

## Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation

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### SUMMARY

We have developed a simple approach for large-scale transgenesis in *Xenopus laevis* embryos and have used this method to identify *in vivo* requirements for FGF signaling during gastrulation. Plasmids are introduced into decondensed sperm nuclei *in vitro* using restriction enzyme-mediated integration (REMI). Transplantation of these nuclei into unfertilized eggs yields hundreds of normal, diploid embryos per day which develop to advanced stages and express integrated plasmids nonmosaically. Transgenic expression of a dominant negative mutant of the FGF

receptor (XFD) after the mid-blastula stage uncouples mesoderm induction, which is normal, from maintenance of mesodermal markers, which is lost during gastrulation. By contrast, embryos expressing XFD contain well-patterned nervous systems despite a putative role for FGF in neural induction.

Key words: transgenic, nuclear transplantation, FGF, mesoderm, neural induction, *Xenopus*, sperm, gastrulation, REMI

### INTRODUCTION

The vertebrate embryo is formed by a series of inductive events that lead to the formation of different cell types, the formation of organ rudiments and the elaboration of the body axis. In *Xenopus laevis* embryos, mesoderm is initially induced and crudely patterned in the marginal zone at the blastula stage by signals from the vegetal hemisphere. During gastrulation, signals produced by the dorsal mesoderm elaborate full mesodermal patterning and induce neural tissue in the overlying ectoderm (for reviews see Harland, 1994; Kimelman et al., 1992; Slack, 1994).

Members of the fibroblast growth factor (FGF) family have potent inducing activities in the *Xenopus* embryo. Several FGFs and FGF receptors are expressed during blastula and gastrula stages (reviewed in Slack and Tannahill, 1992) and FGFs can induce mesoderm when added to explanted animal cap ectoderm (Kimelman and Kirschner, 1987; Slack et al., 1987). The requirement for FGF signaling has also been demonstrated *in vivo* by introducing RNA encoding a dominant negative mutant of the FGF receptor into *Xenopus* embryos (Amaya et al., 1991). Embryos injected with XFD RNA fail to express several immediate early mesoderm markers and develop with severe mesoderm defects, strongly suggesting that FGF receptor signaling is required for primary mesoderm induction (Amaya et al., 1993; Cornell and Kimelman, 1994; LaBonne and Whitman, 1994).

A role for FGF signaling in later patterning events also seems likely. Several FGFs are expressed in the embryo during gastrulation (Isaacs et al., 1992; Tannahill et al., 1992). eFGF, for example, is expressed in the blastopore lip and later in the

dorsoposterior mesoderm. Furthermore, in the presence of eFGF, dissociated gastrula mesoderm cells retain mesodermal character otherwise lost in culture (Isaacs et al., 1994). From this observation, Isaacs and colleagues conclude that eFGF can maintain an otherwise labile state of mesoderm during gastrulation. The term mesoderm maintenance has been used to describe the process by which mesodermal gene expression is dynamically regulated during gastrulation. FGF can also induce neural differentiation when added to dissociated gastrula ectodermal cells (Kengaku and Okamoto, 1993). FGF can posteriorize intact anterior neural tissue induced by noggin (Lamb and Harland, 1995) or follistatin (Cox and Hemmati-Brivanlou, 1995) as well as intact anterior neural plate tissue isolated from gastrula and early neurula stage embryos (Cox and Hemmati-Brivanlou, 1995). Therefore it has been suggested that FGF signaling is partly responsible for neural induction *in vivo*, or more specifically, for the transforming signal believed to posteriorize neural ectoderm at gastrula and early neural stages (for review, see Doniach, 1995).

Here we have investigated whether signaling through the FGF receptor is required *in vivo* for processes occurring after primary mesoderm induction, such as maintenance of mesodermal fate and induction and patterning of neural tissue. To address the question of whether FGF signaling is necessary in the embryo for these later aspects of development, we needed to express the dominant negative FGF receptor only after mesoderm induction had occurred (so that the failure to induce mesoderm did not obscure specific later effects). It was also necessary to express the mutant receptor in all cells. Plasmid injections that place XFD expression under transcriptional control and restrict its expression until after the mid-blastula

transition (when zygotic transcription begins) cannot be effectively used since plasmids are unequally distributed in embryonic cells and thus are mosaically expressed. Therefore we have developed an efficient method for transgenesis in *Xenopus* that, unlike plasmid injection, produces stable, non-mosaic expression of cloned genes in embryos. The approach allows the production, in one day, of hundreds of transgenic embryos that develop normally to advanced stages and express inserted genes at high frequencies. We have found that, unlike plasmid-injected embryos, transgenic embryos show correct temporal and spatial regulation of integrated promoter constructs. This is a substantial improvement upon a previous transgenesis method that generated aneuploid embryos that rarely developed past the neurula stage of development (Kroll and Gerhart, 1994).

We have used this procedure to investigate whether signaling through the FGF receptor is required for mesodermal maintenance and neural induction in the gastrula and postgastrula embryo. We find that when XFD is expressed during gastrulation, mesoderm maintenance is severely inhibited. Expression of the pan-mesodermal marker *Xbra* is lost by mid-gastrulation and embryos develop without a notochord or somites. The requirement for FGF signaling in this process appears to be limited to the gastrula stages, since XFD expression after gastrulation does not severely affect mesoderm development. Although FGF was previously shown to induce neural tissue in vitro, we found that neural induction and patterning was not affected in embryos expressing XFD at any time during early development. This suggests that FGF receptor signaling is not necessary for this process in the embryo.

## MATERIALS AND METHODS

### Plasmid construction

pRLCAR has been previously described (Kroll and Gerhart, 1994). pCARGFP was generated by inserting a 3270 bp *KpnI-SalI* fragment from the *Xenopus* cardiac actin promoter (DNA #254) (Mohun et al., 1986) into *KpnI-HindIII*-digested pCSKA (Condie et al., 1990) to produce pCarA. GFP was then introduced into *HindIII-EcoRI*-digested pCarA as an 810 bp *HindIII-EcoRI* fragment from Nc7 containing the 5' and 3'  $\beta$ -globin UTR sequences flanking the coding sequences of GFP. Nc7 contains a 775 bp *NcoI-XbaI* PCR fragment generated by amplifying the coding sequences for GFP from TU#65 (Chalfie et al., 1994) using the following primers: 5'-CAAC-CATGGGTAAAGGAGAAGAACTTTTC-3' and 5'-AATACGACT-CACTATAG-3' and inserting this fragment into a modified pSP64T vector.

The plasmid, 3.8N $\beta$ T (kindly provided by Paul Krieg), contains a 3.8 kb promoter fragment inserted into pBLCAT3. pCSnucGFP (kindly provided by Adrian Salic) consists of a mutant version of GFP (Ser-65 to Thr) (Heim et al., 1995) which was PCR amplified to introduce *Clal* and *XhoI* sites at the ends of the gene and cloned into a *Clal-XhoI*-digested version of pCS2+ (Turner and Weintraub, 1994) containing a nuclear localization signal of the SV40 large T antigen. pCSKd50 was generated by inserting the 1347 bp *HindIII-EcoRI* fragment from d50/Xss (Amaya et al., 1991) into *HindIII-EcoRI*-digested pCSKA. pCSKXFD and pCSKHAV $\emptyset$  were generated by inserting the 1430 bp *NcoI-EcoRI* fragment from XFD/Xss (Amaya et al., 1991) and the 1421 bp *NcoI-EcoRI* fragment from HAV $\emptyset$  (Amaya et al., 1993), respectively, into *NcoI-EcoRI*-digested pCSKd50. pCSd50 was generated by inserting the 1347 bp *HindIII-*

*EcoRI* fragment from d50/Xss and inserting it into *HindIII-EcoRI*-digested pCS2+ (Turner and Weintraub, 1994). pCMVXFD and pCMVHAV $\emptyset$  were generated by inserting the 1430 bp *NcoI-EcoRI* fragment from XFD/Xss or the 1421 bp *HindIII-EcoRI* fragment from HAV $\emptyset$  into pCSd50 that was also digested with *NcoI-EcoRI*. pCARXFD and pCARHAV $\emptyset$  were generated by inserting the 1760 bp *Sall-NotI* fragment from pCSKXFD or the 1751 bp *Sall-NotI* fragment from pCSKHAV $\emptyset$  into *Sall-NotI*-digested pCarA.

XFD, d50 and  $\beta$ -gal RNA were synthesized in the presence of cap analog and injected into 2-cell stage embryos as previously described (Amaya et al., 1991, 1993).

### Sperm nuclei and high speed extract preparation

Sperm nuclei were prepared as previously described (Murray, 1991), except that the protease inhibitors leupeptin and phenylmethylsulfonyl fluoride were used only in the wash immediately following the lysolecithin treatment. Concentration of sperm stock was determined by Hoechst (Sigma) staining and counting in a hemocytometer. From one male, we typically obtained 0.5 ml of  $1 \times 10^8$  sperm/ml stock. Sperm were stored at 4°C and used for transplantations for up to 48 hours.

High speed interphase extract preparation was performed as follows. Crude cytosstatic factor-arrested extract was prepared from eggs collected from 8-12 frogs as previously described (Murray, 1991), except that cytochalasin was omitted. After the extract was clarified (see, Murray, 1991), CaCl<sub>2</sub> was added to 0.4 mM and the extract was incubated at room temperature for 15 minutes (the CaCl<sub>2</sub> releases the CSF arrest and allows the extract to progress into interphase). Then the extract was centrifuged at 70,000 revs/minute for 1.5 hours at 4°C in a TL100.3 rotor and a Beckman tabletop TL-100 ultracentrifuge. Cytosolic layer was collected through the top of the tube, transferred to fresh tubes and spun at 70,000 for an additional 20 minutes at 4°C. Aliquots of 25  $\mu$ l were frozen in liquid nitrogen and stored at -80°C until use. 1-2 ml of high speed interphase extract was typically obtained from preparations of this scale.

### Transgenesis by sperm nuclear transplantation into unfertilized eggs

A transplantation apparatus was constructed by connecting a line of 3/16 inch tubing from the house vacuum outlet to a three-way valve (Western Analytical Products, Murietta, CA; #001102). Another line connects the house air outlet to a T-connector that split the air flow into an exhaust line and another line connecting to the three-way valve. A third line of 1/32 inch tubing connects the three-way valve to the needle. For fine control of positive pressure into the needle, a screw clamp was placed on the exhaust line. Coarse adjustment of positive pressure was performed by gradually opening or closing the valves (on the three-way valve) connected to the house air and/or vacuum lines.

Preparation of transplantation needles and agarose-coated injection dishes (from 60 mM Petri dishes) has been previously described (Kroll and Gerhart, 1994). Needles had a 200-400  $\mu$ m wide bore drawn to a 60  $\mu$ m tip. To prevent shearing of nuclei, the inside surface of the needles was coated with Sigmacote (Sigma SL-2) by attaching approximately 1 cm Tygon tubing (14-169-1A; Fisher) to the end of a plastic pipette (200  $\mu$ l) tip. Sigmacote was drawn into the tip, then tubing was attached to the needle and the pipette plunger depressed to force Sigmacote through the needle. Needles were rinsed with water before use.

2-4 adult female frogs were injected in the dorsal lymph sac with 500-800 U Human Chorionic Gonadotropin (Sigma) and incubated at 15°C for 12-16 hours before transplantations. Transplantation apparatus was adjusted so that there was a very low positive needle pressure through the needle and a meniscus (of water) in the needle did not appear to move.

Plasmids were linearized for transgenesis using *XbaI* or *NotI*. Linearized plasmids were purified for transgenesis using the GeneClean

II kit (Bio 101) and eluted in dH<sub>2</sub>O at a concentration of 200-250 ng/μl.

A REMI reaction was prepared by adding 4 μl sperm stock (~4×10<sup>5</sup> nuclei) to 5 μl linearized plasmid (200-250 ng/μl). After 5 minutes incubation at room temperature, 0.5 μl of a 1:5 dilution of *Xba*I or *Not*I (the enzyme used for plasmid linearization), 2 μl 100 mM MgCl<sub>2</sub> and 25 μl high speed extract were added. The reaction was mixed gently using a clipped yellow tip and pipetting up and down, and the mixture was incubated 10 minutes at room temperature; sperm were visibly swelled if diluted into Hoechst as before and observed with a 10×-20× objective.

While sperm were swelling in reaction mixture, eggs were collected from individual frogs and dejellied in 2.5% cysteine hydrochloride in 1× MMR (pH 8.0); the healthiest eggs were then drawn into a wide-bore pasteur pipette and transferred to fill the injection dish. After about 5 minutes in 0.4× MMR + 6% Ficoll, the eggs pierced easily.

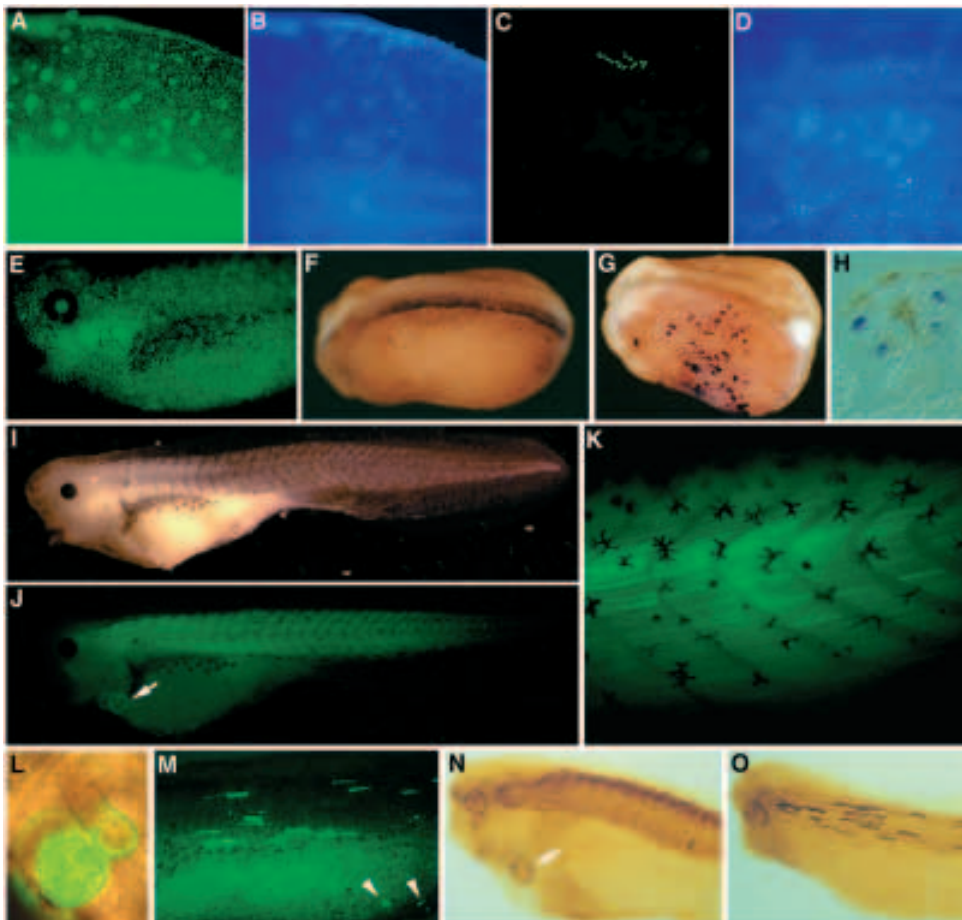
Sperm were diluted into sperm dilution buffer (250 mM sucrose, 75 mM KCl, 0.5 mM spermidine trihydrochloride, 0.2 mM spermine tetrahydrochloride; titrated to pH 7.3-7.5 with NaOH) using dilutions of 1:25-1:100 (such that the final dilution was 1:250-1:1000 or a concentration of 1-2 sperm per 10-15 nl injection volume). MgCl<sub>2</sub> was added to 5 mM. Sperm suspensions were mixed thoroughly with a cut yellow tip before each manipulation. A piece of Tygon tubing attached to a yellow tip was used as previously described for Sigma-coting needles to draw up the dilute sperm suspension and back load a transplantation needle. Sperm nuclei were transplanted into each unfertilized egg in an approximately 10 nl volume. This was done by establishing a steady, slow flow of sperm solution (about 20-25 nl/second) from the transplantation needle and piercing each egg with a single, sharp motion then withdrawing more slowly. A hole about the diameter of the needle tip was visible on the egg and remained

open for several seconds after injection indicating solution had been delivered. All eggs were thus activated by the injection, but only the embryos that cleaved normally (from one fifth to one third of the injected eggs) were selected for further analysis. These represent the embryos that received one nucleus per egg. Eggs failing to receive a nucleus did not cleave or formed pseudocleavages. Eggs injected with too much volume also sometimes failed to cleave and had mottled animal hemisphere pigmentation. Eggs injected with more than one nucleus divided abnormally into three or more cells and generally failed to gastrulate properly, showing incomplete blastopore closure.

When the selected transplant embryos reached the 4-cell to 16-cell stage, they were transferred (at ~10 embryos/well) to 6-well tissue culture containing 0.1× MMR + 6% Ficoll +50 μg/ml gentamycin. When embryos reached stage 12, media was replaced with 0.1× MMR + 50 μg/ml gentamycin without Ficoll. For further information about this method, see Amaya and Kroll (1996).

**In situ hybridizations, immunostaining and β-galactosidase staining**

Antisense RNA probes were prepared as described by Harland (1991) using digoxigenin-11-UTP (Boeringer), except for the anti-FGF receptor probe which was labeled with fluorescein-12-UTP (Boeringer). Templates used for making most probes have been described previously: *Brachyury* (Smith et al., 1991), *noggin* (Smith and Harland, 1992), collagen type II (Amaya et al., 1993), *Otx 2* (Lamb et al., 1993), *En 2* (Bolce et al., 1992), *Krox-20* (Papalopulu et al., 1991), neural-specific β-tubulin (Oschwald et al., 1991), *Pax 3* (Espeseth et al., 1995) and *Hox B9 (Xlhbox 6)* (Doniach et al., 1992). For the 2.9 kb antisense FGF receptor probe, the plasmid E2 (Musci et al., 1990) was linearized with *Xba*I and transcribed with T3 RNA polymerase. Single and double in situ hybridizations were performed



**Fig. 1.** Non-mosaic plasmid expression in transgenic embryos from sperm nuclear transplantations. (A,E,F,H,J,K,L,N) Non-mosaic transgene expression; (C,G,M,O) Mosaic expression typical of embryos directly injected with plasmid. (A-E) Trunks of transgenic (A,E) or plasmid-injected (C) 2-week-old tadpoles expressing pCMVnGFP. (B,D) DAPI staining of regions shown in A and C. (A) Fin nuclei are visible as small spots; the brighter spots also seen are ciliary tuft cells that naturally autofluoresce to varying degrees in older tadpoles and which also express GFP here. (C,D) 150 pg plasmid was injected at the two cell stage. (F-H) Expression of N-tubulin promoter/ CAT plasmid in tailbud stage transgenic (F, H) or plasmid-injected (G) embryos. (H) Cross-section showing CAT expression in primary neurons. (I-O) Expression of pCARGFP (I-M) and pRLCAR (N-O) in tadpoles. Transgenic expression in somitic (J,K,N) and heart muscle (L, white arrows in J and N) is seen. (L) The bright-field image and GFP expression in the heart are overlaid. Mosaic expression following DNA injection of pCARGFP (M) and pRLCAR (O) is seen. (M) White arrowheads mark ectopic expression of injected CARGFP plasmid in the ventral, posterior trunk.

essentially as described by Harland (1991) with the modifications of Doniach and Musci (1995), except that most steps were done in 2 ml screw cap Eppendorf tubes or glass vials rather than on slides. When double *in situ* hybridizations were performed, the alkaline phosphatase (AP)-conjugated anti-fluorescein antibody (Boehringer) was added first and the embryos were stained with BCIP (Sigma). To ensure that transgenic embryos expressing FGF receptor constructs could be clearly distinguished from non-transgenic embryos, staining reactions were kept short and were monitored carefully. Then the AP-conjugated anti-digoxigenin antibody (Boehringer) was added and the embryos were stained with magenta phos (Biosynth). Histological sections were done as previously described (Papalopulu and Kintner, 1993).

CAT expression was assayed by either whole-mount immunohistochemistry (Fig. 1N,O) (Kroll and Gerhart, 1994) or by *in situ* hybridization with an antisense CAT probe (Fig. 1F-H). X-gal staining for  $\beta$ -galactosidase activity (Amaya et al., 1993) has been described previously.

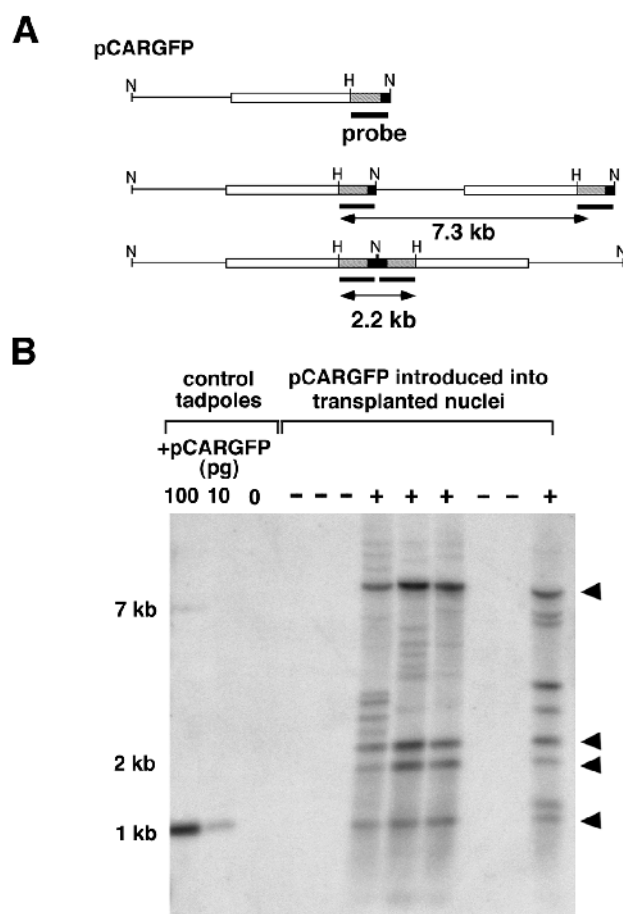
### Photography and imaging

The images shown in Fig. 1E,I,J,L were obtained with an 3CCD color video camera using Northern Exposure software (Phase Three Imaging). The images in Fig. 1E and J are 40-frame on-chip integrations, using a 5 $\times$  objective on a Zeiss Axiophot microscope. In Fig. 1L, a bright-field image has been overlaid on a 25-frame integration through a fluorescein band pass filter; a 10 $\times$  objective was used.

The images in Fig. 1A-D,K were obtained using a Photometrics cooled CCD camera containing a 1024 squared pixel array chip. Universal Imaging Metamorph software was used to control image acquisition from a Zeiss Axiovert microscope. For Fig. 1K, a 10 $\times$ /0.5 objective and fluorescein band pass filter were used to detect wild-type GFP. The image is a 1 second exposure with 2 $\times$ 2 binning and has been pseudocolored. In Fig. 1A-D, a 10 $\times$ /0.5 objective was used to detect a mutant (S65T, see above), nuclear-localized form of GFP protein (750 ms exposure) or DAPI (200-400 ms exposure). These images are also pseudocolored. All remaining images of embryos were obtained using a Wild M420 dissecting microscope and Ektachrome 64T slide film.

### Southern blotting and immunoblotting

Genomic DNA was prepared from nuclear-transplantation-derived tadpoles using the QIAamp Tissue Kit (Qiagen), digested with *Hind*III for 25-30 hours, separated on a 0.7% agarose gel in 1 $\times$  TAE at 10 V for 24 hours (<1 V/cm) and blotted onto Magnagraph nylon membrane (Fisher). The filter was hybridized with random-primed probe consisting of a 1.1 kb *Not*I-*Hind*III fragment from pCARGFP. A Molecular Dynamics phosphorimager was used for quantitation. To determine the plasmid copy number present in each GFP-expressing tadpole, the intensity of all bands recognized by the probe was converted to an absolute plasmid copy number by comparison to 10 pg and 100 pg pCARGFP standards on the blot (Fig. 2B). 10 pg of pCARGFP (7,320 bp) is  $1.4 \times 10^6$  copies. Copy number values were then divided by the number of cell equivalents of genomic DNA loaded per lane (determined by spectrophotometry and converted using the value of 6.3 pg genomic DNA per diploid cell) to determine how many copies per cell of pCARGFP were present in each transgenic embryo. The GFP-expressing tadpoles shown in Fig. 2 contain 8, 34, 20 and 14 copies of pCARGFP per cell respectively. Each of these copy numbers can be divided by the number of novel end fragments seen in that tadpole sample to determine the average number of pCARGFP copies present at each chromosomal integration site; this value ranges from 2 to 6 for the tadpoles described above. As expected, we find that for each tadpole this value (copies per integration site, calculated by the number of novel bands seen) correlates with the ratio of intensities of summed common ('concatemer') to novel ('end') bands. Additionally, if the fragments of novel size represent junctions between integrated plasmid and chromosomal



**Fig. 2.** Tadpoles produced by sperm nuclear transplantation contain integrated plasmid. (A) Schematic of linearized pCARGFP plasmid: cardiac actin promoter (open box), GFP sequences (gray box), SV40 polyadenylation site (solid box) and bacterial sequences (thin line). Below, products expected after pCARGFP concatemerization in the embryo. N, *Not*I. (B) Southern blot of genomic DNA from 1-month-old tadpoles produced using pCARGFP nuclear transplantations (lanes 4-11). Tadpoles expressing GFP non-mosaically (+) and tadpoles not expressing GFP (-) are designated. pCARGFP was detected in *Hind*III (H)-digested genomic DNA using probe sequences designated in A. Lanes 1-3, pCARGFP plasmid was added to genomic DNA from control tadpoles (not produced using nuclear transplantation) just prior to *Hind* III digestion.

DNA, we expect that for each unique band the ratio of its individual copy number value to the cell equivalents loaded in that lane will be one. Consistent with this interpretation, we find that the average of values calculated for each of the unique bands in Fig. 2 is 0.83.

Preparation of protein from transplantation-derived embryos and immunoblotting were performed as previously described (Amaya et al., 1993).

## RESULTS

### Diploid transgenic embryos produced using sperm nuclear transplantation

We have developed a new approach for generating transgenic *Xenopus* embryos that stably express cloned genes. Linearized plasmid DNA is introduced into sperm nuclei using restriction-enzyme-mediated integration (REMI) (Kuspa and Loomis,

1992; Schiestl and Petes, 1991). To do this, nuclei and plasmid are coincubated briefly, then restriction enzyme is added and the nuclei are swelled and partially decondensed in an interphase egg extract. Single nuclei are then transplanted into unfertilized eggs, producing normal, diploid tadpoles that develop to advanced stages and express inserted genes non-mosaically. Unfertilized eggs transplanted with sperm nuclei cleave normally at a frequency of 20-40%. Thousands of nuclear transplantations can be performed by one person in a few hours, yielding several hundred embryos. Between 5% and 40% of these cleaving eggs develop normally beyond feeding tadpole stages, depending on the quality of the unfertilized eggs. We commonly obtain tadpoles 1-2 months old and are currently raising metamorphosed froglets to sexual maturity.

Transgenic embryos from gastrula through tadpole stages express plasmid-encoded genes nonmosaically (Fig. 1). We introduced plasmids containing the simian cytomegalovirus (CMV) (Turner and Weintraub, 1994) promoter or the *X. borealis* cytoskeletal actin promoter (CSK) (Cross et al., 1988; Harland and Misher, 1988) into sperm nuclei. The resulting transgenic embryos expressed both of these promoters ubiquitously, starting at the late blastula and early gastrula stages, respectively. 2-week-old transgenic tadpoles expressing pCMVnGFP, which contains the CMV promoter linked to nuclear localized green fluorescent protein (GFP), are shown in Fig. 1A,B,E. GFP was expressed in all cells that co-stained with DAPI, a DNA marker. By contrast, embryos derived from fertilized eggs injected with the same plasmid always showed mosaic expression, and usually expressed GFP in only a small fraction of cells (Fig. 1, C, D).

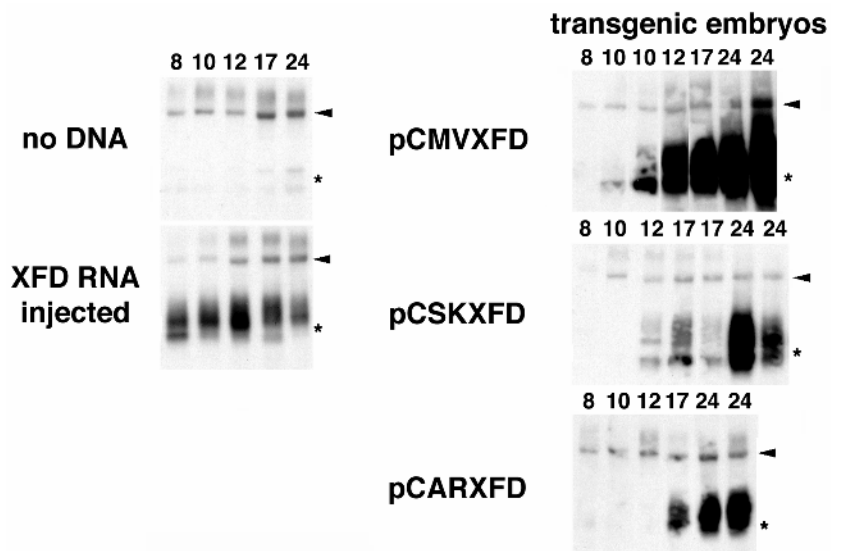
We used transgenesis to integrate plasmids containing promoters that are spatially restricted. First, a plasmid containing a *Xenopus* neural-specific  $\beta$ -tubulin (N-tubulin) promoter linked to chloramphenicol acetyl transferase (CAT)(Paul Krieg, unpublished) was introduced into embryos. The resulting embryos expressed CAT in the primary neurons of the embryo as expected for this promoter (Fig. 1F,H). By contrast, embryos injected with the plasmid directly not only expressed CAT mosaically, but without any apparent tissue specificity (Fig. 1G). Next, a *Xenopus* muscle-specific actin promoter (Mohun et al., 1986) linked to CAT (pRLCAR) or GFP (pCARGFP) was introduced into embryos. Non-mosaic and regionally restricted expression of CAT or GFP, respectively, was observed in transgenic tadpoles carrying these plasmids. Expression was correctly restricted to the somites and cardiac muscle (Fig. 1J,K,N). Tadpoles expressing pCARGFP were raised for several months with GFP expression monitored periodically. We found that non-mosaic, myotome-specific GFP expression was stable over this period. We are currently raising these tadpoles to adults. Direct injection of pRLCAR and pCARGFP plasmids into fertilized eggs resulted in embryos showing highly mosaic expression (Fig. 1M,O). While expression of reporter genes from the cardiac actin promoter was usually regionally specific in plasmid-injected embryos, we occasionally identified a few cells expressing reporter genes outside of the expected domains of heart and

somatic muscle (see arrows in Fig. 1M). We never saw transgenic embryos that showed this non-restricted expression. From these findings, we conclude that expression in transgenic embryos promotes better spatial regulation of cloned promoters than expression of non-integrated plasmids.

A high frequency of nuclear transplantation derived tadpoles expressed plasmid-encoded genes. For example, 36% of tadpoles generated by nuclear transplantations of pRLCAR-treated sperm nuclei expressed CAT in all myotome cells and 53% of the tadpoles expressed it in at least half of the myotomal tissue (Table 1). Embryos that did not express the plasmid in every muscle cell typically expressed in all cells on one side, possibly because of plasmid integration after DNA replication of the first cell cycle. In addition, we found that transgenic embryos showed some variability in the level of transgene expression from one embryo to another, perhaps due to the variable number of transgene copies integrated per embryo (see below). Restriction enzyme was not absolutely required to obtain transgenic embryos. It did, however, enhance the frequency with which nonmosaic expression was obtained in tadpoles. The frequency of tadpoles expressing pRLCAR in all cells dropped by two-fold (from 36% to 19%) when restriction enzyme was omitted from the reaction. Similarly, the frequency of expression of a CSK promoter  $\beta$ -galactosidase reporter plasmid (Vize et al., 1991) dropped by threefold in tadpoles when we omitted enzyme from the reaction (data not shown).

**One month old tadpoles derived from sperm nuclear transplantations contain integrated plasmid**

Transgenic tadpoles raised to 1-2 months of age continue to express introduced genes nonmosaically. To determine



**Fig. 3.** Developmental profile of truncated FGF receptor protein translation in XFD RNA-injected and transgenic embryos. pCSKXFD, pCMVXFD or pCARXFD (right panels) or no DNA (left panel, top) were introduced into embryos using nuclear transplantation. In parallel, 1-2 ng of XFD RNA was injected into 2-cell-stage embryos. Protein samples were collected at stages shown. The endogenous FGF receptor (marked by an arrowhead) and XFD (\*) were detected by western blotting of single embryos with an FGF receptor antibody. Between four and six embryos were analyzed at each developmental stage for each sample type. Where some variability in levels of XFD protein per embryo was seen, more than one sample is shown to reflect the range of protein level detected.

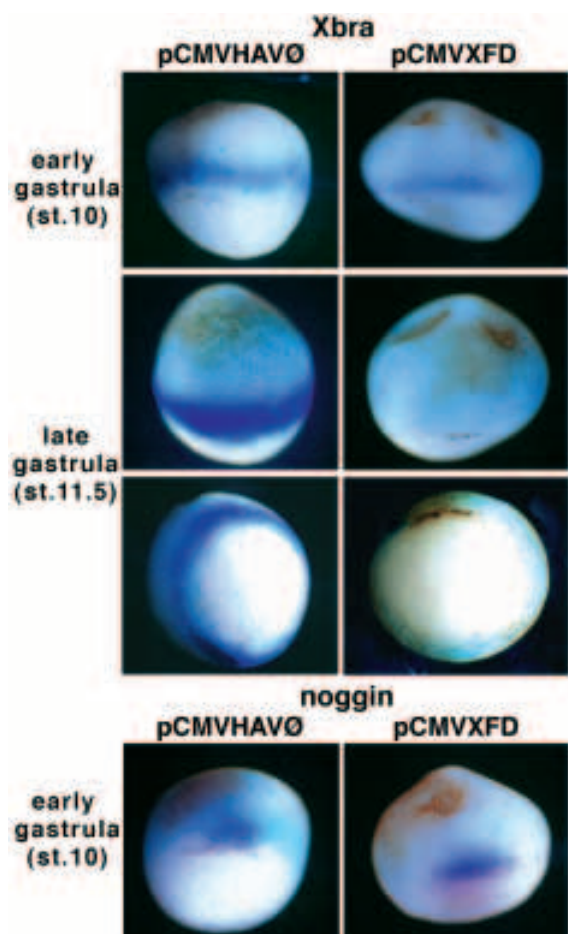
whether pCARGFP transgenic embryos contained integrated plasmid, Southern blots of genomic DNA from one month old tadpoles were probed with a 1 kb fragment (consisting of GFP sequences) from one end of the plasmid (Fig. 2A). Embryos were raised for a month before isolating genomic DNA since most unintegrated plasmid would be lost by this stage. After injection of DNAs into early embryos, little or no plasmid can still be detected in single month-old tadpoles on Southern blots (Kroll, 1994). Here probe sequences were found in transplantation-derived tadpoles that expressed pCARGFP but were absent from sibling tadpoles that did not express the plasmid (Fig. 2B). Fragments of 2.2 kb and 7.3 kb were present in all GFP-expressing tadpoles. These fragments represent back-to-back (2.2 kb) or tandem (7.3 kb) concatemers of the plasmid DNA (Fig. 2A). Additional fragments of 1.0 kb and 1.8 kb were also found in all GFP-expressing tadpoles. Because these fragments are common to all GFP-expressing tadpoles, they are likely to be products of concatemerization which have been modified by recombination within the embryo.

In addition to these four common fragments, each GFP-expressing tadpole contained several other fragments recognized by the probe, probably representing junction sites between plasmid ends and chromosomal sequences. We determined (see Materials and Methods) that transgenic tadpoles contained between 5 and 35 copies of pCARGFP per cell, integrated as single copy or short (2-6 copy) concatemers at 4-8 sites in the genome.

#### Transgenic and RNA-injected embryos show different developmental profiles of truncated FGF receptor protein translation

We compared the temporal profile of XFD protein translation in RNA-injected embryos to that of transgenic embryos expressing XFD from the cytoskeletal actin (pCSKXFD), cytomegalovirus (pCMVXFD), or muscle actin (pCARXFD) promoters. Single transgenic or RNA-injected embryos were harvested at several developmental stages and the levels of endogenous and truncated FGF receptor were detected on Western blots (Fig. 3) using an FGF receptor antibody (Amaya et al., 1993).

Embryos injected with XFD RNA already contained substantial levels of XFD protein by the mid-blastula stage (stage 8) (Fig. 3, lower left panel), relative to the endogenous FGF receptor level. The level of XFD protein in RNA-injected embryos remained fairly constant during gastrulation and neurulation, decreasing slightly by tailbud stages. By contrast with RNA-injected embryos, we did not detect XFD protein in any trans-



**Fig. 4.** Mesoderm induction and maintenance in transgenic embryos expressing XFD or HAVØ from the CMV promoter. Ubiquitous expression of mutant FGF receptors (light blue) and expression of Xbra and noggin (dark blue) was detected by in situ hybridization. Xbra expression at early gastrula (row 1) and late gastrula (rows 2 and 3) stages and noggin expression at the early gastrula stage (row 4) are shown. Rows 1, 2 and 4, dorsal view; row 3, vegetal view. Note: whole-mount in situ hybridization techniques in *Xenopus* fail to detect endodermal gene expression (Frank and Harland, 1992; Lemaire and Gurdon, 1994). Therefore, although transgenic embryos express genes non-mosaically, one cannot detect transgene expression in the endoderm.

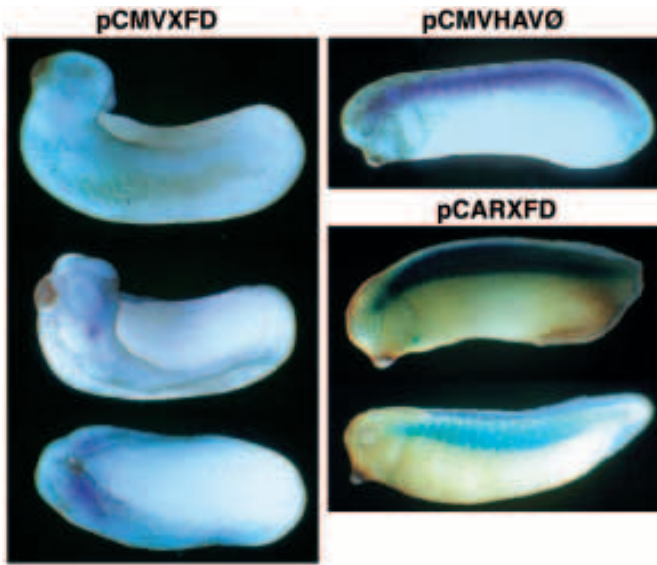
**Table 1.** Effect of restriction enzyme on frequency of plasmid expression in transgenic stage 35 tadpoles

	Enzyme†	Tadpoles	Tadpoles expressing CAT in >50% of somite cells (% of tadpoles scored)	Extent of CAT expression*			
				95-100%	50-95%	10-50%	1-10%
Exp. 1	+	118	65 (55)	43 (36)	22 (19)	0 (0)	1 (1)
	-	50	18 (36)	11 (22)	7 (14)	5 (10)	2 (4)
Exp. 2	+	74	38 (51)	26 (35)	12 (16)	2 (3)	4 (5)
	-	117	32 (27)	18 (15)	14 (12)	0 (0)	7 (6)

pRLCAR was introduced into embryos using nuclear transplantation and somite-specific expression of CAT was determined by immunocytochemistry.

†0.5 µl of a 1:5 dilution of Not I was added to each reaction mixture used for transplantations designated + and omitted for transplantations designated -.

\*Embryos expressing CAT were classified into four categories. The CAT-expressing region of each pRLCAR-expressing tadpole was scored as a percentage of the total somitic tissue in the embryo. The number of embryos in each category is shown. The percentage of the total number of tadpoles obtained in each set of transplantations (+ or - restriction enzyme) is shown in parentheses.



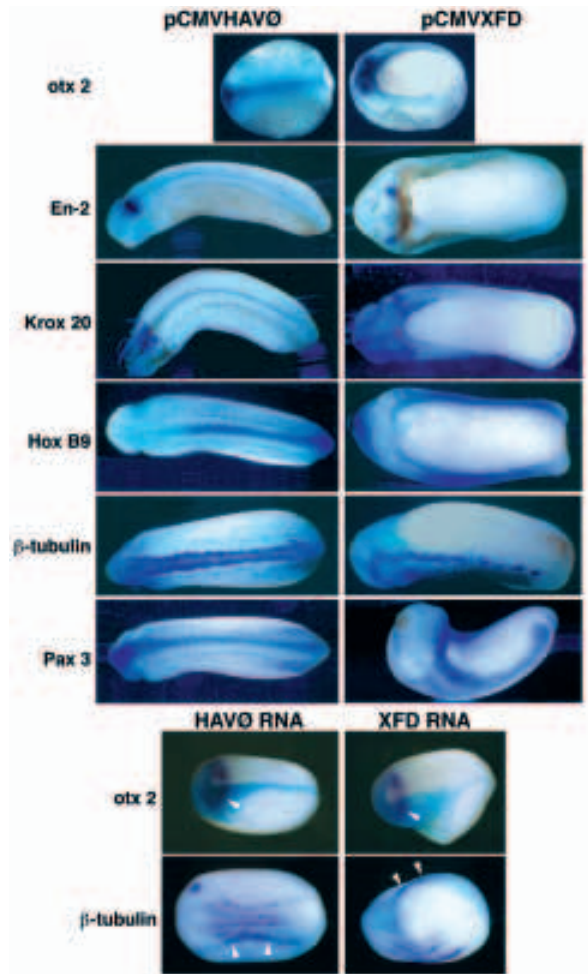
**Fig. 5.** Requirement of FGF signaling during gastrulation for axial mesoderm differentiation. Left and top right panels: transgenic embryos expressing XFD or HAVØ from the CMV promoter. Ubiquitous expression of mutant FGF receptors (light blue) and expression of collagen type II (purple) was detected by in situ hybridization. Right bottom panel: transgenic embryos expressing XFD from the cardiac actin promoter. In situ hybridization detects somite-specific expression of XFD in both embryos. The top embryo in the panel is also stained for collagen type II expression.

genic embryo at stage 8. Protein was first detected in transgenic embryos expressing pCMVXFD, pCSKXFD and pCARXFD at the early gastrula (stage 10), late gastrula (stage 12) and neurula (stage 17) stages, respectively, consistent with the expected timing of expression from these promoters. In stage 10 transgenic embryos expressing pCMVXFD, the level of truncated receptor protein was substantially in excess of the endogenous receptor protein. Therefore, this plasmid appeared most suitable for identifying requirements for FGF signaling during the gastrula stages.

**FGF receptor signaling is required for mesoderm maintenance and differentiation**

Embryos injected with XFD RNA fail to express several immediate early mesoderm markers, including *Xbra*, a gene normally expressed throughout the marginal zone, *Xpo*, a ventrally and laterally expressed gene, and *Xnot*, a dorsal-specific marker (Amaya et al., 1993; von Dassow et al., 1993). These embryos also exhibit a number of later defects in morphogenesis and differentiation. These defects may be a secondary consequence of the block to primary mesoderm induction, since mesodermal tissue plays an essential role in morphogenesis and neural induction. Alternatively, these defects may represent a continued requirement for FGF receptor signaling during gastrulation and postgastrula stages. Because embryos injected with XFD RNA express XFD protein even before primary mesoderm induction, it is not possible to use this approach to determine whether later patterning events require FGF signaling.

To address this issue, we restricted XFD expression to after the mid-blastula stage by placing it under the control of the CMV promoter. Nonfunctional FGF receptors (d50 or HAVØ)



**Fig. 6.** Neural patterning in transgenic embryos expressing the dominant negative FGF receptor. Upper panel: in situ hybridization was used to detect ubiquitous expression of mutant FGF receptors (light blue) and coexpression of neural markers (purple) in transgenic embryos expressing pCMVHAVØ or pCMVXFD. Neural marker expression patterns: *otx 2* (forebrain and midbrain); *En-2* (midbrain-hindbrain boundary); *Krox 20* (hindbrain rhombomeres 3 and 5); *Hox B9* (spinal cord); *Pax 3* (dorsal hindbrain and spinal cord) and N-tubulin (neurons). Lower panel: embryos co-injected with HAVØ or XFD RNA (1-2 ng) and with  $\beta$ -galactosidase RNA (0.2 ng) at the 2-cell stage were raised to neurulae.  $\beta$ -galactosidase activity (blue), detected using X-gal staining, marks injected regions. Expression of neural-specific  $\beta$ -tubulin or *otx 2* was detected by in situ hybridization (purple). Arrowheads denote regions of overlap (dark blue).

were also introduced into embryos as controls. At the early gastrula stage (stage 10), experimental and control embryos were indistinguishable. By stage 11, gastrulation movements in pCMVXFD transgenic embryos appeared to arrest (data not shown). When raised to later stages, pCMVXFD transgenic embryos developed morphological abnormalities similar to those seen in XFD RNA-injected embryos (Figs 5, 6) (Amaya et al., 1991). Like XFD RNA-injected embryos, pCMVXFD transgenic embryos had severe defects in the formation of trunk and tail structures, while head structures were fairly normal. However, the defects in the transgenic embryos were more severe and less variable than those typically seen in

RNA-injected embryos. While XFD RNA-injected embryos usually showed differing degrees of gastrulation defects and usually contained some axial tissue (Amaya et al., 1991, 1993), transgenic embryos did not show this variability. This difference is probably seen because transgenic embryos express XFD stably in all cells, whereas injected RNA partitions with time such that in later embryos protein expression is somewhat mosaic (Amaya et al., 1993).

To determine whether the morphological defects seen in pCMVXFD transgenic embryos were due to a failure of mesoderm induction versus maintenance or differentiation, we assayed expression of early and late mesodermal markers by whole-mount double *in situ* hybridization. In all cases, we identified the transgenic embryos by probing for expression of introduced FGF receptors (light blue stain), and then by probing for expression of mesodermal markers (dark blue or purple stain) (Figs 4, 5). Expression of the endogenous FGF receptor was not detected under the assay conditions used (see Experimental Procedures). In early gastrulae, we found that *Xbra* expression was induced in transgenic embryos expressing either XFD or control constructs (Fig. 4). By contrast, XFD RNA-injected embryos did not express *Xbra* at stage 10 (Amaya et al., 1993). *Noggin* expression was also unaffected in transgenic early gastrulae expressing either pCMVXFD or pCMVHAVØ. Expression of *noggin* and *Xbra* was expected since transgenic embryos at this stage were just beginning to translate XFD protein (Fig. 3). Nevertheless, this finding was significant since it showed that pCMVXFD transgenic embryos can be used to assay the requirements for FGF receptor signaling after mesoderm induction has occurred.

While mesoderm induction occurs in transgenic early gastrulae expressing XFD, mesoderm failed to be maintained in these embryos, as shown by the loss of *Xbra* expression by mid-gastrulation (Fig. 4). Control pCMVHAVØ transgenic embryos continued to express *Xbra* throughout gastrulation. When raised to tailbud stages most XFD-expressing transgenic embryos did not discernably stain for collagen type II, a marker of differentiated axial and paraxial mesoderm and floorplate (Fig. 5) (Amaya et al., 1993; Bieker and Yazdani-Buicky, 1992). This suggested that both notochord and somites were completely absent. In some embryos, small spots of staining were found, but these were always at the anterior end of the embryo in or adjacent to the head. This anterior mesoderm may be less sensitive to inhibition by the dominant negative FGF receptor during gastrulation.

We also examined whether FGF signaling continued to be required after gastrulation for the differentiation of the most abundant type of mesodermal derivative, namely muscle. To do this, we used the muscle-specific actin promoter to direct expression of XFD to predifferentiated myotomal tissue after the gastrula stage. We found that, whereas transgenic embryos that expressed XFD during gastrulation developed abnormally and lacked mesoderm, transgenic embryos expressing XFD from the muscle actin promoter developed normally. These embryos contained differentiated somites of apparently normal morphology and expressed XFD at high levels in the formed somites (Fig. 5). Somite tissue expressing XFD was recognized by both the 12/101 antibody (data not shown) (Kintner and Brockes, 1984) and by *in situ* hybridization for collagen type II (Fig. 5). Therefore, by both

morphological and molecular criteria, patterning and differentiation of muscle occurred in embryos expressing XFD after the gastrula stages. This result is not surprising as recent work has shown that FGF receptor signaling must be down-regulated for terminal differentiation of muscle (Itoh et al., 1996).

### Neural induction and patterning occurs in embryos lacking FGF receptor signaling

Perturbation of FGF signaling specifically during gastrulation in transgenic embryos dramatically affected mesoderm maintenance. Since neural tissue is induced by dorsal mesoderm during the gastrula stages (for review, see Hamburger, 1988), and FGF has recently been shown to induce and pattern neural tissue *in vitro* (for review, see Doniach, 1995), we wished to determine whether FGF receptor signaling in the gastrula is necessary for proper induction and patterning of the neural plate.

We produced transgenic embryos expressing XFD and then assayed the expression of neural markers by double *in situ* hybridization. Transgenic embryos expressing both dominant negative receptor and control plasmids showed robust expression of all neural markers tested (Fig. 6). Since transgenic and RNA-injected embryos expressing the dominant negative FGF receptor often have fairly normal heads, we were not surprised to find that anterior neural markers were expressed normally in these embryos. However, the finding that XFD-expressing embryos contained a complete range of neural pattern including the posterior neural markers *Hox B9*, *Pax 3* and N-tubulin was unexpected. In fact, staining for the dorsoposterior neural marker, *Pax 3*, was usually stronger in the experimental embryos than in the controls. All of these markers were expressed along the anterior-posterior extent of the trunk, although the XFD-expressing transgenic embryos had severe morphological defects and contained no axial mesoderm, as assayed by collagen type II expression.

Transgenic embryos do not accumulate detectable levels of XFD protein until the late blastula stage. To eliminate the possibility that FGF receptor signaling before the early gastrula stage is sufficient to induce and pattern the neural plate, we also injected embryos with XFD RNA at the 2- to 4-cell stage in the dorsal animal sector. By injecting RNA at these stages, XFD protein is expressed well before neural induction and patterning occurs in the embryo. Since XFD RNA injection results in a mosaic pattern of protein expression (Amaya et al., 1993), we used  $\beta$ -galactosidase RNA as a lineage reporter for the XFD RNA. We have shown previously that co-injection of XFD RNA and  $\beta$ -galactosidase RNA results in a close correlation between the translation of both proteins (Amaya et al. 1993). At the neurula stage, the embryos were fixed and stained for  $\beta$ -galactosidase activity to identify cells that received the injected RNA. Then the neural markers *otx 2*, N-tubulin (Fig. 6) and *Pax 3* (data not shown) were assayed by *in situ* hybridization. We commonly found that the injected region of the embryo overlapped with the region of expression of these neural markers. Thus injection of XFD RNA into presumptive neurectoderm cells did not prevent expression of *otx 2*, N-tubulin or *Pax 3*. We therefore conclude that induction and patterning of neural tissue in the *Xenopus* embryo does not require signaling through the FGF receptor.



## DISCUSSION

### The role of FGF in mesoderm maintenance

We have identified *in vivo* requirements for FGF receptor signaling in *Xenopus* that would not have been easily apparent from transient expression of injected RNA or DNA. Our findings show that continued signaling through the FGF receptor during gastrulation is essential for mesodermal patterning. We infer that maintenance of mesoderm in the gastrulating embryo is crucial for the formation of differentiated mesoderm like notochord and somites, and that primary mesoderm induction alone is not sufficient to maintain mesoderm patterning. These data are consistent with work showing that FGF is necessary to maintain mesodermal gene expression in dissociated gastrula stage marginal zone cells (Isaacs et al., 1994; Schulte-Merker and Smith, 1995).

Although there appears to be a requirement for continuous FGF signaling during gastrulation, it is not clear how the cellular response to FGF is mediated by downstream genes during gastrulation. The FGF-activated gene *Xbra* is essential for mesoderm maintenance and formation given that overexpression of *Xbra* causes ectopic mesoderm formation in embryos (Smith et al., 1991; Cunliffe and Smith, 1992), and that homozygous mutants for brachyury in mouse (Chesley, 1935) and zebrafish (Halpern et al., 1993) have defects in posterior mesoderm (loss of the tail and notochord). However, *Xbra* is unlikely to be the only target of FGF signaling since, at least in zebrafish, embryos expressing the dominant negative FGF receptor have more severe mesoderm defects (all structures posterior to the otic vesicle missing, including all somites) than do brachyury mutants, which typically contain 17-19 somites (Griffin et al., 1995).

The morphology of transgenic embryos expressing XFD closely resembles that of XFD RNA-injected embryos. However, in transgenic embryos, mesoderm induction occurs normally. This suggests that morphogenetic defects seen in these embryos directly result from blocking FGF signaling during gastrulation and do not occur as a secondary consequence of blocking mesoderm induction.

### FGF and neural induction

In contrast with its role in mesoderm maintenance during gastrulation, we found that signaling through the FGF receptor was not required *in vivo* for neural induction or patterning. Embryos expressing XFD contained normally patterned neural tissue along the entire anterior-posterior axis. Posterior neural tissue was present in these embryos even though the trunk region was highly morphologically abnormal and contained no muscle or notochord. This finding was particularly unexpected given that recent work (reviewed in Doniach, 1995) has shown that FGFs can induce and pattern neural tissue when added to gastrula ectoderm. Recently Launay et al. (1996), have also suggested that FGF receptor signaling is required for neural induction in *Xenopus*. In this paper, the authors show that expression of the dominant negative FGF receptor (XFD) in animal caps blocks neural induction by noggin and organizer tissue. This seems to be in contrast with our findings and we do not presently understand why the animal cap assay gives different results than experiments in the whole embryo. However, we note that Launay et al. (1996) have obtained results in embryos that are consistent with our interpretation.

Specifically, they find that injecting XFD RNA into early embryos does not significantly reduce later N-CAM staining (Fig. 2 of Launay et al., 1996), suggesting that FGF receptor signaling is not necessary for neural induction in the embryo. Experiments in other laboratories have also failed to uncover a requirement for FGF receptor signaling during neural induction or patterning in the intact embryo. For example, neurons expressing dominant negative FGF receptor protein are often found in embryos injected with XFD RNA at the 32-cell stage (T. Lamb and R. Harland, personal communication). In addition, experiments by Schulte-Merker and Smith (1995) show that animal caps expressing noggin and XFD RNA express NCAM, a neural marker.

Taken together these results suggest that, although FGF has the capacity to induce and pattern neural tissue in isolated ectoderm, signaling through the FGF receptor does not appear to be required for proper neural induction and patterning in the intact embryo. However, several other explanations are possible. First, the FGF pathway may be activated during normal neural induction but could be compensated by another pathway in embryos expressing XFD. Second, neural induction or patterning in the embryo may be mediated by an endogenous receptor that couples to the same intracellular signaling pathway as the FGF receptor. For example, members of the nerve growth factor family of neurotrophins, which are important for nervous system development in mammals, signal through Trk receptor tyrosine kinases and, like FGF, can activate the Ras/Raf/MAP kinase signaling pathway (Barbacid, 1995 and references therein). It is thus possible that FGF stimulates neural induction and patterning in animal cap tissue by mimicking the *in vivo* neural inducer through activation of tyrosine kinase pathways. Finally, the truncated FGF receptor used for our experiments (a mutation of FGF receptor-1) is capable of blocking mesoderm induction in animal cap tissue by all FGF receptor ligands that have been tested including FGF-2 (Amaya et al., 1991), eFGF (Isaacs et al., 1994) and most recently FRL-1 and FRL-2 (Kinoshita et al., 1995). However, the FGF pathway that induces neural tissue in the embryo may utilize an as of yet unidentified FGF ligand and/or receptor combination which is not sensitive to blocking by the dominant negative FGF receptor that we have used. For example, several new members of the FGF receptor family recently identified by Kengaku and Okamoto [1995] could potentially mediate the neural-inducing signal.

The fact that XFD transgenic embryos contain a nervous system that is well-patterned is also somewhat unexpected since, by mid-gastrula stages, these embryos lack the axial and paraxial mesoderm which would normally induce neural tissue. This may indicate that the transient presence of dorsal mesoderm at the early gastrula stage is fully sufficient to form a completely patterned nervous system and that shortly thereafter the dorsal mesoderm becomes dispensable for neural induction. Alternatively, neural-inducing capability may be present in other dorsal marginal zone tissues which are not affected by expression of the dominant negative receptor and which are not reflected by loss of *Xbra* expression in our experiments. For example, the anterior mesoderm that expresses *gooseoid* and *noggin* at stage 10 (Vodicka and Gerhart, 1995) is fairly insensitive to expression of the dominant negative FGF receptor (Amaya et al., 1993). Additionally, the endodermal epithelium in the dorsal marginal zone region has been shown to have organizer proper-

ties, including the ability to organize a secondary axis after transplantation to the ventral side of the embryo (Shih and Keller, 1992). Because this tissue is prospective endoderm rather than mesoderm, its maintenance may be unaffected by the failure of FGF signaling during gastrulation and it may be capable of inducing a normal nervous system in our embryos. The only discernable alteration of neural patterning seen in XFD transgenic embryos is an apparent dorsalization of the neural tube, as suggested by up-regulation of the dorsal neural tube marker, *Pax 3*, and absence of the floorplate marker, collagen type II. We do not know whether this effect suggests a direct role for FGF signaling in dorsal-ventral patterning of the neural tube or whether the apparent dorsalization is due to the absence of a notochord in these embryos (Clarke et al., 1991).

### Transgenesis in amphibians

The amphibian transgenesis procedure that we have described provides a new and versatile tool for studying embryonic patterning. While we have applied this method to dissect the FGF signaling pathway, this approach should be of general utility for analyzing specific postblastula functions of any gene. We can now introduce ectopic or dominant negative activities either into every cell of an embryo or, by using tissue- or temporally specific promoters, into a defined subset of cells. We can also transplant transgenic tissues into non-transgenic embryos to produce, in principle, any specific combination of wild-type and genetically modified cells. We may therefore be able to use the accessibility and ease of manipulation of frog embryos to pinpoint the molecular basis of early developmental decisions in a manner not currently possible in other vertebrates. Tissue-specific promoters are correctly regulated in transgenic embryos, even in cases where these promoters are expressed both ectopically and mosaically after plasmid injection. This specificity should allow reliable mapping of the upstream regions of frog promoters and also promoters from mouse and other vertebrates. Some mouse, zebrafish and frog promoters are regulated appropriately in other vertebrates (Brakenhoff et al., 1991; Morasso et al., 1995).

The amphibian transgenesis procedure described here compares favorably to transgenesis procedures used in other vertebrates. Large numbers of transgenic embryos can be produced inexpensively within a day and, since the embryos are not chimeric, breeding of animals is not required to obtain non-mosaic, ubiquitous expression. We are currently combining this transgenesis approach with ribozyme and antisense technology to allow functional knockout of genes in cases where dominant negative mutations of gene products have not yet been identified. We have additionally found that sperm nuclear transplantation into oocytes can be used to simply produce normal embryos from oocytes handled *in vitro* (Amaya and Kroll, 1996). The combination of this approach with antisense oligonucleotide injections (Heasman et al., 1991) should also facilitate functional 'knockouts' of maternal genes in frog embryos.

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