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cDNA Cloning and Distribution of the *Xenopus* Follistatin-Related Protein

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Recently, several proteins which have a follistatin module have been isolated. One of them, the follistatin-related protein (FRP), is encoded by TSC-36 (TGF-β-stimulated clone 36) in mouse, originally isolated as a cDNA clone up-regulated by TGF-β1 in mouse osteogenic MC3T3E1 cells. To determine the physiological role of FRP in early *Xenopus* embryonic development, we cloned the *Xenopus* FRP (xFRP) cDNA. The resulting cDNA clone was a secreted glycoprotein consisting of 299 amino acid residues with about 70% similarity to the mammalian and avian FRPs. Northern blotting analysis revealed that xFRP gene expression started at stage 10, the onset of gastrulation, gradually increased during the blastula and neurula stages and was sustained through the tail-bud stage. Whole-mount *in situ* hybridization analysis showed the localization of xFRP mRNAs in the Spemann organizer, notochord, neural floor plate, hypochord and somite. The similarities with the pattern of expression of *Xenopus* follistatin mRNA suggests that xFRP may play a role in neuralization.

The recently cloned TSC-36 (TGF-ß-stimulated clone 36) gene was originally isolated as a cDNA that was up-regulated by TGF-ß1 in mouse osteogenic MC3T3E1 cells [1], and contains a follistatin module. The TSC-36 protein is a secreted protein of 306 amino acids and four potential N-glycosylation sites and an apparent molecular mass of 38 kDa. TSC-36 is a member of the follistatin-related-protein (FRP) family which includes rat FRP, human FRP [2] and chicken Flik (follistatin-like) [3]. Although the temporal and spatial expression of the Flik gene in chicken embryogenesis is fundamentally similar to follistatin [3,4], studies suggest that Flik and follistatin are differently modulated, and no function has been assigned to FRP to date.

Follistatin, initially isolated from follicular fluid as an inhibitor of follicle-stimulating hormone (FSH) secretion [5,6], is an activin-binding protein [7]. Follistatin was found to have a neutralizing effect on various activin activities, including its dorsal mesoderm inducing activity [8,9,10], and has been known as an activin antagonist [11,12,13]. During early *Xenopus* development, follistatin (XFS319) is expressed in the Spemann organizer, prechordal and chordal mesoderm, and was found to have an excess XFS319 direct neural inducing activity in *Xenopus* embryos [14]. These findings suggest an important role for follistatin in anteroposterior axis formation. Follistatin has a four closely related cystein-rich domains, previously reported as follistatin modules [15]. Follistatin modules have been recognized in several proteins including agrin, the well investigated mediator of the motor-neuron-induced aggregation of acetylcholine receptors [16,17,18], and SPARC (osteonectin), the secreted glycoprotein which modulates cell shape and cell-matrix [19,20].

In this study, we cloned the *Xenopus* FRP homologue (xFRP) and determined its expression to elucidate the physiological function of FRP in *Xenopus* embryogenesis.

MATERIALS AND METHODS

Xenopus embryos. Eggs of *Xenopus laevis* were obtained by injecting 600 IU of human chorionic gonadotropin (Gestron, Denkaseiyaku, Japan) into *Xenopus*. Fertilized eggs were dejellied with 4% cysteine hydrochloride in the Steinberg's solution (pH 7.8), washed with sterilized Steinberg's solution (pH 7.4), and cultured at 20°C. The embryos were staged according to Nieuwkoop and Farber [21].

Construction and screening of a cDNA library. Total RNA was extracted from 170 *Xenopus laevis* stage 24-26 embryos by the acid guanidine thiocyanate-phenol-chloroform method [22]. Poly A(+) mRNA was purified by Oligotex dT30 (Super) (Takara Co. Japan) according to the manufacturer's protocols. cDNAs corresponding to

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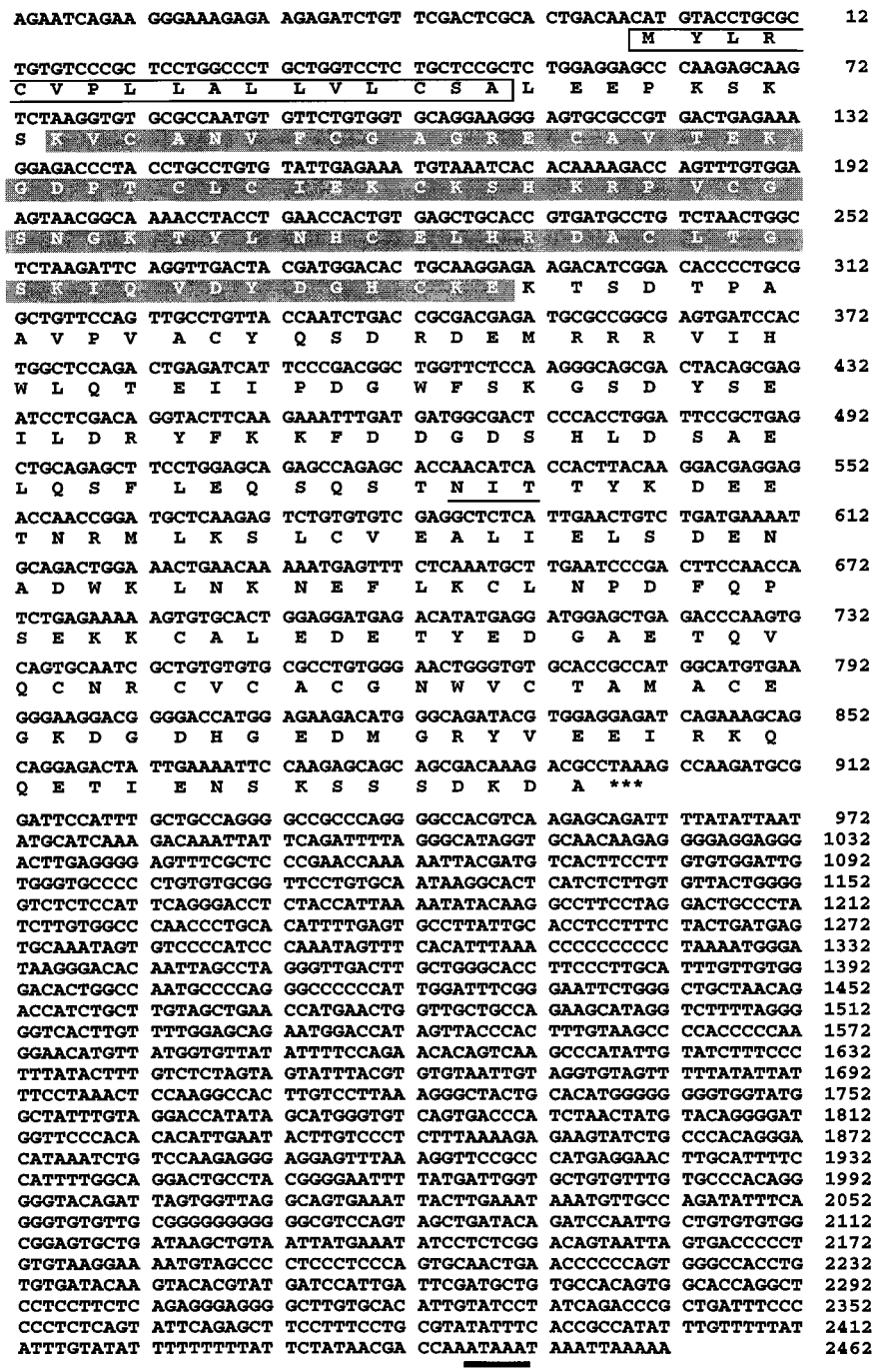

FIG. 1. Nucleotide and predicted amino acid sequences derived from *Xenopus* FRP cDNA clone. Numbering of nucleotides starts from the A of the initiation codon. The putative signal sequence at the NH2-terminus is boxed. The follistatin module is shadowed. A potential N-linked glycosylation site is underlined. The stop codon is indicated by asterisks and the polyadenylation signal is heavily underlined.

the resulting mRNAs were synthesized using a cDNA Synthesis Kit (Pharmacia Biotech) and ligated to EcoRI/NotI adaptor (Life Technologies, Inc.). cDNAs longer than 1 kb were selected by Sephacryl S-500 chromatography, cloned into the λZAPII vector, and packaged with an in vitro packaging extract (Stratagene). About 8×10^5 plaques were screened to isolate xFRP cDNA clones. Filters were hybridized in hybridization buffer (6 × SSC, 0.2% Denhardt's solution, 0.1% SDS, 100 mg/ml salmon sperm DNA) with a 32P-labeled TSC-36 cDNA fragment at 65°C overnight, then washed twice in 1 × SSC and 0.1% SDS at 65°C. Positive clones were isolated, and the clones containing inserts longer than 2 kb were excised by the ExAssist/SOLR System (Stratagene) in the form of pBluescript SK according to the manufacturer's protocols. Clones X-21, and X-29 were used for further analysis.

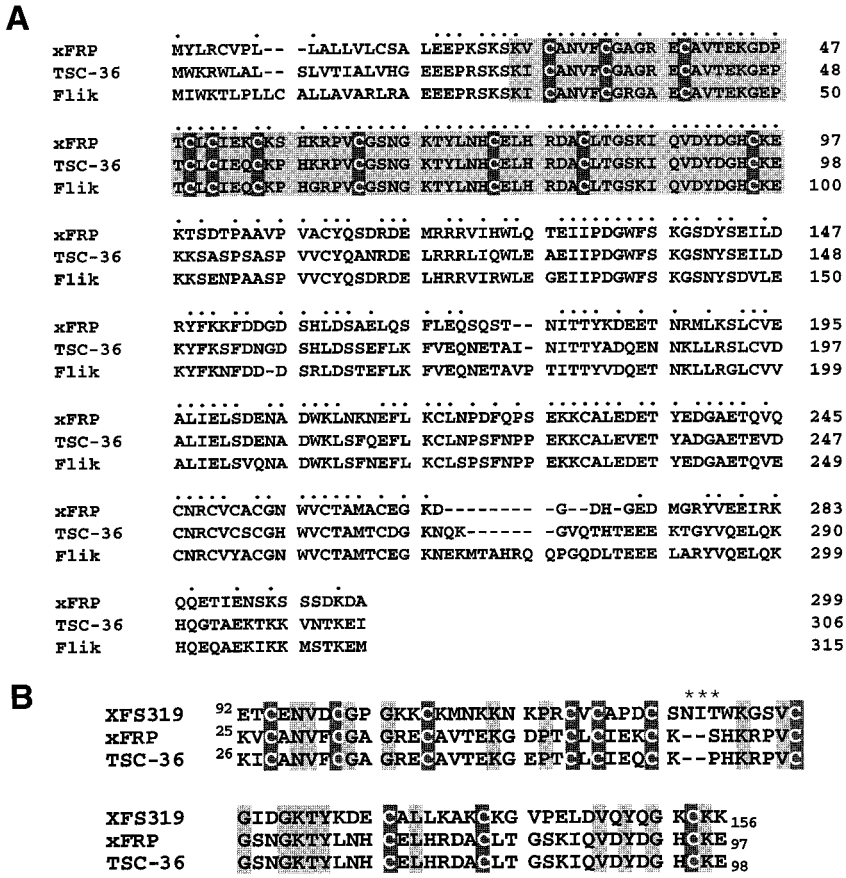

FIG. 2. (A) Alignment of the amino acid sequence of xFRP, TSC-36, and Flik. Identical residues are indicated by dots. The dash (−) represents a gap inserted for maximal alignment. The follistatin module is shadowed, and the included cystein residues are heavily shadowed. (B) Alignment of the follistatin modules of XFS319, xFRP, and TSC-36. Identical residues are shadowed. The potential N-linked glycosylation site of XFS319 is indicated by asterisks.

Sequence analysis. Both strands of the isolated cDNA clones and constructed subclones were sequenced with a DNA sequencer model 373A using Dye Deoxy Terminator Cycle Sequencing Kits (Applied Biosystems). Nucleotide sequences were analyzed by the Gene Works 2.5.1 program (Intelligenetics Inc.).

Northern blotting. Poly A(+) mRNA was directly prepared from lysates of embryos in each stage using oligo-d(T) cellulose (TOYOBO, Japan) with proteinase K treatment. One μg poly A(+) mRNAs were denatured at 70°C for 5 min in 50% formamide, 20% formaldehyde and 20 mM MOPS. These samples were electrophoresed in an agarose/formaldehyde denaturing gel and transferred to Hybond N+ nylon membrane (Amersham Corp.). The membrane was hybridized with ^{32}P-labeled probes in hybridization buffer (50% formamide, 5 × SSPE (pH 7.4), 5 × Denhardt's solution, 0.1% SDS, 100 mg yeast tRNA) at 42°C for 12 hours. A 1.1 kb xFRP fragment named HcΔ at nucleotide position 1366-2470 was radiolabeled and used as a probe. Washing was performed in 1 × SSPE and 0.1% SDS at room temperature. The same membrane was reprobed with a ^{32}P-labeled cDNA fragment of *Xenopus* ODC [23].

Whole-mount in situ hybridization. Albino *Xenopus* embryos at various stages were fixed in MEMFA for 5 hours and then placed in 100% methanol. Digoxigenin (DIG)-labeled RNA probes in sense and antisense orientations were prepared from the xFRP gene fragment Hc? subcloned into pBluescript II SK-, with the DIG RNA Labeling Kit (Boehringer Mannheim). Whole-mount *in situ* hybridization was performed by Harland's method [24] with the following modifications. Embryos were washed with maleic acid buffer (MAB; 100 mM maleic acid, 150 mM NaCl, pH 7.5) instead of the PBS-based solutions, and incubated in MAB with anti-DIG antibody (Boehringer-Mannheim) and 20% bovine serum. Stained samples were dehydrated with ethanol, transferred to xylene, embedded in paraffin, cut into 10-μm sections with a microtome, and examined in detail under microscope.

RESULTS AND DISCUSSION

A *Xenopus* tail-bud (stage 24-26) cDNA library was screened using a 2637-bp cDNA fragment of mouse TSC-36 digested with EcoRI as a probe to obtain *Xenopus* FRP cDNA clone highly homologous to the mouse TSC-36. About 30 positive clones were isolated from a total of 8×10^5 independent clones. The X-21 clone contained the entire open reading frame encoding 299 amino acid residues, the 5′-noncoding region of 15 bp nucleotides and the 3′-noncoding region with the

polyadenylation signal and poly A(+) tail. The X-29 clone contained 40 bp of 5′-nonreading region and the same open reading frame as X-21. The nucleotide sequence surrounding the ATG codon at positions 1-3 conformed to the initiation site consensus sequence identified by Kozak [25]. Moreover, there was no other proximal ATG codon and 16 hydrophobic amino acids followed the ATG codon. Therefore the initiation site was assigned to this codon and based on the high similarity with mammalian FRPs, the resulting cDNA was named xFRP (Fig. 1).

The alignment of the deduced amino acid sequence showed that the identity of xFRP to mouse TSC-36, human FRP and chicken Flik was 70%, 71% and 68%, respectively. The follistatin domain located in the NH2-terminal region showed particularly high homology of about 95%, and the positions of the characteristic cystein residues were completely conserved among these homologues (Fig. 2A). xFRP protein contained putative signal sequence as the other homologues, but had only one potential N-glycosylation site whereas the mammalian FRPs and chicken Flik had 4 and 2 sites, respectively. Alignment of the follistatin modules showed that FRP lacked 2 amino acids residues (Asn-Ile) at the potential N-glycosylation site of follistatin (Asn-Ile-Thr) (Fig. 2B). This may reflect some functional differences between these two proteins.

Northern blotting analysis of *Xenopus* embryos from stage 6 to 46 was carried out using the 1.1-kb xFRP fragment (HcΔ) as a probe. The xFRP gene transcripts were detected as a single band at approximately 2.5 kb position. Expression was observed faintly at stage 11, increased gradually during the proceeding of gastrulation, peaked at stages 18 to 20, and then sustained through tail-bud and tadpole stages (Fig. 3). The temporal expression pattern suggested two peaks at stages 18 to 20 and stages 42-46, but it was unclear. The total tendency of these observations was similar to the XFS319 expression.

The spatial distribution of the xFRP gene was analyzed by whole-mount *in situ* hybridization of various embryonic stages using DIG-labeled antisense RNA probe corresponding to the xFRP cDNA fragment HcΔ (Figs. 4, 5). The first detectable expression of xFRP RNA was slightly observed in the Spemann organizer region at stage 10, as in the case of XFS319. This faint expression became sharpened in the central axis region of the mesoderm during gastrulation, and clear expression was maintained in the notochord until late neurula (stage 22). The hypochord cells were also slightly stained. In ectoderm, simultaneous to the expression in chordal mesoderm, faint expression was detected in the predicted floor plate, the region adjacent to the chordal mesoderm, and this expression also slightly intensified and was defined in the induced floor plate. In the anterior neural tissues, xFRP RNAs were detected in the floor region of the hindbrain. In the late neurula stage (stage 18 to 20), weak expression was also observed in somites adjacent to the notochord. After stage 23, xFRP expression decreased in the notochord, but simultaneously increased in the hypochord, then strong expression in the floor plate and the hypochord was maintained until stage 32. At the late tail-bud stage, the expression in the trunk region was considerably weakened, and observed to gradually move toward the anterior and posterior regions. As a result, xFRP transcripts remained in the notochord, floor plate, hypochord and somite of the tail region and their counterparts and mesenchymal tissues of the head region at stage 36. These findings were coincident in some points with the XFS319 RNA distribution reported previously [14]. Initially, xFRP and XFS319 were expressed in the Spemann organizer at the onset of gastrulation, were continuously expressed in the prechordal and anterior chordal mesoderm at gastrula stage, then expression of both genes was observed in the hypochord and the floor of the hindbrain and spinal cord at the neurula stage. The differences between the expression of the two genes were as follows. (1) xFRP was expressed in the entire notochord from the beginning of notochord differentiation, but XFS319 expression was located in the anterior notochord at first, successively extended posteriorly, and then included the whole notochord by stage 21. (2) In the neural tissues, xFRP RNA was precisely localized in the floor region of the predicted hindbrain and spinal cord, in spite of the broad XFS319 expression in the forebrain, midbrain and hindbrain. (3) xFRP RNA expression was observed weakly in the somite, but XFS319 was not. However, XFS319 was expressed distinctly in the pronephros, whereas xFRP was not. (4) xFRP expression faded in the notochord as the vacuolization of the notochord proceeded, whereas XFS319 expression was maintained in the notochord even at the tadpole stage.

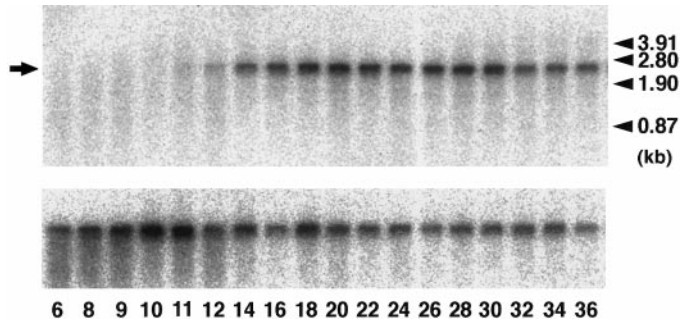

FIG. 3. Expression of xFRP mRNA during *Xenopus* development. Northern blot anaysis was performed using one mg of stage-specific mRNA per lane. The blotting membrane was probed with ^{32}P-labeled xFRP cDNA (upper) or *Xenopus* ODC cDNA (lower). Arrow indicates xFRP. Embryonic stages are indicated by numbers according to Nieuwkoop and Farber [21].

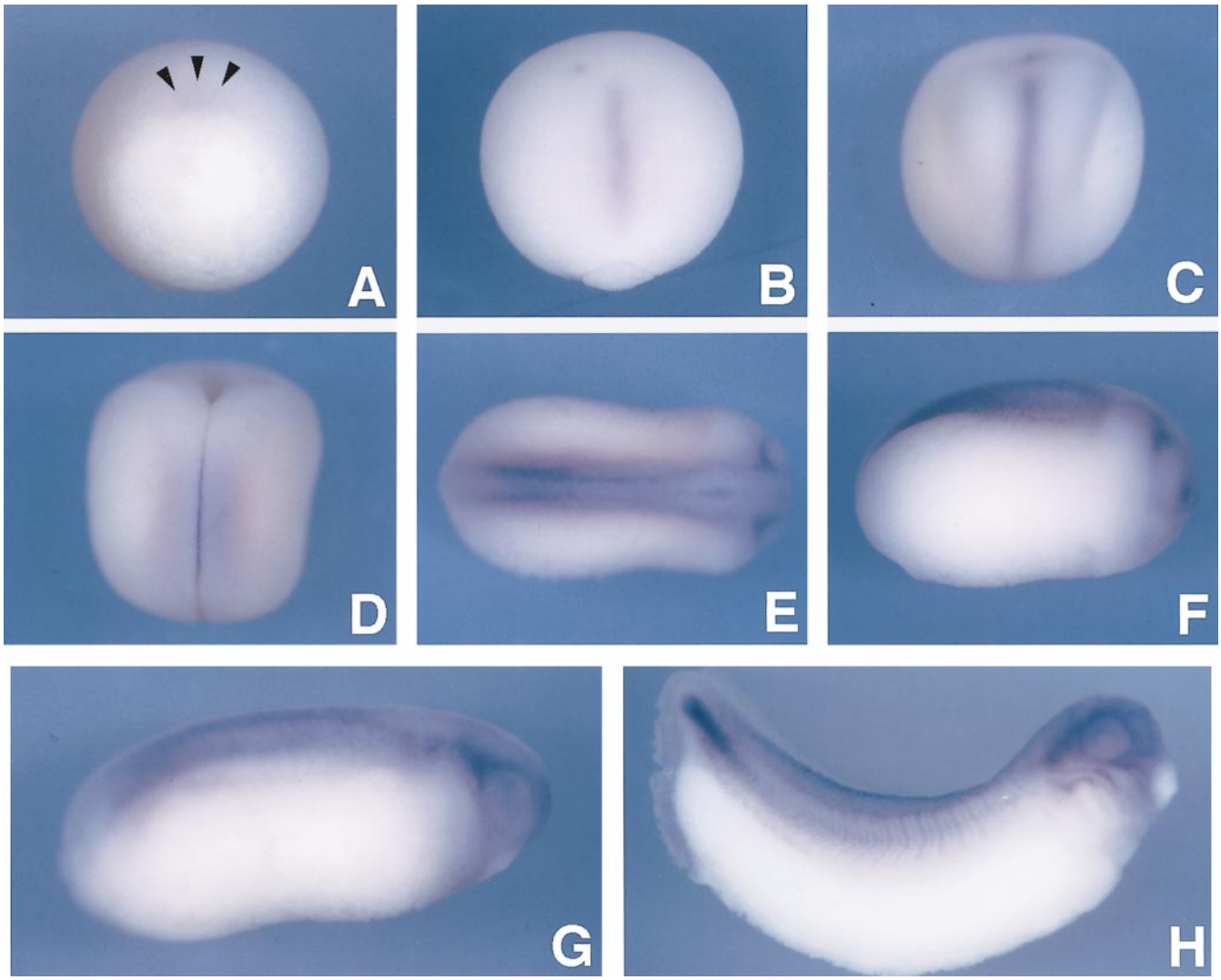

FIG. 4. Spatial distribution of xFRP mRNA during *Xenopus* embryogenesis. Whole-mount *in situ* hybridization was performed with DIG-labeled antisense xFRP RNA. (A) Stage 10 embryo, vegetal view. xFRP RNA was slightly detected in the organizer (arrowheads). (B) Stage 12 embryo, dorsal view. Signals were detected in prechordal and chordal mesoderm. (C) Stage 15 embryo, dorsal view. (D) Stage 18 embryo, dorsal view. (E) Stage 21 embryo, dorsal view. (F) Stage 21 embryo, lateral view. (G) Stage 24 embryo. (H) Stage 30 embryo.

Since the spatial pattern of xFRP gene expression was similar that of XFS319, we carried out microinjection of synthesized xFRP mRNA to test the neuralizing activity of xFRP. xFRP mRNA and control mRNA (β-globin) were transcribed *in vitro*, dissolved in Gurdon's buffer (88 mM NaCl, 1 mM KCl, 15 mM Tris-HCl, pH 7.5), and microinjected into blastomeres of the dorsal-vegetal or ventral-vegetal side of *Xenopus* 8-cell stage embryos at a rate of 0.1-1 ng mRNA per blastomere. Injected embryos were cultured for 1 week, but no apparent changes were observed in the injected embryos, compared with the controls or untreated embryos. To clarify the effect of injected xFRP mRNA in molecular marker gene expression, after the injection of mRNA into the animal side of the 2-cell stage embryos, the animal caps from these embryos at stage 9 (blastula) were dissected and total RNA from the caps cultured for 1-2 days was extracted. RT-PCR analysis with the RNA showed that xFRP mRNA injection caused no apparent up- or down-regulation of various neural and notochordal marker genes such as *N-CAM*, *HNF3-β*, *Pintallavis*, *Xwnt4*, *netrin-1*, *Xshh*, and *Xnot*.

TSC-36 was originally isolated as a factor induced by TGFβ1, and several studies suggested that TSC-36 did not mimic the growth inhibitory activity of TGFβ1 nor TGFβ1 signaling [2]. Interestingly, TSC-36 was extinguished in the *v-ras* transformed MC3T3 cells, but recovered to a normal level in the flat revertant [1],

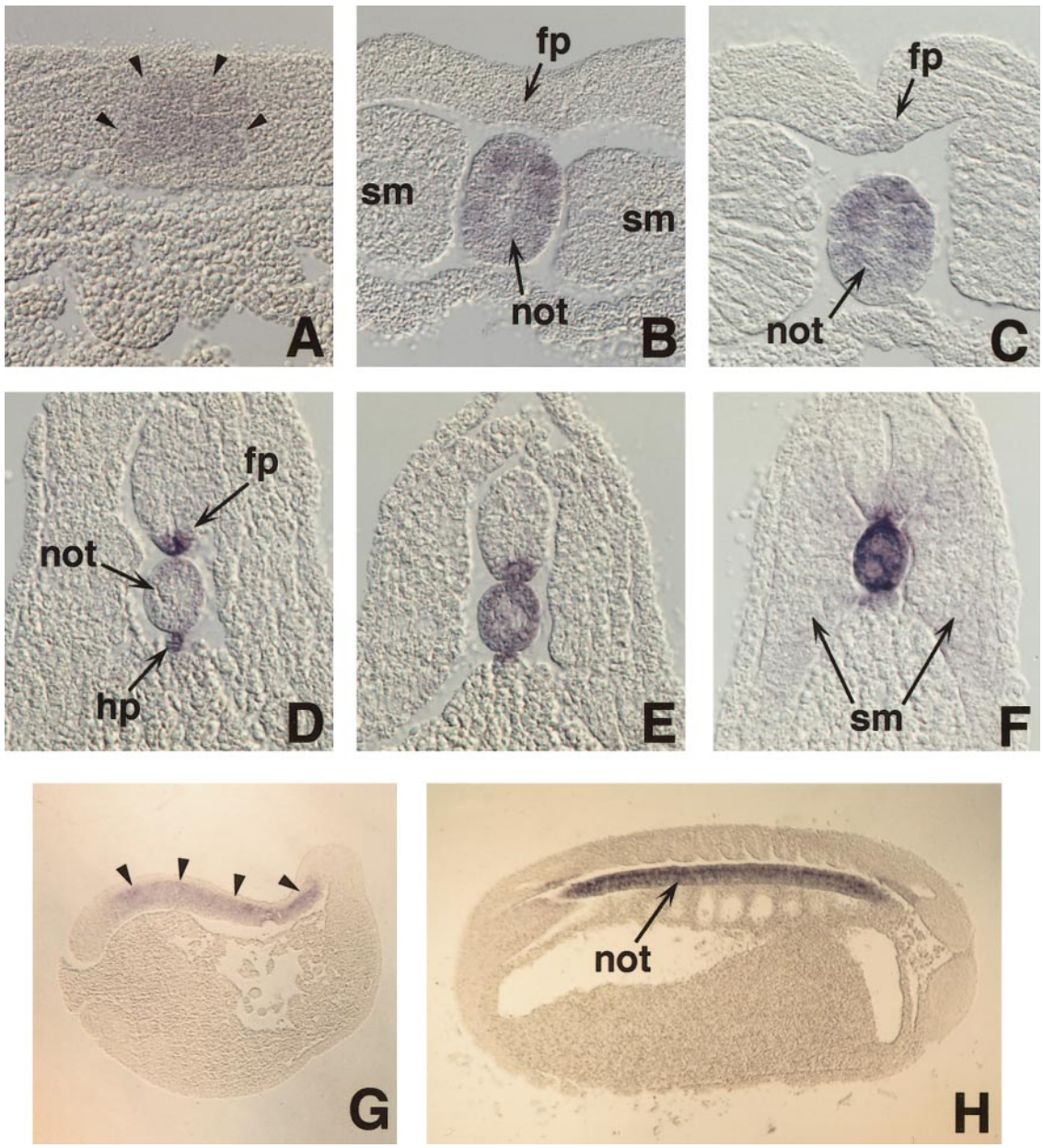

FIG. 5. Transverse or vertical sections of the embryos stained by whole-mount *in situ* hybridization. Dorsal side is at the top in each figure. (A) Transverse section of stage 12 embryo. Faint signal is localized in the chordal mesoderm (arrowheads). (B, C) Transverse sections of stage 14 and 17 embryos, respectively. Notochord and predicted neural floor plate are stained. (D, E, F) Transverse sections of stage 30 embryo. The middle, anterior, and posterior region of the trunk region. xFRP mRNA is localized in notochord, neural floor plate, and hypochord. Faint signal is also observed in somites. xFRP expression fades in the middle region of notochord (D). (G) Vertical section of stage 12 embryo. Prechordal and chordal mesoderm are clearly stained (arrowheads). (H) Vertical section of stage 22 embryo. xFRP is expressed throughout the notochord. Abbreviations: not, notochord; fp, floor plate; hp, hypochord; sm, somite.

indicating that activated *ras* or *ras* signaling might down-regulate TSC-36 gene expression. In *Xenopus* development, *ras* mediates the signaling between FGF receptor and *Raf-1*, and excess *ras* expression causes ectopic mesoderm formation [26, 27, 28]. Considering the relationship between TSC-36 and *ras*, the fact that the xFRP gene is expressed in mesodermal tissues is apparently inconsistent with the mesoderm inducing activity of *ras*. Moreover, in the *Xenopus* embryo animal-cap assay, TGFβ1 has no mesoderm inducing activity [29]. It must be clarified whether xFRP is induced by TGFβ1 in *Xenopus* and whether xFRP binds to any growth factor to elucidate the biological functions of xFRP.

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