' sequence, we used RACE procedures (Frohman et al., 1988) to amplify this segment from neurula stage mRNA. A 400 bp PCR product was obtained and sequenced on both strands after ligation into pBlue-script SK. It contained part of the first Ig-like domain over-

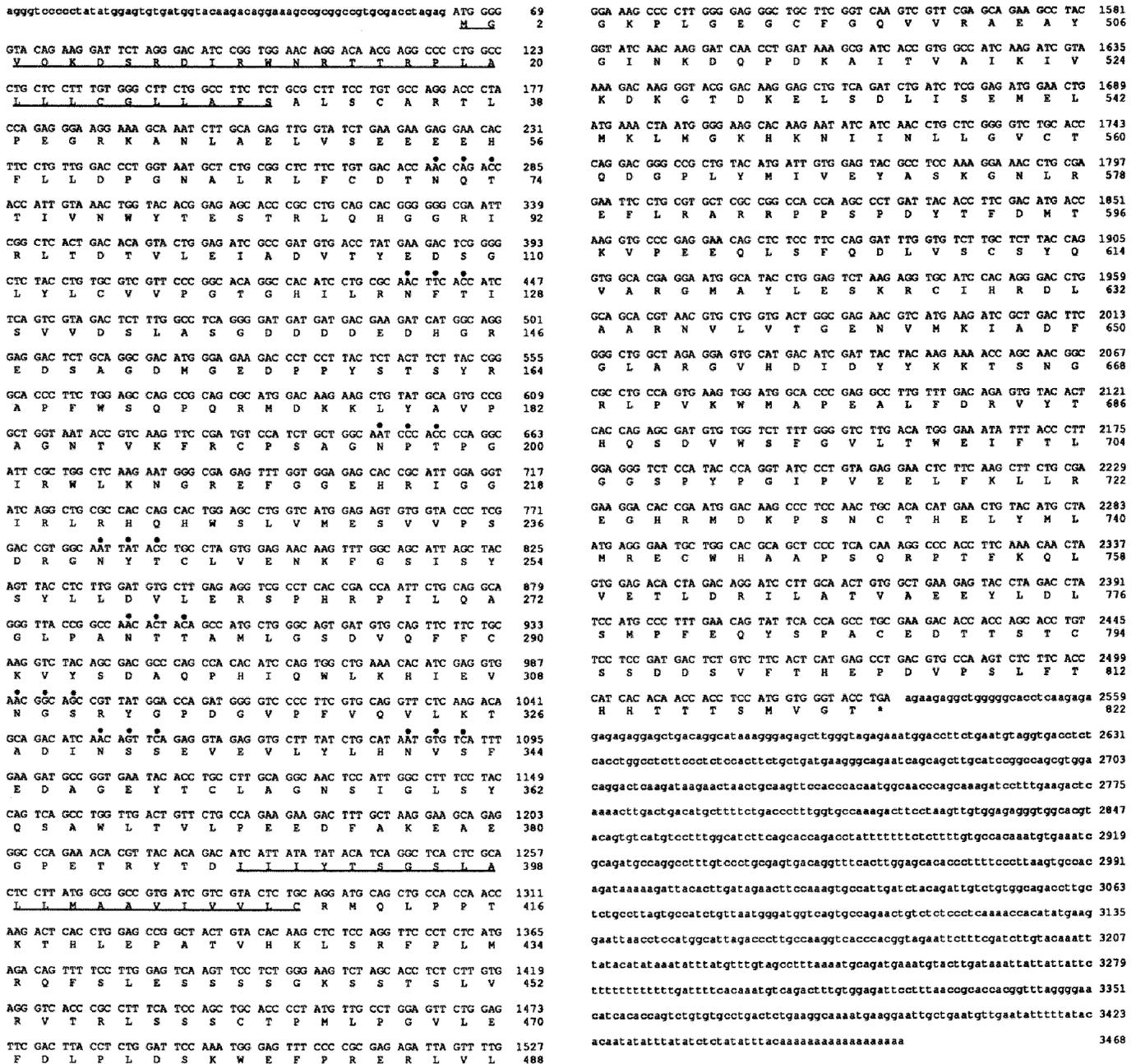


Fig. 2. Nucleotide and deduced amino acid sequences of the PFR4 cDNA clone. The complete sequence was obtained from two overlapping cDNA clones. Features in the sequence are indicated as in Fig. 1. The nucleotide sequence has been deposited in the EMBL data base with accession number X 65059.

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PFR1  MLSWRHLVFWAMLVMATLSAA-----RPAPTLPE-QVSPKAKVEVESYSAHGD
PFR4  .GVOKDSRDIRWRNRTRRPL.LLLCGLLAFSAALSC.R...GNLAELVSE.E.HFLLDP.N

PFR1  LLQLRCRLRDDVHSINWEKDGVLQAEIETNRTRITGAEVEVRDAVQEDSGLYACMTHRPSGT
PFR4  A.R.F.D-TNQTIV..YTESTR.QHGG.I.LTDTVL.IA.VTY.....L.VVP-GT.H

PFR1  ETTFFAVNVSDRIPSV[EDDDDDDEK]--SSSEEKAEENSK-PNPV-APFWAHPEKMEKKLH
PFR4  ILRN.TIS.V.SLA.G[....E.]HGRED.AGDMG.DPPYSTSYR....SQ.QR.D...Y

PFR1  AVPAAKTVKFRCPAGGTPSPTLRWLKNGKEFKPDHRIGGYKVRYQTWSIIMDSVVP SDKG
PFR4  ....GN.....SA.N.T.GI.....R..GGE.....IRL.H.H..LV.E.....R.

PFR1  PYTCLVENNYGSINHTYQLDVVERSHPRLQAGLPANQTPVVGSNVDFVCKVYSDPQPH
PFR4  N.....KF...SYS.L..L.....T.AML..D.Q.F.....A...

PFR1  IQWLKHVTVNGSKYSGDGLPLVQVLKAAGVNTTDKEMEVLHLRNVSFEDAGEYTCLAGNS
PFR4  .....IE....R..P..V.F.....T.DI.SS--.V...Y.H.....

PFR1  IGISHHSAWLTVV-EAISENPVIMTSPLYLEIIYCTGAFLISCMVTVIIYKMKNTTKK
PFR4  ..L.YQ.....LP.EDFAKEAEGPETR.TD....TS.SLALLMAA.I.VLCR.QLPPT.

PFR1  TDFNSQPAVHKLAKSFPLQRQVSADSSSSMSSGVMVLRPSRLSSSGSPMLTGVSEYELPE
PFR4  .HLEP-AT....SR-...M..F.LE...GK.STS...VT.....CT...P..L.FD..L

PFR1  DPRWEFSRDRLLIGKPLGEGCFGQVVMGEAIGLDKEKPNRVTKVAVKMLKSDATEKDLSD
PFR4  .SK...P.E..V.....RA..Y.IN.DQ.DKAIT..I..IV.DKG.D.E...

PFR1  LISEMEMMKMIGKHKNIINLLGACTQDGPLYVIVEYASKGNLREYLRARRPPGMEYCYNP
PFR4  .....L..LM.....V.....SPD.TFDM

PFR1  IHVSKDMLSFKDLVSCAYQVARGMEYLASKKCIHRDLAARNVLVTVEDSVMKIADFGLARD
PFR4  TK.PEEQ...Q.....A..E..R.....GEN.....G

PFR1  IHHIDYKKTNGRLPVKWMapeALFDRIYTHQSDVWSFGVLLWEIFTLGGSPYPGVPVE
PFR4  V.D.....S.....V.....T.....I...

PFR1  ELFKLLKEGHRMDKPGNCTNELYMMMRDCWHAVPSQRP TFKQLVEDLDRIVAMTSNQEYL
PFR4  .....R.....S..H...L..E...A.....T...L..VAE...

PFR1  DLSMPVDQYSPGFPDTRSSSTCSSGEDSVFSDPLPDEPCLPKYQHANGGLKKR
PFR4  ....FE....ACE.-T....-D....T.E.AV--.S.FT-H.TTSMVGT
    
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Fig. 3. Comparison of deduced amino acid sequences of the PFR1 and PFR4 cDNA clones. Dashes indicate the absence of amino acid residues at the corresponding position. Dots in PFR4 sequence represent residues that are identical to the corresponding residues in PFR1. The putative signal-peptide sequences and transmembrane regions are underlined. The open brackets surround the acidic regions. Arrows demarcate the tyrosine kinase domains. The potential ATP-binding residues are indicated by asterisks and the tyrosine kinase insertion sequences are indicated in italics.

lapping exactly the 5' end of the partial clone. A likely initiation methionine, preceded by an in-frame stop codon, is located at position 64 of the nucleotide sequence followed by a putative signal peptide. Thus, the open reading frame of 2466 nucleotides encodes 822 amino acids (Fig. 2) corresponding to a putative M_r of approximately 92×10^3 . It shows 59% identity overall with PFR1 at the amino acid level, and identities with human FGFR-1/FLG, FGFR-

2/BEK, FGFR-3 and FGFR-4 are 59%, 60%, 64% and 66% respectively. Based on this sequence comparison, we designate the cDNA clone PFR4, since it likely represents the *Pleurodeles* homolog of FGFR-4.

As with other FGF receptor sequences, both PFR1 and PFR4 contain three Ig-like domains in their extracellular regions (Fig. 3). A region composed of 8 (PFR1) or 6 (PFR4) consecutive acidic amino acid residues is located

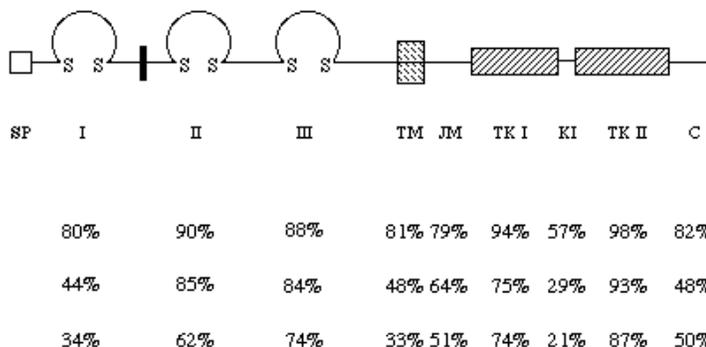


Fig. 4. Structural similarities between PFR1, human FLG, PFR4 and human FGFR-4. Different regions of the FGF receptor are illustrated. The open box is the putative signal peptide; the loops represent the Ig-like domains; the black box represents the acidic region and the hatched box represents the transmembrane region; the tyrosine kinase domains are represented by dotted boxes. The degree of amino acid identity is indicated under each specific region including the Ig-like domains (I, II and III), the transmembrane region (TM), the juxtamembrane region (JM), the tyrosine kinase domains (TK I and TK II), the kinase insert sequence (KI) and the C-terminal region (C).

between the first and the second Ig-like domains. The transmembrane region of both molecules contains 21 amino acids. In the intracellular part of both molecules, the tyrosine kinase domain is preceded by a long juxtamembrane region which is composed of 76 residues in PFR1 with a deletion of 2 amino acids, as compared with that in human FGFR-1/FLG. PFR4 contains 74 residues in this region as does its human homolog FGFR-4. The tyrosine kinase catalytic domains are particularly conserved between PFR1 and FGFR-1/FLG (Figs 3, 4). Like other FGF receptors, the tyrosine kinase domain of both PFR1 and PFR4 is split by an insert of 14 amino acid residues which is poorly conserved. Another feature is that the C-terminal region is much more conserved between PFR1 and FGFR-1/FLG than between PFR1 and PFR4 (Figs 3, 4).

Developmental expression of PFR1 and PFR4 mRNAs

Northern blot and RNAase protection analyses were performed to determine the expression of the two FGF receptor mRNAs during various developmental stages in *Pleurodeles*. Total cellular RNA was extracted from 5 embryos at various stages of development, from fertilized eggs until tail-bud stage. For northern blot analysis, they were probed under high-stringency conditions with an *EcoRI/NdeI* fragment of PFR1 containing the entire coding region except the first Ig-like domain, and an *EcoRI* fragment of PFR4 which contains the extracellular and the juxtamembrane regions.

The PFR1 probe hybridized to a single transcript in full-grown oocytes (not shown) and from fertilized egg to tail-bud stages (Fig. 5A). The results show that PFR1 is present as a maternal mRNA, and that it is expressed throughout early development until at least the late tail-bud stage. PFR1 mRNA levels remain constant during early development except that a slight decrease was reproducibly observed at the early gastrula stage when the intensity of PFR1 mRNA was normalized to the GAPDH mRNA, a typical "house-keeping" gene (Fort et al., 1985). Based on relative mobility to RNA markers run in parallel, the PFR1 transcript was estimated to be 4.2 kb in size. In addition, no obvious change in mRNA size was noticed during development.

For hybridization with PFR4, either the same blots or a new preparation of total cellular RNA were used. Both methods gave the same results. As shown in Fig. 5B, the PFR4 mRNA was first detected at the late blastula stage, and was then continually expressed as a single transcript of 4.0 kb. When compared to GAPDH mRNA, which was present at all stages examined, one can notice a high level of PFR4 mRNA expression at the late gastrula stage. No PFR4 mRNA was detected by northern analysis from fertilized eggs to early blastula stages. Thus, unlike PFR1 mRNA, PFR4 is not a maternally derived mRNA. Accumulation of PFR4 transcripts at the late blastula stage suggests that this mRNA is zygotically expressed in the embryo.

We also performed RNAase protection experiments to determine more accurately the developmental expression of PFR1 and PFR4 mRNA, because the zygotic expression of PFR4 mRNA is initially not expected. The PFR1 probe is 387 bp in length and encodes amino acids 54-181 (see Fig.

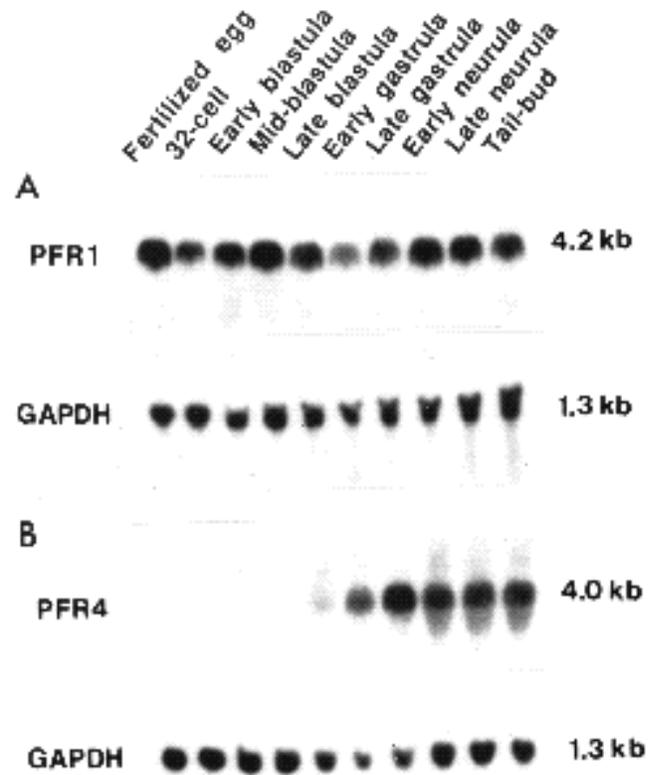


Fig. 5. Developmental expression of PFR1 and PFR4 mRNAs. Total RNA from the indicated stages was subjected to northern analysis. The blots were probed with an *EcoRI/NdeI* fragment of the PFR1 clone and with an *EcoRI* fragment of the PFR4 clone. A transcript of 4.2 kb is observed with PFR1 probe at all stages examined (A). Notice that PFR4 mRNA is observed from the late blastula stage onward as a transcript of 4.0 kb (B). The blots were also probed with a full-length GAPDH cDNA to compare the equivalence of RNA loaded on each lane.

3), PFR4 probe is 462 bp in length and encodes amino acids 82-235 (see Fig. 3). A 284 bp GAPDH probe was included in each hybridization to control for RNA equivalence. The results confirm those obtained by northern blots. When the intensity of the protected fragments from both FGF receptors mRNAs was normalized to the protected fragments of GAPDH mRNA, we found that the level of PFR1 mRNA varied little from fertilized egg to tail-bud stage except for a slight decrease between late blastula and early gastrula stages (Fig. 6A) as observed in northern analysis. The PFR4-specific protected fragments were detected from late blastula stage onward (Fig. 6B). This protected fragment appeared at the mid-blastula stage upon longer exposure (not shown). As in northern hybridizations, the GAPDH-specific protected fragments remained relatively constant at different stages. Therefore, the absence of PFR4 mRNA expression before late blastula stage is indeed due to the lack of its transcription.

Regional distribution of PFR1 and PFR4 mRNAs

The differential expression of PFR1 and PFR4 mRNA suggests that the two receptor mRNAs are independently regulated during early development. We further looked for

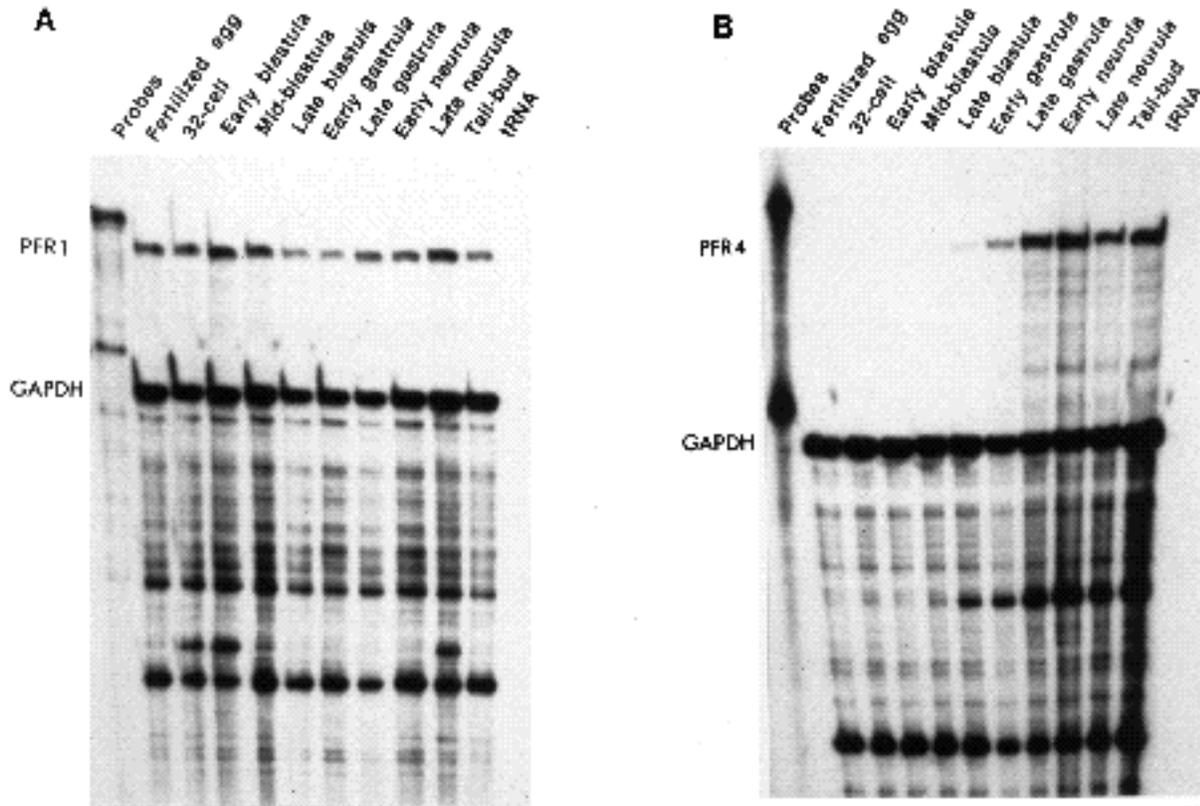


Fig. 6. RNAase protection analyses of the developmental expression of PFR1 and PFR4 mRNAs. Total RNA was isolated from five embryos of each stage. Samples were hybridized with 5×10^5 cts/minute of PFR1 or PFR4 probes; 5×10^4 cts/minute of the GAPDH probe was included in each sample to control RNA equivalency. The specific protected fragments of PFR1 and PFR4 transcripts were normalized to those of GAPDH. PFR1 mRNA is expressed at constant levels from fertilized egg to tail-bud stage with a slight decrease between late blastula and early gastrula stages (A); PFR4 mRNA is detected from late blastula stage onward (B). The autoradiograms were exposed for 48 hours (A) and 72 hours (B).

the distribution of both receptor mRNAs in different regions of the early gastrula and at the early tail-bud stages.

Regional explants from early gastrula were dissected from animal pole, dorsal and ventral marginal zones and the endodermal mass as illustrated in Fig. 7A. Explants of dorsal and ventral marginal zones were dissected according to the lineage fate map of the early gastrula (Delarue et al., 1992) to avoid contamination by ectodermal cells. Northern blot or RNAase protection analyses were performed using RNA extracted from 20 explants of each region. In both cases, the level of PFR1 and PFR4 mRNAs was normalized to the amount of GAPDH mRNA. Northern blot analysis revealed that PFR1 mRNA was expressed at similar levels in animal pole, dorsal and ventral marginal zones, with low level of expression in the endoderm (Fig. 7B, C). PFR4 mRNA was abundantly expressed in the animal pole; however, the transcripts were almost undetectable by northern blot in the endoderm (Fig. 7B). Interestingly, the normalized levels of PFR4 mRNA indicate that the ventral marginal zone expresses more than two-fold the amount of transcript compared to the dorsal marginal zone (Fig. 7C).

We further determined the pattern of expression of PFR1 and PFR4 mRNAs at tail-bud stage by northern blotting using RNA extracted from different embryonic tissues

including epidermis, neural tube, somites, lateral plates and endoderm. We found that PFR1 transcripts were mostly distributed in the neural tissue. Mesodermal tissues like somites and lateral plates expressed the transcripts at a similar level (Fig. 8A). The transcripts were also detected in the endoderm. However, the normalized levels of PFR1 mRNA indicate that the relative amount of the transcripts decline dramatically in the epidermis compared to the ectoderm of the early gastrula (Fig. 8A). When the blot was probed for PFR4 transcripts, we found that, although the transcripts were present in each tissue, they were most strikingly localized to the neural tissue (Fig. 8B), which expressed over 50% of the transcripts. Lateral plate mesoderm was found to express more than two-fold the amount of transcripts compared to the somites (Fig. 8B). This result is consistent with the dorso-ventral difference of PFR4 mRNA localization observed at the early gastrula stage. In addition, as with PFR1 mRNA, PFR4 mRNA also undergoes a dramatic decline in the epidermis. When the amounts of both mRNAs are normalized to that of GAPDH mRNA, we conclude that epidermis expresses a very low level of both PFR1 and PFR4 mRNA at tail-bud stage. Furthermore, when explants from animal pole, dorsal and ventral marginal zones of the early gastrula were isolated and allowed to differentiate in vitro until tail-bud stage, the same results

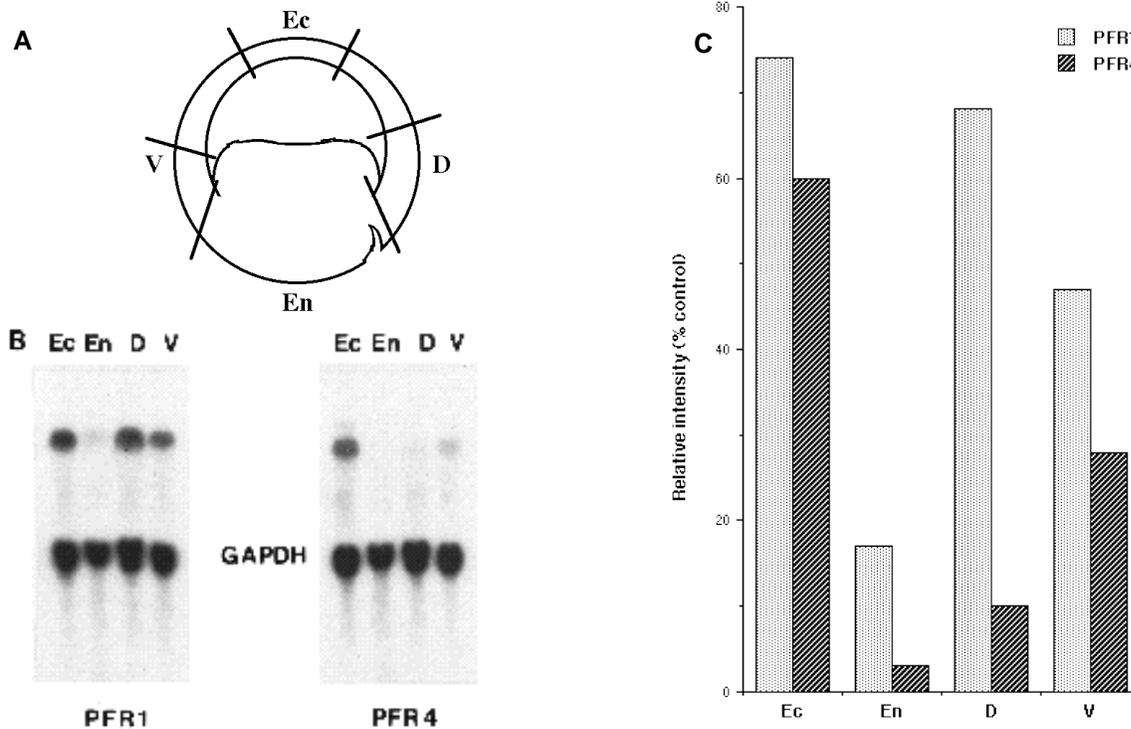


Fig. 7. Regional distribution of PFR1 and PFR4 mRNAs. (A) Schematic illustration of dissected regions from animal cap (Ec), dorsal (D) and ventral (V) marginal zones and endodermal mass (En) at early gastrula stage. (B) Northern analysis of PFR1 and PFR4 mRNA expression in each region. The samples were divided into two equal parts, each part was probed with PFR1 and PFR4, respectively, the blots were also hybridized with the GAPDH probe to control RNA equivalency. (C) Histogram of the relative distribution of PFR1 and PFR4 mRNA in different regions. Autoradiograms were scanned by laser densitometer and the relative intensity of PFR1 and PFR4 transcripts were normalized to the amount of GAPDH mRNA. PFR1 mRNA is distributed uniformly in the embryo with a low level of expression in the endoderm; PFR4 mRNA is mainly distributed in the ectoderm; more transcripts were detected in the ventral marginal zone than in the dorsal marginal zone.

were observed as in tissue dissection experiments (not shown).

In another set of experiments, we investigated the location of PFR1 and PFR4 transcripts along the anterior-posterior axis. Early tail-bud embryos were sectioned into three parts corresponding to head, trunk and tail regions. Total RNA extracted from these regions were divided into two equal fractions, and each fraction was analyzed by northern blotting to compare the expression of the two FGF receptors. We found that both head and tail regions expressed PFR1 transcripts with similar levels, which was at least twice as much as in the trunk (Fig. 8A). Interestingly, PFR4 mRNA was expressed according to an anterior-posterior gradient; the head region had higher amount of transcripts than in the trunk, whereas the transcripts were barely detectable in the tail region (Fig. 8B). Hybridization of the blots with GAPDH probe showed an equivalent amount of total RNA loaded for each region (Fig. 8A, B).

Regulation of PFR1 and PFR4 mRNAs by mesoderm-inducing factors

We addressed the question of whether mesoderm-inducing factors can modulate the level of expression of PFR1 and PFR4 mRNAs in isolated animal cap explants. Animal cap explants were dissected from the late blastula stage and treated with several growth factors reported to have meso-

derm-inducing activity in *Xenopus* (Slack et al., 1987; Kimelman and Kirschner, 1987; Rosa et al., 1988; Smith et al., 1990). These growth factors have a similar effect in *Pleurodeles* ectodermal cells when tested in vitro. Activin A induces the formation of dorsal structures such as notochord and muscles whereas FGF induces mostly ventral-type mesodermal structures. However, both TGF-1 and TGF-2 fail to induce mesoderm differentiation in isolated ectodermal explants. In addition, activin A can induce isolated ectodermal cells to spread and migrate on fibronectin-coated surfaces (D. L. S., unpublished observations).

In our present analysis, 20 animal caps explanted at late blastula stage were cultured in the presence or absence (control) of growth factors for 24 hours (see Materials and methods), at which time control embryos reached late gastrula stage. Total cellular RNA extracted from both control and treated animal caps was probed with PFR1 or PFR4, followed by GAPDH to control for RNA equivalence. When the levels of PFR1 and PFR4 mRNA were normalized to the amount of GAPDH mRNA, we observed that PFR1 mRNA was generally unaffected by these factors, although a slight increase was sometimes observed by treatment with bFGF and activin A when the explants were cultured for 24 hours (Fig. 9A). However, when cultured until tail-bud stage, we observed a significant decrease of PFR1 mRNA in untreated explants compared to the level

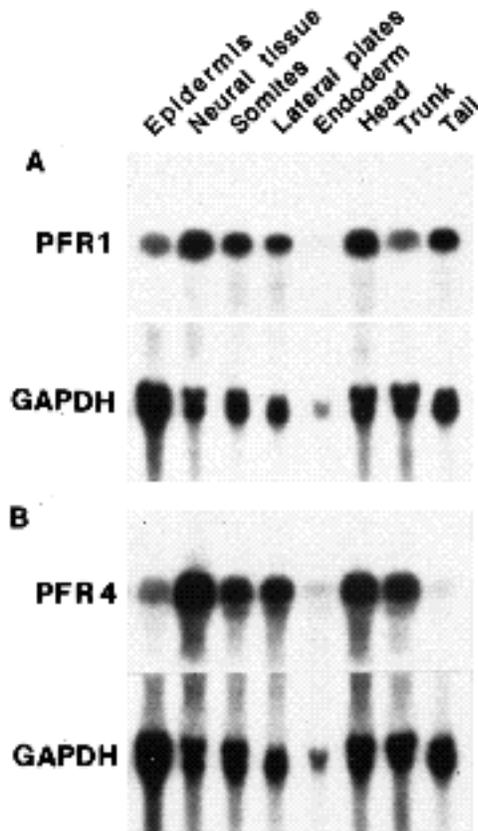


Fig. 8. Tissue distribution of PFR1 and PFR4 mRNAs at tail-bud stage. Total RNA was extracted from different tissues including epidermis, neural tissue, somites, lateral plate and endoderm, as well as from sectioned regions corresponding to head, trunk and tail. The samples were divided into two equal fractions for northern blotting. Each fraction was probed for PFR1 (A) and PFR4 (B), respectively, followed with GAPDH to control the relative amount of total RNA loaded for each tissue. Although the endoderm lane was relatively underloaded in this experiment, PFR1 and PFR4 transcripts were detectable.

observed at early gastrula stage, while animal caps treated with bFGF and activin A maintained PFR1 mRNA at the initial level (not shown). Similar results were observed with the *Xenopus* homolog of FGFR-1 (Musci et al., 1990; Friesel and Dawid, 1991). PFR4 mRNA exhibited a completely different pattern of regulation in response to mesoderm-inducing factors. We reproducibly observed that bFGF and activin A strongly decreased PFR4 mRNA levels in treated animal cap explants cultured for 24 hours (Fig. 9B), while PFR4 mRNA level in untreated explants increased gradually during this period to reach a maximal level at the late gastrula stage. The mRNA level then declined as mentioned earlier. TGF-1 had no effect while an inhibitory effect was sometimes observed by treatment with TGF-2. We have performed three different northern blots with RNA extracted from treated animal caps. Based on these results, when the total mRNA level in each condition was normalized to the amount of GAPDH mRNA, we observed that PFR4 mRNA level was reduced at least five-fold by both bFGF and activin A. To study the time

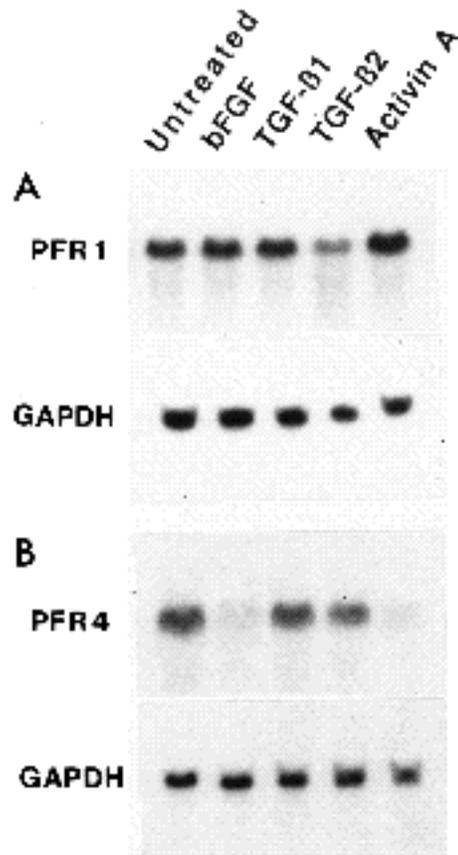


Fig. 9. Regulation of PFR1 and PFR4 mRNA levels in animal cap explants cultured for 24 hours in the presence or absence of mesoderm-inducing growth factors. Total RNA from 20 explants was prepared for northern blot analysis to estimate the PFR1 (A) and PFR4 (B) mRNA levels. The same filters were also hybridized with the full-length GAPDH cDNA. Note that PFR1 mRNA levels are not much affected by the growth factors (A), whereas bFGF and activin A strongly down-regulate PFR4 mRNA levels (B).

course of this down-regulation, animal cap explants were dissected at early gastrula stage and cultured for various time intervals in the presence or absence of bFGF and activin A. The results indicated that FGF decreased PFR4 mRNA level by approximately 50% after 1 hour of treatment; however, activin A required at least 6 hours of treatment to down-regulate the receptor mRNA (not shown).

Discussion

We have analysed two *Pleurodeles* cDNA clones (PFR1 and PFR4) that encode two distinct FGF receptors sharing high amino acid sequence identity with other members of the FGF receptor superfamily. The deduced sequences reflect characteristics expected of FGF receptors, including three extracellular Ig-like domains, an unusual acidic region, a long intracellular juxtamembrane region and a tyrosine kinase domain split by an insertion sequence; all features of which are common to the superfamily of FGF receptors. Recently, coding sequences for FGF receptors

with tyrosine kinase activity have been identified in different species. They are classified into several groups based on sequence homology. Dionne et al. (1990) have described two distinct but related human genes (named FLG and BEK, respectively) that bind both aFGF and bFGF when expressed in NIH-3T3 cells. More recently, human FGFR-3 (Keegan et al., 1991) and FGFR-4 (Partanen et al., 1991) have been identified. The predicted sequence of PFR1 has a higher level of amino acid identity to FLG than to other FGF receptors, both in overall sequence (85%) and in the tyrosine kinase catalytic domain (96%). This suggests that PFR1 is likely to represent the *Pleurodeles* homolog of FGFR-1/FLG. In addition, a high level of identity exists between PFR1 and chicken and mouse FGFR-1 (Lee et al., 1989; Reid et al., 1990) as well as the recently identified *Xenopus* FGF receptor (Musci et al., 1990; Friesel and Dawid, 1991). Comparison of PFR4 with the four reported human FGF receptors reveals that it is more similar to FGFR-4 (66% overall identity), thus we classify PFR4 as the *Pleurodeles* homolog of FGFR-4.

The expression of PFR1 and PFR4 mRNA in early *Pleurodeles* embryos was analysed by northern blot and RNAase protection. PFR1 mRNA is shown to be expressed throughout early developmental stages at a constant level and maternal mRNA for PFR1 is present in full-grown oocytes. This observation is in agreement with the timing of mesoderm induction which begins at the 64-cell stage, a period where zygotic transcription does not occur (Newport and Kirschner, 1982). Because FGF receptors are active before the mid-blastula transition (Gillespie et al., 1989), it is possible that they are produced by translation of maternal mRNAs. A similar conclusion has been proposed for FGF receptor expression in early *Xenopus* embryos (Musci et al., 1990; Friesel and Dawid, 1991). In addition, binding studies demonstrate that animal pole blastomeres from *Xenopus* blastula have high affinity binding sites for radioiodinated FGF (Gillespie et al., 1989). Although there is no evidence concerning the distribution of FGF-binding proteins in *Pleurodeles* embryos, we can reasonably expect that the situation is similar between these two species. Thus the localization of FGF-binding sites and of receptor mRNA in early embryos may reflect a possible role for FGF receptors in mesoderm induction.

Another interesting observation made in this study is that, in contrast to PFR1 mRNA, PFR4 mRNA is not maternally derived and its expression begins at the late blastula stage as shown by northern blot and RNAase protection analyses. The question then arises as to what the role played by PFR4 in mesoderm induction may be since this phenomenon begins long before the stages when PFR4 mRNA is first expressed. In *Xenopus* embryos, it has been shown that animal cap cells expressing a dominant negative mutant form of the FGF receptor fail to respond to induction by FGF (Amaya et al., 1991). When compared to the human sequence, both the *Xenopus* FGF receptor and the *Pleurodeles* PFR1 appear most similar to FGFR-1/FLG. Thus it is likely that the presence of PFR1 and/or other maternally derived FGF receptors at cleavage stages confer competence to animal cap cells for responding to FGF. The expression of another FGF receptor, PFR4, at the end of the period of competence of animal cap cells may provide

an additional signalling pathway to refine the formation of different mesodermal derivatives. Although this hypothesis requires further investigation, the observation that ventral marginal zone expresses more than two-fold the amount of PFR4 transcripts than dorsal marginal zone at the early gastrula stage is of particular interest given that FGF induces ventral or intermediate types of mesoderm (Slack et al., 1988). At present, four members of the FGF family (aFGF, bFGF, hst/K-FGF and int-2) have been shown to have mesoderm-inducing activity (Slack et al., 1987; Paterno et al., 1989); thus it is important to identify the *in vivo* ligand(s) for PFR1 and PFR4. The presence of PFR1 and PFR4 mRNAs throughout early stages of development is consistent with the pleiotrophic effects of FGFs because members of the FGF family affect a wide variety of biological events including cell growth and proliferation, neurite outgrowth, nerve cell survival and differentiation, and several FGF genes are expressed at various stages of embryogenesis (reviewed in Goldfarb, 1990). Thus, it is likely that PFR1 and PFR4 may participate in other FGF-mediated developmental processes such as in mesodermal cell migration, neurogenesis or angiogenesis after the period of competence of mesoderm induction. The observation that PFR1, and especially PFR4 mRNA, are predominantly localized to the neural tissue at the tail-bud stage is in accordance with this conclusion. Indeed, the differential expression patterns of both receptors mRNAs in the embryonic tissues as well as along the anterior-posterior axis of the embryos are of particular interest. They imply that PFR1 and PFR4 may mediate different functions of FGFs during morphogenesis. In support of this, similar results have been obtained concerning FGF receptors expression during murine development (Orr-Urtreger et al., 1991; Peters et al., 1992).

The regulation of PFR1 and PFR4 mRNAs was analysed using animal cap explants isolated from late blastulae and cultured in the presence of growth factors. PFR1 mRNA levels were maintained by mesoderm-inducing factors, in contrast, PFR4 mRNA levels in animal cap explants were inhibited significantly by treatment with bFGF and activin A. These results suggest that PFR1 and PFR4 mRNAs may be differentially regulated *in vivo*. It is likely that the maintenance of PFR1 mRNA level in animal cap explants by bFGF and activin A is a consequence of mesoderm formation induced by these factors. An indirect evidence supporting this proposition comes from the results obtained by culture of animal cap, dorsal and ventral marginal zones explants from early gastrula to tail-bud stage, in this case, both dorsal and ventral marginal zones explants maintained PFR1 mRNA to the initial level, whereas animal cap explants had a barely detectable level of the receptor mRNA (not shown in this study). Thus, our results concerning the regulation of PFR1 mRNA are similar to previous observations that suggest that mesoderm-inducing factors may positively regulate and maintain FGFR-1 mRNA in *Xenopus* animal cap explants (Musci et al., 1990; Friesel and Dawid, 1991). As for PFR4 mRNA, its down-regulation by mesoderm-inducing factors is more complex. FGF and activin A might down-regulate the PFR4 mRNA level through independent mechanisms, since the effect is more rapid with bFGF which decreases the PFR4 mRNA level

after 1 hour of treatment, whereas activin A requires at least 6 hours of treatment. Thus it is likely that bFGF may exert a direct negative effect on PFR4 mRNA expression, and that activin A, however, down-regulates PFR4 mRNA expression as a result of mesoderm induction. Nevertheless, we can not exclude the possibility that inhibition of PFR4 mRNA by bFGF may represent an early response to mesoderm induction. Recently, several lines of evidence suggest that terminal muscle differentiation is accompanied by a coordinate down-regulation of FGF receptors (Olwin and Hauschka, 1988; Moore et al., 1991). More recently, FGFR-4 has been cloned in mouse (Stark et al., 1991) where expression of this receptor is restricted to early myotomal cells in addition to other embryonic tissues, but it is not expressed in myotubes. The PFR4 mRNA is expressed primarily in the ectoderm of early gastrulae. Our northern blot and RNAase protection experiments detect only low levels of PFR4 transcripts in the mesoderm and, more specifically, in the dorsal marginal zone of the early gastrula, which differentiates as notochord and muscles. Most interestingly, we have found that lateral plate mesoderm expressed more than two-fold the amount of PFR4 transcripts than did the somitic mesoderm. Moreover, activin A can induce dorsal-type mesoderm such as notochord and muscles (Smith et al., 1991), and a high concentration of FGF also induces muscles (Slack et al., 1988). Taken together, the decrease of PFR4 mRNA in FGF- and activin-A-treated animal cap explants may probably reflect a concomitant down-regulation of the receptor as a result of mesoderm formation.

In summary, we have described the cloning and regulation of two FGF receptors in the amphibian embryo. PFR1 is a *Pleurodeles* homolog of previously identified *Xenopus* FGF receptor (Musci et al., 1990; Friesel and Dawid, 1991). Its expression and regulation are similar to the *Xenopus* receptor. However, the cloning and expression of the amphibian homolog of FGFR-4 have not been reported before. The observations reported in this study concerning the distinct patterns of expression for these receptors, demonstrate the likely complexity of FGF receptors regulatory loops. These results suggest that PFR4 may have a different function from PFR1 in embryogenesis. Recent studies demonstrate considerable differences in the regulation of members of the FGF receptor family in human fetal tissues (Partanen et al., 1991). Although further study will be necessary to understand the tissue distribution of PFR1 and PFR4 in amphibian embryos, the present studies strongly suggest that members of the FGF receptor family are differentially regulated during early amphibian development.

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