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Targeted expression of the dominant-negative FGFR4a in the eye using *Xrx1A* regulatory sequences interferes with normal retinal development

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SUMMARY

Molecular analysis of vertebrate eye development has been hampered by the availability of sequences that can selectively direct gene expression in the developing eye. We report the characterization of the regulatory sequences of the *Xenopus laevis Rx1A* gene that can direct gene expression in the retinal progenitor cells. We have used these sequences to investigate the role of Fibroblast Growth Factor (FGF) signaling in the development of retinal cell types. FGFs are signaling molecules that are crucial for correct patterning of the embryo and that play important roles in the development of several embryonic tissues. FGFs and their receptors are expressed in the developing retina, and FGF receptor-mediated signaling has been implicated to have a role in the specification and survival of retinal cell types. We investigated the role of FGF signaling mediated by FGF receptor 4a in the development of retinal cell types in *Xenopus laevis*. For this purpose, we have made transgenic *Xenopus* tadpoles in which the dominant-negative FGFR4a (ΔFGFR4a) coding region was linked to the newly characterized regulatory sequences of the *Xrx1A* gene. We found that the expression of ΔFGFR4a in retinal progenitor cells results in abnormal retinal development. The retinas of transgenic animals expressing ΔFGFR4a show disorganized cell layering and specifically lack photoreceptor cells. These experiments show that FGFR4a-mediated FGF signaling is necessary for the correct specification of retinal cell types. Furthermore, they demonstrate that constructs using *Xrx1A* regulatory sequences are excellent tools with which to study the developmental processes involved in retinal formation.

Key words: FGF receptor, Photoreceptors, Retina, Transgenic, *Xenopus*, *Xrx1A*

INTRODUCTION

Rx is a paired-like homeobox gene that has been identified in several vertebrate species, including *Xenopus*, mouse, chicken, medaka, zebrafish and human (Casarosa et al., 1997; Furukawa et al., 1997a; Loosli et al., 2001; Mathers et al., 1997; Ohuchi et al., 1999). It is first expressed in the anterior neural region of developing embryo, in cells that are destined to become eyes and ventral hypothalamus (Casarosa et al., 1997; Furukawa et al., 1997; Mathers et al., 1997; Mathers and Jamrich, 2000). *Rx* has a crucial function in eye development: its overexpression in *Xenopus* leads to overproliferation of the neuroretina, and targeted elimination in mice results in a lack of eye formation (Mathers et al., 1997; Zhang et al., 2000). Furthermore, a naturally occurring mouse mutation *eyeless* (*ey1*) displays severe eye abnormalities as a result of a mutation in the coding region of the murine *Rx* (*Rax* – Mouse Genome Informatics) gene (Tucker et al., 2001), and the temperature-sensitive mutation *eyeless* in medaka is caused by an insertion in the *Rx3* gene (Loosli et al., 2001).

As *Rx* shows specific expression in the retinal progenitor cells, its regulatory sequences would be uniquely suited to direct gene expression in the developing retina. These sequences could be used to specifically alter gene expression in the developing eye. In this study, we used a transgenic approach in *Xenopus laevis* to identify the regulatory sequences of the *Xrx1A* gene. Having identified these sequences, we used them to demonstrate the crucial role of FGF signaling in the correct specification of retinal cell types.

Fibroblast Growth Factors (FGFs) are a family of signaling molecules that are expressed in a wide range of tissues in partially overlapping patterns. FGFs have been implicated to

have roles in the formation of mesoderm and neuroectoderm. Their exact role during embryogenesis is not fully understood, partially because of several contradictory findings.

In *Xenopus* there are at least five FGF receptors (Friesel and Brown, 1992; Gillespie et al., 1989; Golub et al., 2000; Hongo et al., 1999; Shiozaki et al., 1995) and it would not be surprising if additional members of this gene family are discovered in the future. The dominant-negative forms of these receptors have proved to be efficient inhibitors of FGF signaling pathways and have been used as the primary means to demonstrate the dependence of biological processes on FGF signaling. In several studies the dominant-negative FGF receptor 1 (XFD, ΔFGFR1) was used to block FGF receptor 1 (FGFR1)-mediated FGF signaling. From injections of mRNA encoding ΔFGFR1 into *Xenopus* embryos, it was demonstrated that FGFs are involved in mesodermal induction (Amaya et al., 1991; Amaya et al., 1993; Cornell and Kimelman, 1994; LaBonne and Whitman, 1994). This conclusion was supported by transgenic expression of this dominant-negative mutant receptor in *Xenopus* embryos (Kroll and Amaya, 1996). The effects of FGF signaling on neural induction are less clear. FGFs can induce neural differentiation in dissociated ectodermal cells (Kengaku and Okamoto, 1993), and expression of neural markers in ectodermal explants (Barnett et al., 1998; Launay et al., 1996; Sasai et al., 1996). However, no significant effects on neural induction were observed when ΔFGFR1 was expressed in transgenic *Xenopus* embryos (Kroll and Amaya, 1996). More recently a dominant-negative FGF receptor 4a (ΔFGFR4a) has been used to investigate the role of FGF signaling in embryogenesis. This dominant-negative receptor is a better inhibitor of FGF signaling than ΔFGFR1 (XFD) and inhibition of FGFR4a results in different effects on embryogenesis than does the inhibition of the FGFR1 mediated pathway (Hongo et al., 1999). In contrast to the overexpression of ΔFGFR1, the overexpression of ΔFGFR4a has been shown to impede neural induction in *Xenopus* embryos, demonstrating that individual FGF signaling pathways have distinct roles in the formation of different tissues (Hardcastle et al., 2000; Hongo et al., 1999). These studies highlighted the acute need to evaluate each FGF signaling pathway separately, because pathways mediated by different receptors might have different effects on embryologic events. In this paper, we analyze the effects of the elimination of FGF signaling through FGFR4a on the development of retinal cell types.

During eye development, the initially undifferentiated, seemingly homogeneous, retinal progenitor cells develop into a layered array of seven cell types with different capabilities. These include the light sensitive photoreceptor cells, the bipolar interneurons that transmit electrical stimulus from the photoreceptor to the ganglion cells, and the ganglion cells that transmit the information from the eye to the brain. The formation of these cells types, and their correct proportionality, is necessary for the proper function of the vertebrate eye. FGF molecules and their receptors are expressed in developing retina (de Iongh and McAvoy, 1993; Gao and Hollyfield, 1995; McFarlane et al., 1998; Patstone et al., 1993), and several studies have investigated their role in proliferation, survival and differentiation of retinal cells (Guillemot and Cepko, 1992; Park and Hollenberg, 1989; Sievers et al., 1987). It was shown that inhibition of the FGFR1-mediated pathway in *Xenopus* embryos resulted in a 50% loss of photoreceptor and amacrine cells, with a concomitant increase of Müller cells (McFarlane et al., 1998). The role of FGF signaling through receptors FGFR1 and FGFR2 in the survival of murine photoreceptor cells has also been investigated (Campochiaro et al., 1996). These authors demonstrated that inhibition of FGF signaling using ΔFGFR1 and ΔFGFR2 resulted in a progressive death of photoreceptor cells. The role of FGFR4a-mediated FGF signaling on retinal development has not yet been examined. In this study, we have used the regulatory sequences of the *Xrx1A* gene to direct ΔFGFR4a expression in retinal progenitor cells of transgenic *Xenopus* embryos. This approach has a significant advantage over the injection of dominant-negative receptor mRNA into *Xenopus* embryos, as it does not interfere with neural induction. This allows us to monitor the role of FGFR4a-mediated signaling on the development of retinal cells after the retinal progenitor cells were formed. Using transient transgenic lines of *Xenopus laevis*, we have found that the specification of retinal cells, as well as eye development on the whole, is severely affected in transgenic embryos expressing the ΔFGFR4a construct. These embryos have abnormal retinal layering and the population of photoreceptor cells is either absent or significantly reduced. At the same time the percentage of Müller glial cells is significantly increased.

MATERIALS AND METHODS

Transgenesis

Transgenic *Xenopus laevis* embryos were generated by restriction enzyme-mediated integration (REMI) or by intra-cytoplasmic sperm injection (ICSI) with similar results. The transgene DNA was either linearized or released from the vector by restriction digestion, and purified from agarose gel using the QIAEX II kit (Qiagen) or the Bio 101 Geneclean Spin kit (Qbiogene). REMI was performed as described (Kroll and Amaya, 1996; Amaya and Kroll, 1999), except that the amount of restriction enzyme used (*Not*I) was reduced to 0.15 units and 2 μl of egg extract was used for each transgenesis reaction. Sperm nuclei were permeabilized using lysolecithin, and later using digitonin. The protocol for ICSI was performed as described by Sparrow et al. (Sparrow et al., 2000), with minor modifications, using snap frozen sperm nuclei. 400,000 sperm nuclei (in 4 μl) were incubated with 250-500 ng transgene DNA (in 2.5 μl water) at room temperature for 15 minutes. The reaction mixture was diluted with 22.5 μl sperm dilution buffer (SDB), then 2.5 μl of the diluted mixture was transferred to 230 μl SDB for injection. Eggs were injected in 0.4×MMR (Marc's Modified Ringer's) containing 6% (w/v) Ficoll. Properly gastrulating embryos were raised in 0.1×MMR until approximately stage 42 and then transferred to dechlorinated tap water. Tadpoles were anesthetized in 0.01% 3-aminobenzoic acid ethyl ester (Sigma) and monitored for GFP expression using a fluorescent stereoscope. Developmental stages of embryos were determined according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967).

Constructs for transgenesis

GFP reporter plasmids pCS2mt+SGP and pCS2mt+UGP were kindly provided by Dr Mike Klymkowsky (Rubenstein et al., 1997). The plasmid phs3LSN, containing a minimal heat shock protein gene promoter, was a gift from Dr Janet Rossant. A *Hin*dIII-*Asp*718 fragment containing GFP from pCS2mt+SGP was subcloned into pBluescriptIIKS to generate pBS-GFP for further transgene construction. A detailed description of the *Xrx1A* deletion constructs is in the legend of Fig. 3. *ΔFgfr4a* was prepared by digestion of *Fgfr4a* cDNA with *Bgl*II and self ligation, removing the 216 amino acid

kinase domain (Golub et al., 2000). To prepare *Xrx1A-ΔFgfr4a*, the *ΔFgfr4a* coding region was amplified from *ΔFgfr4a* cDNA by PCR (forward: 5′-CCCATGATCACATGTCTGGATCCATAAG-3′; and reverse: 5′-GATCATCGATAAGTCCCAAGTTCACTGTG-3′), digested with *Bcl*II and *Cla*I, and ligated to the *Bam*HI and *Cla*I sites of the GFP construct, pCS2mt-UGP (Rubenstein et al., 1997). The *ΔFgfr4a-GFP* fusion was then subcloned into the *Hin*dIII and *Not*I sites of construct 1 (replacing the GFP cassette) in two sequential steps (as *Hin*dIII-NotI, and *Hin*dIII fragments). DNA was prepared for transgenesis by digestion with *Sac*I, or *Sac*I and *Not*I. In *Xrx1A* regulatory sequence analysis experiments, negative expression results with transgene constructs 6, 8, 9, 10 and 11 were confirmed by PCR using the GFP-specific primer 5′-GAATTGGGACAACTCCAGTG-3′, and the *Xrx1A* regulatory element specific primers 5′-GAAC-GACACAAAGGACACAG-3′ (239-220) or 5′-GTACAAAGGTA-GAGAAGCAG-3′ (666-647).

In experiments involving transgenesis using ΔFGFR4a, a transgene DNA for GFP driven by cardiac actin promoter (*Car-GFP*) was included in the transgenesis reaction to aid in the identification of transgenic embryos. The definitive genotype of embryos was confirmed by PCR using the GFP-specific primer 5′-GAATT-GGGACAACTCCAGTG-3′ and the FGFR4a primer 5′-CAGTT-GGCTTCATCTTCGGATAAC-3′. All of these studies were performed using transient transgenic animals. Each construct was injected at least three times, and constructs labeled as positive generated GFP-positive tadpoles in every trial.

The sequence of the 3.4 kb *Sst* I-*Pst*I fragment that was used for the deletion analysis of the *Xrx1A* regulatory sequences can be found at GenBank Accession Number AY250711.

In situ hybridization and immunostaining

Whole-mount in situ hybridization was performed as described by Smith and Harland (Smith and Harland, 1991). Staining of paraffin sections, frozen sections and whole embryos with antibodies was performed as previously described (El-Hodiri et al., 1997). Primary and secondary antibodies were used at the following dilutions: mouse anti-rhodopsin (Adamus et al., 1991), 1:100; rabbit anti-calbindin (Swant), 1:400; rabbit anti-Islet-1, 1:100; mouse anti-glutamine synthetase (Chemicon), 1:100; HRP-conjugated goat anti-mouse IgG (Sigma), 1:100; Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch), 1:200; and Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch), 1:200. Nuclei were counterstained with either 5 μg/ml Hoechst 33258 (Sigma) or 2 μM Topro-3 (Molecular Probes). Stained sections were examined and images recorded using either a Zeiss 510 LSM confocal microscope, or a Leica MPS52 fluorescent microscope and Diagnostic Instruments digital camera. For the analysis of the percentage of specific retinal cell types, the glutamine synthetase-positive and Islet1-positive cells were counted in transgenic embryos at stage 42 on 12 μm serial sections across the retina.

TUNEL staining

Apoptotic cells were detected by whole-mount TUNEL staining following a previously described protocol (Hensey and Gautier, 1997) with some modifications. The embryos were rehydrated in PBS/0.5% Tween-20 (2×20 minutes), then washed in PBS and terminal deoxynucleotidyl transferase (TdT) buffer. The labeling reaction was carried out in TdT buffer containing 0.5 μM digoxigenin-11-dUTP (Roche) and 150 U/ml TdT (Invitrogen). After termination of the reaction and PBS washes, the embryos were washed in PBS/0.1% TritonX-100/0.2% BSA (PBT×B buffer) and blocked in PBT×B buffer with 20% goat serum. The embryos were then incubated with 1:2000 anti-digoxigenin antibody coupled to alkaline phosphatase (Roche), and then washed in PBT×B buffer (4×30 minutes). The stain was developed in alkaline phosphatase buffer using nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrates. The stained embryos were dehydrated in ethanol, counterstained with Eosin, embedded in paraffin wax and 10 μm sections cut. Sections were de-waxed in xylene and mounted with Permount (Fisher). Stained cells were counted in every second section across the entire retina.

RESULTS

Identification and characterization of *Xrx1A* regulatory sequences

In order to identify *Rx* regulatory sequences, we initially isolated an 11 kb clone from a *Xenopus* genomic library by screening it with an *Xrx1A* cDNA probe (Mathers et al., 1997). A 3.4 kb *Sst*I-*Pst*I fragment of this clone contained part of the 5′UTR of the cDNA clone and an additional 3.2 kb of upstream DNA. This fragment was cloned upstream of a Green Fluorescent Protein (GFP) reporter gene (Fig. 3; construct 1) and used to produce transgenic *Xenopus laevis* embryos. Analysis of these embryos showed that they expressed GFP in the anterior neural plate of neurula embryos, and later in the eyes, hypothalamus and ventral forebrain of tailbud embryos (Fig. 1B,E,H,K). The GFP expression pattern in these embryos was practically identical to the endogenous *Rx* expression

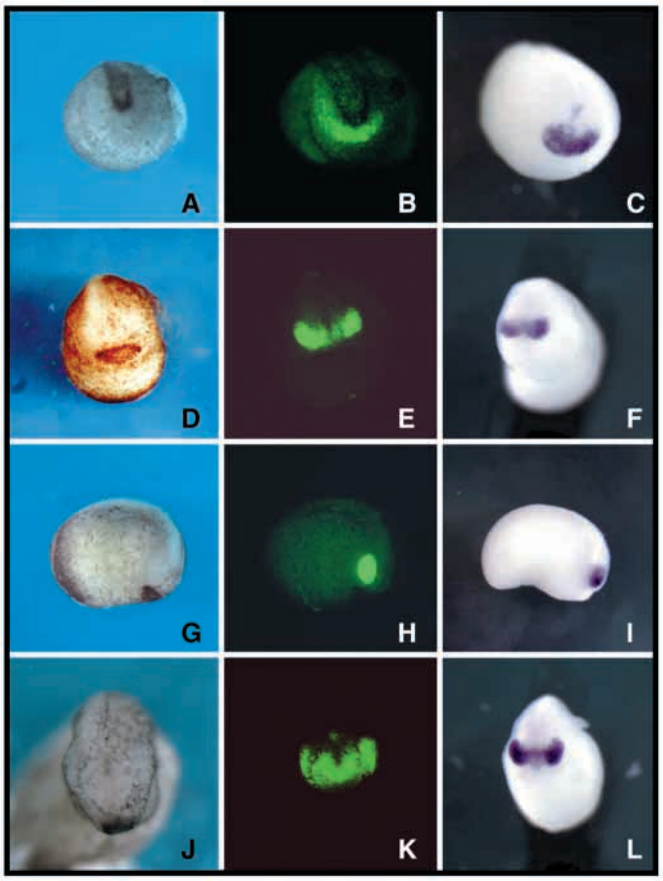

Fig. 1. Transgenic *Xenopus laevis* embryos at different stages carrying *Xrx1A-GFP* construct 1 (A,D,G,J; see Fig. 3), displaying GFP fluorescence (B,E,H,K). (C,F,I,L) In situ hybridization of *Xrx1A* probe to non-transgenic embryos of the same developmental stage to demonstrate the normal expression pattern of the *Xrx1A* gene. A-C, stage 15; D-F, stage 21 (frontal view); G-I, stage 21 (side view); and J-L, stage 28.

pattern as detected by whole-mount in situ hybridization (Fig. 1C,F,I,L). A comparison of the expression pattern of GFP with that of endogenous *Rx* suggests that this construct contained most, if not all, of the *Rx* regulatory sequences necessary to direct gene expression in the retinal progenitor cells and the optic cup. One female frog carrying this construct was raised to sexual maturity. Approximately 50% of the offspring of this frog expressed GFP in the eyes and pineal gland, demonstrating that this transgene could be genetically transmitted.

At later stages, expression of GFP regulated by the 3.4 kb *Sst*I-*Pst*I fragment was extinguished in most of the retinal cell types with the notable exception of the photoreceptor cells (Fig. 2). We used antibodies against rhodopsin, a marker of rods (Adamus et al., 1991), and calbindin, a marker of cones, (Chang and Harris, 1998) to determine whether GFP expression was limited to a certain subpopulation of photoreceptor cells. As shown in Fig. 2B-E, rhodopsin-positive cells displayed GFP fluorescence, showing that these *Rx* regulatory sequences can direct gene expression in the rods. However, not all of the GFP-positive cells expressed rhodopsin, suggesting that cones might also express GFP. Indeed, staining with calbindin antibodies demonstrated that cones also express GFP (Fig. 2F-I). This shows convincingly that all of the photoreceptor cells, cones and rods alike, expressed GFP under the regulation of *Xrx1A* regulatory region.

The *Xrx1A* regulatory regions were further characterized by 5′-deletion analysis. Expression of GFP in the anterior neural plate of stage 20 embryos was unaffected by deletion up to –2726 base pairs (bp; Fig. 3, construct 2), but was weakened by deletion up to –1595 bp (construct 3) and extinguished by deletion up to –1304 bp (construct 4), which suggests that cis-acting elements necessary for the initiation of expression in the anterior neural plate reside between –1304 and –2726 bp.

Although deletion to –1304 bp abolished the early GFP expression that takes place during neurulation, expression of GFP was still present in tadpole eyes, suggesting the presence of a second regulatory region, which controls the late expression of the *Rx* gene. This late expression initiated around stage 35 and was primarily limited to the photoreceptor cells. We made several deletion constructs to identify the sequences responsible for this late onset of GFP activity. We found that deletion to –982 bp (construct 5) had no effect on expression of GFP in the photoreceptor cells. However, a deletion to –606 bp (construct 6) eliminated all activity of the *Xrx1A* enhancer, which suggests that the –982 to –606 bp region contained cis-acting elements required for the photoreceptor activity of the *Xrx1A* promoter.

To test this hypothesis further, we fused the –982 to –606 bp region to a minimal heat shock protein promoter (hsp; construct 7) and produced transgenic animals. This construct was active in photoreceptor cells confirming that the –982 to –606 bp region was sufficient for the activity of the *Xrx1A* promoter in photoreceptor cells. Comparison of expression data from construct 6 with that from construct 7 also indicated that the region from 0 to –606 bp contained the *Xrx1A* core promoter sequences. This was confirmed by the finding that the –982 to –606 bp region was not able to direct GFP expression to the photoreceptor cells in the absence of the heat shock protein promoter (construct 8). The heat shock promoter alone was also inactive when placed in front of the GFP coding region (construct 9).

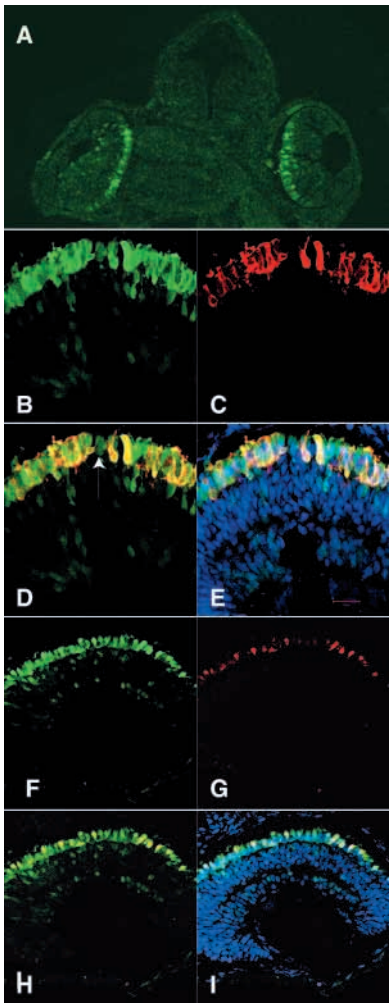

Fig. 2. (A) Section of a stage 40 transgenic *Xenopus laevis* embryo carrying *Xrx1A-GFP* construct 1 (see Fig. 3) and displaying GFP fluorescence in photoreceptor cells. (B) High magnification of a section through an eye of a stage 40 transgenic tadpole shows fluorescence in the photoreceptor layer. (C) The same section stained using antibodies against rhodopsin. (D) Overlap of B and C, demonstrating that the rhodopsin-positive rods (yellow cells) express GFP. However, some rhodopsin-negative cells also express GFP (arrowhead). (E) Additional staining with Topro-3 visualizes other retinal cells. (F) High magnification of a section through an eye of stage 40 transgenic tadpole displays fluorescence in the photoreceptor layer. (G) Staining of the same section with antibodies against calbindin, a marker of cone cells. (H) Overlap of F and G, demonstrating expression of GFP in cone cells (yellow cells). (I) Additional staining of the same section with Topro-3 visualizing other retinal cells.

Further deletions in this crucial region resulted in abolishment of GFP expression. For example, a deletion to –857 bp (construct 10) established that the region from –982 to –857 bp is essential for gene activity. However, this segment alone was not sufficient to activate GFP expression. When this short fragment of DNA was placed in front of the hsp promoter (construct 11), it did not activate the *Xrx1A* promoter. However,

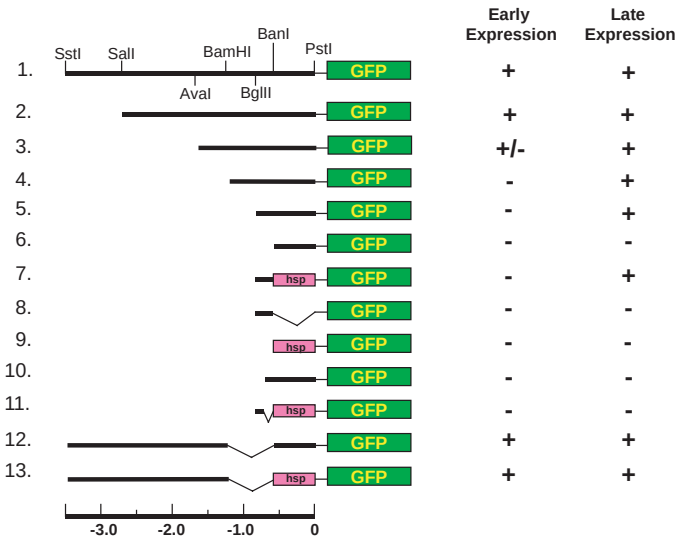

Fig. 3. Schematic diagram of the constructs used to make transgenic *Xenopus* tadpoles for the characterization of *Xrx1A* regulatory sequences. Transgene construct 1 was made by cloning the *Xrx1A* regulatory sequences (*Sst*I-*Pst*I fragment) in front of *GFP* in pBS-*GFP*. The full-length promoter construct 1 was digested with *Not*I and *Sal*I, *Ava*I, *Bam*HI, *Bgl*II, and *Ban*I, respectively, to release the transgene constructs 2, 3, 4, 5 and 6. Transgene construct 9 was obtained by inserting a heat shock protein promoter (hsp) into the *Sma*I site of pBS-*GFP*. The *Bgl*II-*Ban*I fragment from the *Xrx1A* promoter was subcloned into the *Eco*RV site of construct 9 and pBS-*GFP* to generate transgene constructs 7 and 8, respectively. To make transgene constructs 12 and 13, the *Sst*I-*Bam*HI fragment from the *Xrx1A* promoter was subcloned into pBS-*GFP* first, then the hsp promoter (blunt-ended *Hin*dIII-*Nco*I fragment of phs3LSN) or the *Ban*I-*Pst*I fragment from the *Xrx1A* promoter were inserted into the *Eco*RV site between the *Xrx1A* early enhancer and *GFP*. Construct 10, containing nucleotides (nt) –857 to 0 of the *Xrx1A* regulatory sequence, was prepared by PCR of construct 1, with a GFP-specific primer (see below) and the *Xrx1A* promoter specific primer: 5′-GATCGGATCCCTTCCAGCAATCATATCCTA-3′ (–857 to –838). The resulting product was digested with *Pst*I (3′-end of the *Xrx1A* regulatory region) and *Bam*HI (included in the *Xrx1A*-specific primer) and subcloned into pBS-*GFP*. Construct 11, including nt –986 to –838 of the *Xrx1A* regulatory region was prepared by PCR of construct 1 using the following primers: 5′-GATCAGATCTTAGGATATGATTGCTGGAAG-3′ (the complement of the previous primer encompassing nt –857 to –838, but with a *Bgl*II site at the end); and 5′-GATCGGATCCGATCTGTTATCTGGAAAACCCC-3′ (nt –986 to–965 of the *Xrx1A* regulatory sequence and a *Bam*HI site). The PCR product was digested with *Bam*HI and *Bgl*II and subcloned into the *Bam*HI site of construct 9.

the –982 to –606 bp region crucial for the late activation of the *Xrx1A* promoter is not necessary for the early activation of the *Xrx1A* promoter. We have deleted the –1304 to –606 bp sequence from construct 1 (to create construct 12) and found that GFP expression was activated during neurulation, as it was with construct 1. This was the case regardless of whether the putative *Xrx1A* core promoter sequences were used (construct 12) or whether they were replaced by the hsp promoter (construct 13). Interestingly, transgenic animals carrying constructs 12 and 13 displayed GFP fluorescence in their photoreceptor cells, suggesting that the *Sst*I-*Bam*HI fragment contains regulatory elements that can direct or stabilize gene expression in the photoreceptor cells. Therefore it appears that the *Sst*I-*Pst*I fragment contains two independent regulatory sequences that can direct gene expression to the photoreceptor cells.

Effect of FGF signaling on specification of retinal cell types

To investigate the role of FGFR4-mediated signaling in retinal development, we used the newly characterized *Xrx1A* regulatory sequences (a 3.4 kb *Sst*I-*Pst*I fragment from construct 1; Fig. 3) to drive expression of a dominant-negative FGF receptor 4a (Fig. 4A) in the developing retina of *Xenopus*. Initially, a construct was made that linked the coding region of the dominant-negative receptor construct in frame to the coding region of GFP. Unfortunately, this fusion protein failed to fluoresce when exposed to UV light and therefore could not be used to detect transgenic embryos. Based on the suggestion of Bronchain et al. (Bronchain et al., 1999), we inserted a stretch of glycine residues upstream of the GFP-coding region. However, as this did not alleviate the problem, we co-injected *ΔFgfr4a-GFP* with a cardiac actin promoter-*GFP* (*Car-GFP*) construct, which drives expression in the skeletal and heart muscle where *Xrx1A* is never expressed. This allowed us to easily identify transgenic embryos. Definitive genotyping of these embryos was accomplished by PCR. More than 75% of embryos carrying the *Car-GFP* construct were also transgenic for the *ΔFgfr4a-GFP* construct.

A superficial examination of these transgenic animals showed a grossly normal phenotype (Fig. 4B), with eyes that

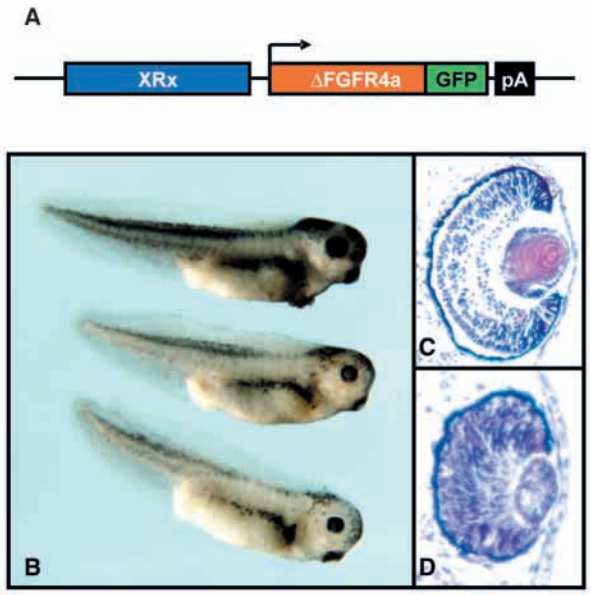

Fig. 4. (A) A schematic diagram of the *Xrx1A-ΔFGFR4a* construct used to make transgenic tadpoles. (B) Comparison of stage 39 transgenic *Xenopus* tadpoles carrying the *Xrx1A*-ΔFGFR4a construct (lower two tadpoles) with the sibling that does not carry this construct (upper tadpole). (C) Hematoxylin and Eosin (H&E)-stained section of an eye from a stage 39 *Xenopus* tadpoles that do not carry the *Xrx1A-ΔFGFR4a* construct. (D) H&E-stained section of an eye from a stage 39 transgenic tadpole carrying the *Xrx1A-ΔFGFR4a* construct, demonstrating disturbed retinal layering.

were slightly smaller than normal. Their retinas contained about 20% fewer cells than the wild-type retinas. However, a more detailed histological analysis of the eyes of these transgenic tadpoles revealed significant changes in retinal development. Most importantly, the transgenic eyes did not display normal retinal layering and some cell types appeared to be missing (Fig. 4C,D). Based on morphology only, we were not able to observe any photoreceptor cells in most of these embryos. To confirm this observation, we used antibodies against rhodopsin to evaluate these transgenic embryos for the presence of photoreceptor cells. Although these antibodies detected photoreceptor cells in transgenic embryos carrying the *Car-GFP* construct (Fig. 5A), they failed to recognize any photoreceptor cells in 59% of the embryos carrying the *ΔFGFR4a-GFP* construct (10/17; Fig. 5B). In a smaller percentage of embryos (41%, 7/17) some photoreceptor cells were present, but these cells were frequently in abnormal locations (Fig. 5C).

This deficiency of photoreceptor cells could be caused either by a failure of photoreceptor formation or by a failure of photoreceptor precursor survival. To distinguish between these two possibilities, we compared rod development in embryos expressing the ΔFGFR4a-GFP construct with rod development in embryos not expressing this construct. As seen in Fig. 5D-G, there is a steady increase in the number of rod cells in wild-type embryos from stage 33 to stage 38. As mentioned before, there were no rods present at any stage in most of the transgenic embryos expressing ΔFGFR4a. However, in embryos that had some rod cells, the number of rods was significantly reduced at all stages and never reached levels comparable to wild type (Fig. 5H-M). Even two days later, at stage 45, the difference between wild-type and transgenic animals remained dramatic. As shown in Fig. 5N,P, a comparison of anti-rhodopsin-stained eye sections at stage 45 confirmed that the transgenic animals had no rhodopsin-expressing cells. This showed that the differentiation of rod

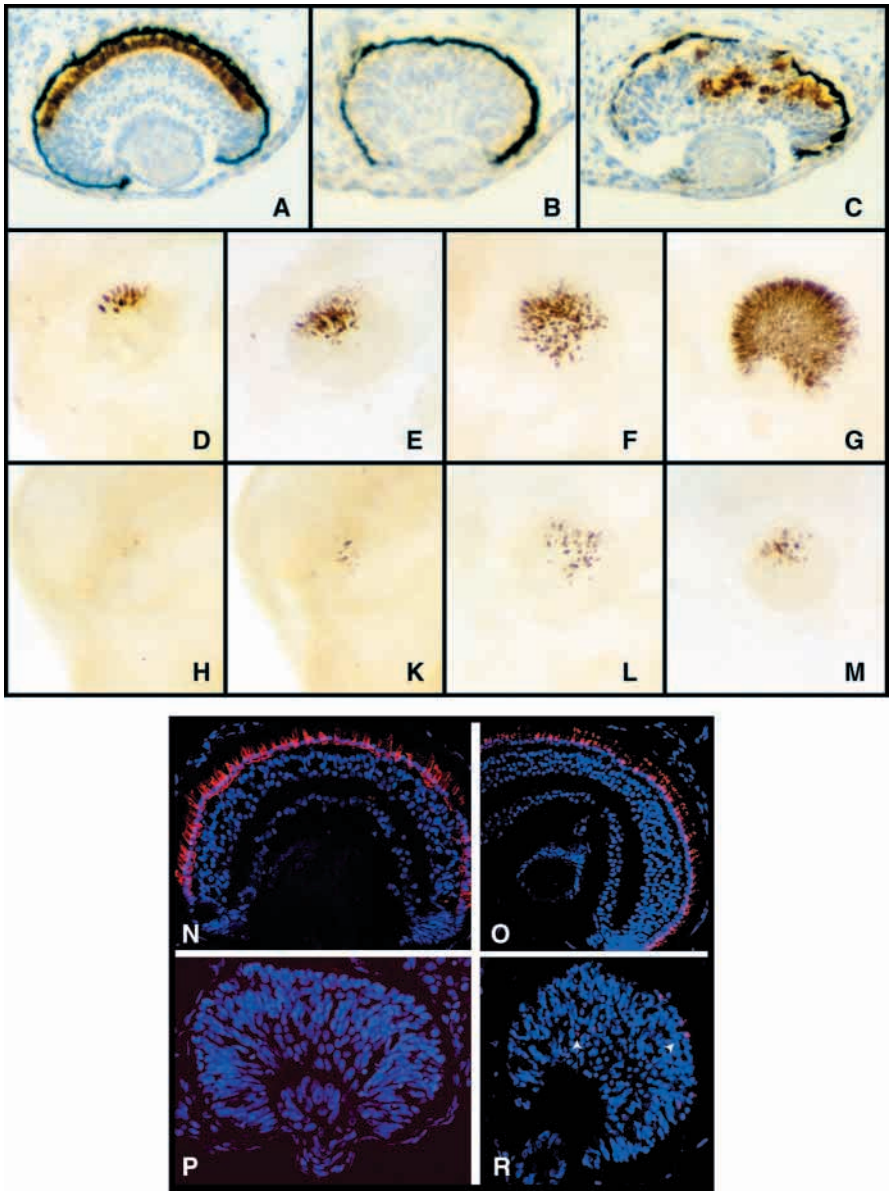

Fig. 5. (A-C,N-R) Immunostaining of sections of tadpole eyes with antibodies against rhodopsin and calbindin. (D-M) Whole-mount staining of tadpoles with antibodies against rhodopsin. (A) Section of a stage 39 tadpole that does not carry the *Xrx1A-ΔFGFR4a* construct stained with antibodies against rhodopsin, demonstrating the presence of photoreceptor cells. (B) Section of a stage 39 tadpole that carries the *Xrx1A-ΔFGFR4a* construct stained with antibodies against rhodopsin. Note the lack of photoreceptor cells. (C) Section of a tadpole carrying the *Xrx1A-ΔFGFR4a* construct stained with rhodopsin antibodies that shows some photoreceptor cells in ectopic position. (D-G) Whole-mount staining of *Xenopus* tadpoles that do not carry the *Xrx1A-ΔFGFR4a* construct with rhodopsin antibodies at different stages, demonstrating the normal accumulation of photoreceptor cells during development. (H-M) Whole-mount staining of transgenic *Xenopus* tadpoles expressing the *Xrx1A-ΔFGFR4a* construct with rhodopsin antibodies at different stages, demonstrating lower numbers of photoreceptor cells in these embryos at all stages. D,H, stage 33; E,K, stage 35; F,L, stage 36; G,M, stage 38. (N) Staining of a section from a stage 46 embryo that does not carry the *Xrx1A-ΔFGFR4a* construct with antibodies against rhodopsin. (O) Staining of a section of stage 45 embryo that does not carry the *Xrx1A-ΔFGFR4a* construct with antibodies against cone-specific calbindin. (P) An eye section from a stage 45 tadpole expressing the *Xrx1A-ΔFGFR4a* construct stained with rhodopsin antibodies. Note the lack of rhodopsin-positive rods. (R) An eye section from a stage 45 tadpole expressing the *Xrx1A-ΔFGFR4a* construct stained with calbindin antibodies. Only few cones are present (arrowheads), some of them in ectopic locations.

cells was not simply delayed but was actually not taking place. The same was true for cone cells. Although wild-type stage 45 embryos showed strong labeling in their retinas when stained with cone-specific calbindin antibodies (Fig. 5O), the transgenic embryos expressing the ΔFGFR4a construct showed only a few labeled cells (Fig. 5R). Like the rods, some of these cone cells were present in ectopic locations.

This experiment demonstrated that the problem in the ΔFGFR4a transgenic retinas was with the initial formation of photoreceptor cells, rather than with their survival. If the expression of ΔFGFR4a affected photoreceptor survival, we would have expected to see normal numbers of photoreceptor cells in early embryonic stages, followed by a reduction in their numbers. This was clearly not the case. To further eliminate the possibility that the lack of photoreceptor cells was caused by their selective cell death, we performed TUNEL assays on stage 29 and stage 35 embryos. As demonstrated in Fig. 6, the rates of retinal cell death in stage 29 embryos were practically identical in single- (58) and double-transgenic animals (54). There was a slight increase in cell death in double-transgenic animals at stage 35 that was not statistically significant. These results suggested that the absence of photoreceptor cells at later stages of development was not caused by increased death of photoreceptor progenitor cells, but rather by the lack of their specification.

The reduction in the number of photoreceptor cells was also not due to a general delay in the differentiation of retinal cells. Staining of transgenic eye sections with antibodies against Islet1, which recognize the ganglion and amacrine cells, or with antibodies against glutamine synthetase, which recognize Müller cells, revealed the presence of these cell types. As shown in Fig. 7, the distribution of these cells within the retina was not normal, but these cells were clearly present in large numbers. Counting of cells in sections of wild-type and transgenic animals revealed that retinas that expressed the ΔFGFR4a construct had twice as many glutamine synthetase-positive Müller cells (21.43%) as did the control retinas (10.62%). By contrast, the percentage of Islet1-positive cells (ganglion cells and amacrine cells) remained constant (20.63% versus 20.14%). This strongly suggests that FGF signaling is one of the key mechanisms controlling the specification of different retinal cell types. It appears that the limiting factor is FGF rather than the FGF receptor, as injection of the wild-type receptor does not seem to have any significant effect on the layering of the retina or the specification of retinal cell types (data not shown).

The presence of photoreceptor cells in some transgenic animals and the uneven morphology of the retina was initially puzzling, but we believe that these variations were due to the mosaic expression of the transgene that was observed in some transgenic animals (data not shown).

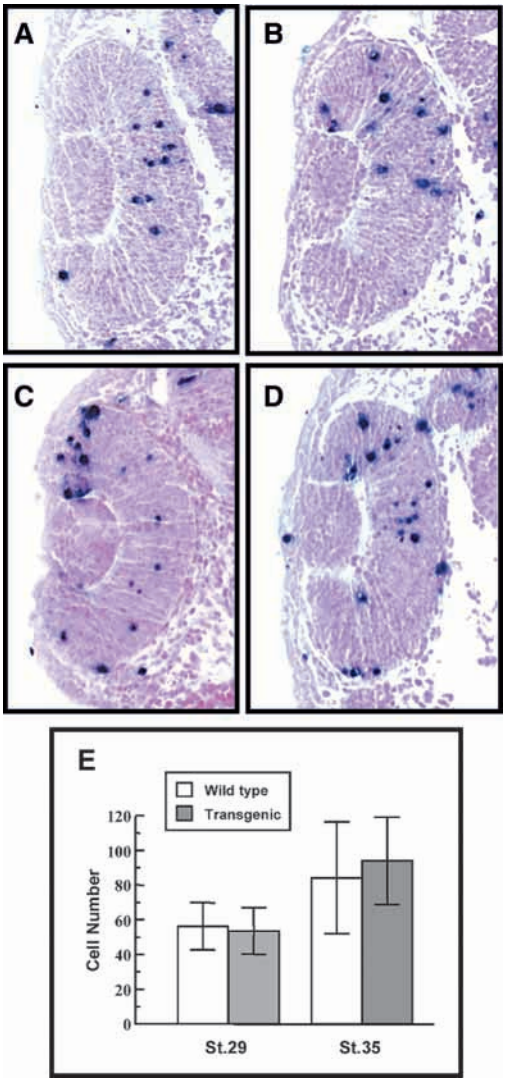

Fig. 6. Levels of apoptosis in retinas of tadpoles carrying *Xrx1A-ΔFGFR4a* transgene. (A,C) Cross-section of TUNEL-stained eyes of wild-type embryos at stage 29 (A) and stage 35 (C). (B,D) Cross-section of TUNEL-stained eyes of embryos carrying the *Xrx1A-ΔFGFR4a* transgene at stage 29 (B) and stage 35 (D). (E) Histogram showing the average number of labeled apoptotic cells on each retina of the wild-type (*car-GFP* transgenic) and transgenic (*car-GFP/Xrx1A-ΔFGFR4a* transgenic) embryos at each stage. Wild type (stage 29, *n*=16 retinas; stage 35, *n*=20 retinas); transgenic (stage 29, *n*=28 retinas; stage 35, *n*=24 retinas).

DISCUSSION

Analysis of vertebrate eye development has been undertaken for more than a century, but has been hampered by the inability to selectively change gene expression in the developing eye. In this paper, we describe the isolation of the regulatory sequences of the *Xrx1A* homeobox-containing gene, which can be used to selectively alter gene expression in the developing retina.

Xrx1A is one of the earliest and most specific markers of retinal development. It is expressed in the anterior neural plate, in cells that will become retinal progenitor cells. We have found that the 3.2 kb 5′ upstream DNA sequence of the *Xrx1A* gene contains regulatory regions sufficient to direct gene expression in the developing retina. Expression of GFP directed by these regulatory sequences starts in the anterior neural plate and persists in the retina, at least until stage 42. Whereas before stage 30 GFP expression was present in all retinal cell types, after stage 30 the GFP expression became progressively restricted to the photoreceptor cells. Therefore this region can account for the early transcription of *Xrx1A* in

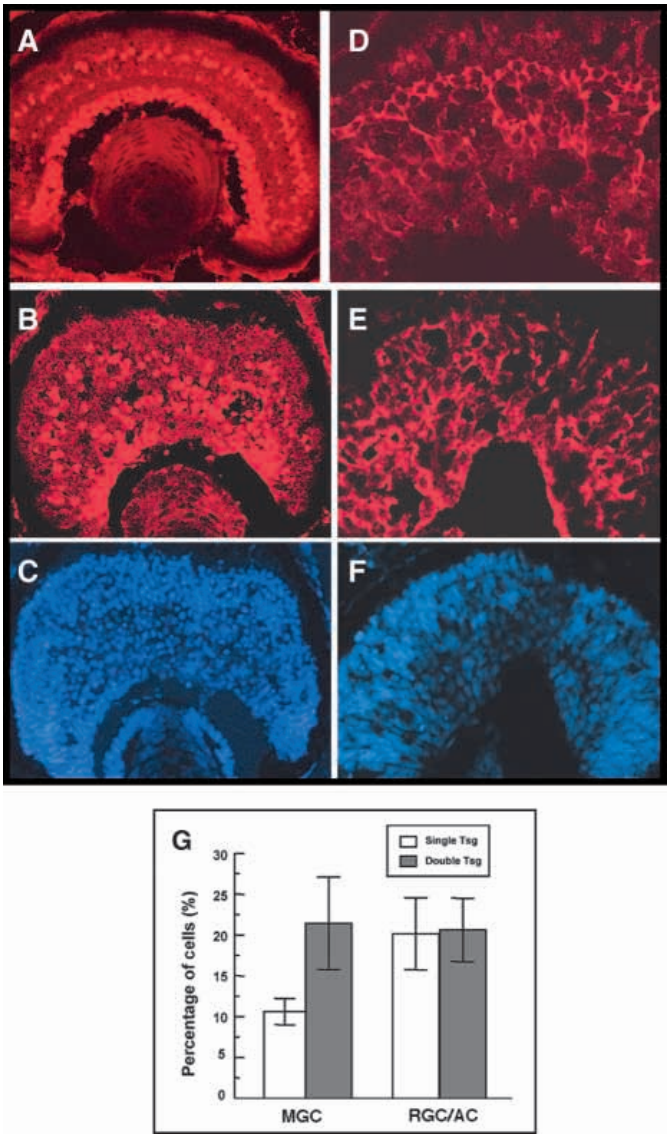

Fig. 7. Comparison of retinal cell distribution in tadpoles carrying and lacking the *Xrx1A-ΔFGFR4a* construct. (A) Immunostaining of an eye section from a stage 45 non-transgenic tadpole with antibodies against Islet1, which recognizes the ganglion and amacrine cells. (B) Immunostaining of an eye section from a stage 45 tadpole that carries the *Xrx1A-ΔFGFR-4a* construct with antibodies against Islet1, demonstrating disturbed layering of retinal cells. (C) Hoechst staining of the section from B. (D) Immunostaining of an eye section from a stage 45 tadpole that does not carry the *Xrx1A-ΔFGFR4a* construct with antibodies against glutamine synthetase, which recognizes Müller cells. (E) Immunostaining of an eye section from a stage 45 tadpole that carries the *Xrx1A-ΔFGFR4a* construct with antibodies against glutamine synthetase demonstrates irregular distribution of Müller cells in the retina of these tadpoles. (F) Hoechst staining of the section from E. (G) Histogram showing the percentage of Müller glial cells and retinal ganglion cells/amacrine cells in the retina of transgenic tadpoles. Müller glial cells and retinal ganglion cells/amacrine cells are identified by immunostaining with antibodies against glutamine synthetase and Islet1, respectively. MGC, Müller glial cells; RGC, retinal ganglion cells; AC, amacrine cells; Single Tsg, *car-GFP* transgenic (MGC, *n*=8 retinas; RGC/AC, *n*=6 retinas); Double Tsg, *car-GFP/Xrx1A-ΔFGFR4a* transgenic (MGC, *n*=10 retinas; RGC/AC, *n*=11 retinas).

the anterior neural plate and retinal progenitor cells (Casarosa et al., 1997; Mathers et al., 1997), and for the late expression in photoreceptor cells (Perron et al., 1998). At later stages, GFP expression was not completely limited to photoreceptor cells, as some cells outside of the photoreceptor layer displayed fluorescence. The identity of these cells is not known at present. One attractive hypothesis is that these are photoreceptor progenitor cells. As we do not have an independent specific marker for progenitors of photoreceptor cells, we cannot currently test this hypothesis.

Deletion analysis of the 3.2 kb segment of upstream sequence revealed that this DNA segment can be divided two distinct regulatory regions. The first region, –2726 to –1304 bp upstream of the *Pst*I site, could activate transcription early in the anterior neural plate, whereas the second region, which is located –982 to –606 bp upstream of the *Pst*I site, could activate transcription late in the development of photoreceptor cells. There appears to be a redundancy in photoreceptor specific regulatory elements, as the first region alone was sufficient for the restriction of GFP expression to the photoreceptor cells (Fig. 3; construct 12 and 13). Therefore both regions can direct gene expression in the photoreceptor cells, but only the first region can direct gene expression in the retinal progenitor cells.

We have taken advantage of these sequences to study the role of FGFR4a-mediated FGF signaling on the development of retinal cell types. For this purpose, we expressed the dominant-negative FGFR4a under the control of the *Xrx1A* regulatory sequences in retinal progenitor cells of transgenic *Xenopus laevis*. Our results showed that FGF signaling mediated by FGFR4a was essential for normal specification of retinal cell types and for the correct development of the entire retina. Specifically, we observed a marked loss of photoreceptor cells in transgenic animals expressing ΔFGFR4a. This demonstrated that FGFR4a-mediated FGF signaling in the retina is required for the specification of photoreceptor cells, as measured by expression of rhodopsin and calbindin. At the same time, we observed an increase in the formation of Müller glial cells, whereas the number of glutamine synthetase-positive ganglion/amacrine cells was not significantly affected. As it was demonstrated that these altered ratios were not due to selective cell death of specific retinal cell types, we conclude that the lack of photoreceptor cells was caused by a failure in photoreceptor specification.

This observation agrees well with other studies that have investigated the role of different FGF molecules in photoreceptor formation. McFarlane et al. demonstrated that inhibition of FGF signaling using a dominant-negative form of XFGFR1 (XFD) resulted in a 50% loss of photoreceptor cells with a concurrent 3.5-fold increase in Müller glial cells (McFarlane et al., 1998), suggesting a shift towards a Müller cell fate in the absence of a Fibroblast Growth Factor receptor signal. In addition, Hicks and Courtois showed that FGF can increase the number of photoreceptor cells in dissociated rat retinal cells (Hicks and Courtois, 1992). The experiments of Campochiaro et al. showed that transgenic mice expressing dominant-negative forms of FGFR1 and FGFR2 in photoreceptor cells displayed progressive photoreceptor degeneration, but did not show abnormalities in the specification of photoreceptor cells (Campochiaro et al., 1996). This result differs from ours in that the development of

photoreceptor cells was not affected, only their survival. However, this is not surprising as the rhodopsin promoter used by Campochiaro et al. activates gene expression only after the photoreceptor cells are differentiated to a significant degree. Taken together, all published observations on the role of FGF signaling in retinal development present a unified picture, in which FGF signaling is necessary for specification and survival of retinal cell types. In addition, we believe that FGF signaling is necessary for the correct layering of the retina.

FGF molecules have also been suggested to play a role in the separation of the neuroepithelium of the optic vesicle into the neuroretina and retinal pigment epithelium (RPE). Hyer et al. found that in the absence of surface ectoderm, the neural and RPE cells were mixed together (Hyer et al., 1998). Exogenously added FGF1 was able to replace the function of the surface ectoderm. In a similar study, Nguyen and Arnheiter demonstrated that FGF1- or FGF2-coated beads could transform retinal pigment epithelium into the neuroretina (Nguyen and Arnheiter, 2000). Based on these observations, we would have expected a conversion of the neuroretina into RPE in our transgenic animals that express the ΔFGFR4a in the developing retina. We did not observe such a conversion. This might be because we have not eliminated FGF signaling mediated by other FGF receptors. Although this is a formal possibility, the current belief is that ΔFGFR4a does not inhibit only one specific FGF signaling pathway, but can also inhibit all other FGF signaling pathways. This is because ΔFGFR4a can heterodimerize with other FGF receptors. If this is indeed the case, a general inhibition of FGF signaling might result from such interactions. However, it was shown by Hongo et al. that although ΔFGFR4a is capable of inhibiting FGF signaling effectively, it is not able to do it completely (Hongo et al., 1999). We may not observe a conversion of neuroretina into RPE because the primary molecule that leads to a separation of neuroretina and RPE is not a FGF molecule. The experiments of Hyer et al., and of Nguyen and Arnheiter, do not exclude this possibility (Hyer et al., 1998; Nguyen and Arnheiter, 2000). Indeed, a targeted elimination of FGF1, or FGF2, or both in mice (Dono et al., 1998; Miller et al., 2000; Ortega et al., 1998) did not result in the retinal phenotype observed by Hyer et al., and by Nguyen and Arnheiter (Hyer et al., 1998; Nguyen and Arnheiter, 2000), indicating that further experimentation will be necessary to fully understand the role of FGF molecules and their receptors in retinal formation.

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