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The development of *Xenopus tropicalis* transgenic lines and their use in studying lens developmental timing in living embryos

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SUMMARY

The generation of reporter lines for observing lens differentiation in vivo demonstrates a new strategy for embryological manipulation and allows us to address a long-standing question concerning the timing of the onset of differentiation. *Xenopus tropicalis* was used to make GFP reporter lines with γ1-crystallin promoter elements directing GFP expression within the early lens. *X. tropicalis* is a close relative of *X. laevis* that shares the same ease of tissue manipulation with the added benefits of a diploid genome and faster life cycle. The efficiency of the *Xenopus* transgenic technique was improved in order to generate greater numbers of normal, adult transgenic animals and to facilitate in vivo analysis of the crystallin promoter. This transgene is transmitted through the germline, providing an accurate and consistent way to monitor lens differentiation. This line permitted us to distinguish models for how the onset of differentiation is controlled: by a process intrinsic to differentiating tissue or one dependent on external cues. This experiment would not have been feasible without the sensitivity and accuracy provided by the in vivo reporter. We find that, in specified lens ectoderm transplanted from neural tube stage donors to younger neural-plate-stage hosts, the onset of differentiation, as measured by expression of the crystallin/GFP transgene, is delayed by an average of 4.4 hours. When specified lens ectoderm is explanted into culture, the delay was an average of 16.3 hours relative to control embryos. These data suggest that the onset of differentiation in specified ectoderm can be altered by the environment and imply that this onset is normally controlled by external cues rather than by an intrinsic mechanism.

Key words: *Xenopus tropicalis*, Crystallin, Lens, Timing, Transgenesis, Differentiation

INTRODUCTION

We have used *Xenopus tropicalis* to prepare transgenic reporter lines to aid in performing embryological manipulations in general, and here, specifically, to address the source of the signal controlling the onset of differentiation in the lens. *Xenopus laevis* has long been favored as a developmental system because of the external development of its large embryos and the ease of surgically manipulating these embryos. The major weakness of this system is that until recently there have been few tools for manipulating *Xenopus* genetically, with the notable exception of the development of transgenic technology (Kroll and Amaya, 1996). There has been little effort expended towards developing genetic tools due to unfavorable traits of *X. laevis* – namely its pseudotetraploid genome and generation time of 1-2 years under normal conditions (Duellman and Trueb, 1986). *X. tropicalis* is the only diploid member of the genus (de Sa and Hillis, 1990) with a genome size roughly half that of mouse (1.7×10^9 bp/haploid nucleus, Tymowska, 1973), and a significantly shorter generation time of 3-5 months. *X. tropicalis* is also tractable to embryonic surgery, making it a much more suitable organism for the preparation of permanent transgenic lines and for other genetic manipulations (Amaya et al., 1998).

The production of transgenic lines in the *X. tropicalis* system has allowed us to rapidly localize the promoter elements of the *γ1-crystallin* gene in a developmental context. The transgenic lens reporter lines also provide an extremely valuable tool for resolving a subtle timing question like the onset of differentiation because they afford a precise, straightforward way to monitor a temporal event that would otherwise be impractical or infeasible to assay by conventional gene expression assays. In addition, the transgene provides a marker for donor tissue in transplant experiments that obviates the need for other host and donor marking schemes.

The timing of major milestones during development, for example the onset of specification and differentiation in particular tissues, has been the subject of extensive discussion though little is known about these processes (Cooke and Smith, 1990). Very early embryonic events, like the timing of gastrulation, are thought to involve maternally derived timing signals (e.g. Howe et al., 1995). Experiments on the timing of mesodermal development after gastrulation in *Xenopus* show that muscle differentiation begins at approximately the same time whether the inductive signal responsible for eliciting

muscle formation is received early or late within the period of mesodermal competence (Gurdon et al., 1985; Steinbach et al., 1998). Later during somitogenesis in the chick, the timing of paraxial mesoderm differentiation seems to correlate with autonomously controlled waves of *c-hairy* expression (Palmeirim et al., 1997).

Can one generalize that all tissue differentiation is controlled by similar tissue-intrinsic timing mechanisms? During organogenesis, many specialized tissues such as the liver and pancreas have been shown to require extrinsic signals to facilitate their differentiation (Gittes et al., 1996; Luo et al., 1995). This suggests that external cues may be more widely used than tissue-intrinsic timers at later stages of development. Currently, however, there is no definitive experiment either in culture or in vivo that shows the presence of an extrinsic timer of differentiation. At much later stages of development, for example during metamorphosis in amphibians, it is well documented that differentiation can be controlled by external cues such as hormones (Shi et al., 1996). While it is clear that careful control of the timing of differentiation must be important for coordinating developmental processes at all stages of development, when tissues first form it has not been clearly determined whether tissue-intrinsic or external cues are more important in establishing this control.

The issue of timing of differentiation can be very effectively studied during formation of the lens of the eye because so much is known about the development of this system, and because of the new techniques for evaluating this process. Lens determination is one of the oldest experimental paradigms of tissue induction (Spemann, 1901). Experiments done almost a century ago showed that the region of ectoderm that comes to overlie the optic vesicle during neurulation is fated to form a lens and will do so even if the optic rudiment is ablated at the earlier neural plate stage (Lewis, 1904). More recent transplant experiments have shown that the presumptive lens ectoderm (PLE), having received early biasing signals from the anterior neural plate, is specified by inductive signals arising in the embryo at late neurula stages (stage 19, all staging according to Nieuwkoop and Faber, 1967). At this stage, the PLE will go on to express early lens markers (e.g. γ1-crystallin) even when explanted and grown in simple physiological salts (Henry and Grainger, 1987, 1990). As in most systems where differentiation has been studied, little is known about how the process is activated.

In this study, we have modified the *Xenopus* transgenic technique to increase its efficiency, and then used this procedure to map the promoter elements for the lens structural gene, *γ1-crystallin*. We then produced stable lines in *X. tropicalis* expressing a GFP reporter from γ1-crystallin promoter elements. These lines have been used to detect the onset of lens differentiation in transplant and explant experiments designed to probe the mechanisms underlying the timing of lens development. We find evidence of extrinsic signals, which are not limited to the eye field, that appear to control the onset of lens differentiation.

MATERIALS AND METHODS

Transgenic technique for *X. laevis* and *X. tropicalis*

The procedures used here were based on the REMI technique of Kroll and Amaya (1996) for generating transgenic *Xenopus laevis* embryos. The procedures for making high-speed egg extract and sperm nuclei were unaltered from the original protocol (Amaya and Kroll, 1999). Nuclear concentrations were determined by labeling nuclei with Hoechst 33342 (Sigma), counting with a hemocytometer and adjusting to 200,000 nuclei/μl. DNA constructs used for transgenesis were linearized and purified using GeneClean (Bio101). For each REMI reaction, 400,000 nuclei were combined with 150-250 ng of DNA in 5 μl total Sperm Dilution Buffer (SDB; Sive et al., 2000). After 5 minutes at room temperature, the following were added: 10 μl SDB, 1 μl high-speed egg extract, 1 μl 100 mM $MgCl_2$, 0.5 μl 1:50 restriction enzyme in SDB, and this was incubated an additional 15 minutes at room temperature. The nuclei were then diluted to 3 nuclei/5 nl in MOH buffer (10 mM KPO_4 pH 7.2, 125 mM potassium gluconate, 5 mM NaCl, 0.5 mM $MgCl_2$, 250 mM sucrose, 0.25 mM spermidine, 0.125 mM spermine) and back-filled into glass needles, broken to 80 μm at the tip. Freshly squeezed eggs were dejellied using 2% cysteine in 1× MMR, pH 7.9, rinsed carefully with 1× MMR (Sive et al., 2000) and loaded into agarose depression dishes containing 6% Ficoll in 1× MMR. Nuclei were delivered at a rate of 18 μl/hour (5 nl/second) using a Harvard 11 infusion pump with an injection of ~1 second/egg (empirically determined to yield ~1 nucleus/egg). Immediately following the injection, the 6% Ficoll solution was replaced with 1/3× Modified Barth's Saline (MBS, Sive et al., 2000) containing 50 μg/ml gentamycin. After 2-3 hours, the normally cleaving embryos were removed to fresh agarose-coated dishes and placed at 16°C overnight.

The primary differences in the procedure used for *X. tropicalis* reflect its smaller egg size and the empirically observed requirement for lower salt media. The injection needles used for *X. tropicalis* were broken to 50 μm at the tip. Eggs were dejellied with 2% cysteine in Ca^{2+}-free 1/3× MBS, rinsed with Ca^{2+}-free 1/3× MBS and loaded into agarose depression dishes filled with 6% Ficoll in 1/3× MBS with 90 μM $CaCl_2$ and 40 μM EGTA. The Ca^{2+} was reduced to prevent embryonic damage from excessive cortical contraction following injection, which was commonly observed using media with higher Ca^{2+} concentrations. Following the transgenic reaction, as described above, the nuclei were diluted to 3 nuclei/2.5 nl in MOH buffer and delivered at a rate of 9 μl/hour (2.5 nl/second) for ~1 second/egg. Following injection, the 6% Ficoll injection medium was replaced with 1/8× MBS (with normal Ca^{2+}) and embryos were cultured at 22°C.

In situ hybridization

Embryos were collected from stage 14 to 33 and analyzed for γ1-crystallin expression. Whole-mount in situ hybridization was performed, as previously described (Sive et al., 2000), using a 600 bp probe from the 3rd exon. Stained embryos were postfixed in MEMFA (Sive et al., 2000) prior to digital imaging.

γ1-crystallin deletion constructs

The γ1-crystallin genomic clone used in this study had been previously isolated (Smolich et al., 1993), using *Rana temporaria* γ2-crystallin probes in homology screens. A 2.2 kb region of the γ1-crystallin promoter, extending 5′ from the first AUG of the coding region, was generated by PCR and cloned into the filled-in *Hin*dIII and *Sma*I sites of a CS2-based vector containing a variant of GFP (P. Bradley and R. M. G., unpublished data; Bronchain et al., 1999). This GFP, referred to as GFP3, has been modified to red-shift its excitation wavelength and increase its solubility, as previously described (Zernicka-Goetz et al., 1996, 1997). The GFP3 AUG in this construct is the first one downstream of the transcriptional start site. This construct, referred to as 2.2γ1-crys/GFP3, was linearized with *Hin*dIII for making transgenic animals.

To produce a nested set of 5′ deletions, the entire construct was excised by *Hin*dIII and *Not*I digestion, subjected to *Sau*3A partial digestion, size selected on an agarose gel and cloned into *Bam*HI- and

*Not*I-digested pBluescript SKII. This resulted in three 5′ deletions containing 1.2 kb, 1.0 kb and 0.8 kb regions of the γ1-crystalin promoter, referred to as 1.2γ1-crys/GFP3, 1.0γ1-crys/GFP3, and 800γ1-crys/GFP3, respectively. These were linearized using *Hin*dIII.

Several smaller deletions of the 800γ1-crys/GFP3 construct were made using the Exonuclease III/Mung Bean nuclease protocol (Promega, Inc.). Deleted clones were analyzed by restriction digestion with *Bam*HI and *Kpn*I, which releases the γ1-crystallin promoter portion of each deletion. Clones having γ1-crystallin promoter regions of 600, 500, 380, 320 and 160 bp (referred to as 600γ1-crys/GFP3, 500γ1-crys/GFP3, 380γ1-crys/GFP3, 320γ1-crys/GFP3 and 160γ1-crys/GFP3) were linearized by the unique 3′ *Not*I site. Sequence analysis of the 380γ1-crys/GFP3, 360γ1-crys/GFP3 and 160γ1-crys/GFP3 constructs was carried out using the Sequenase 2.0 kit (Gibco BRL).

Photomicroscopy

Images of transgenic animals were obtained using either an Olympus SZH10 or Zeiss Stemi SV-11 microscope with fluorescent illumination and fluorescein filter set. Transgenic animals were photographed primarily using the Olympus PM-10 35 mm photo system with 400 ASA slide film. These images were digitized using a Nikon LS-1000 slide scanner and optimized in Adobe Photoshop. Images of embryos after in situ hybridization and of some transgenic embryos, however, were captured using a Hamamatsu 5810 or a Dage 330 cooled CCD camera.

Genotyping of transgenic lines

Animals were raised to late tadpole stages (stage 50-51), anesthetized in 1:2000 (w/v) 3-aminobenzoicacid ethyl ester (MS222) and a ~1 cm long piece of the tail was removed. The tail pieces were homogenized and the DNA purified by standard Proteinase K digestion techniques (Sive et al., 2000). A portion of each sample was analyzed by PCR using the 5′ AAAGGAGAAGAACTTTTCACTGG and 3′ TTATTT-GTATAGTTCATCCATGCC primers that are specific for the GFP3 reporter region of the construct. The remainder of the DNA was digested with *Bam*HI, which cuts once between the promoter regions and the GFP3 reporter cassette, and analyzed by Southern blot hybridization using the 800 bp GFP3 region as a ^{32}P-labeled probe. Concatamers of the transgene digested with *Bam*HI are predicted to yield multiple fragments of 7.6, 6.0 and 4.4 kb (based on the all possible orientations of the trangene within the concatamer) and single copy levels of the end fragments. Densitometric analysis used a 5 kb presumptive end fragment to estimate the number of copies of the transgene.

Lens ectoderm transplants and explants

For transplant and explant experiments, donor embryos expressing the 2.2γ1-crys/GFP3 transgene were produced by out-crossing F_1 transgenic animals to wild type; host embryos for transplants were obtained from wild-type matings. Stage 19 donors were assayed for transgene expression prior to surgery by scoring for diffuse GFP3 expression, which can be detected as early as stage 17, in the anterior neural tube. Stage 14 donors showed no detectable GFP3 expression and thus only 50% of transplants came from a transgenic donor: the remainder were not counted in this study. For transplants, ectoderm from the PLE region on the right side of a host embryo was removed and replaced with homotopic ectoderm from a transgenic donor. The PLE on the donor embryo's left side was kept intact as a control to determine the normal onset of transgene expression. Surgical medium was 1× MBS + 0.05 mg/ml gentamycin sulfate (MBS+gent.). Surgeries were performed using a tungsten needle and transplants were allowed to heal under glass coverslips for 1 hour. Embryos were transferred to 1/2× MBS+gent. overnight, and were then placed in 1/10× MBS+gent. for the duration of the experiment. GFP3 expression was assayed about 12 hours postoperatively, and at regular intervals thereafter, using a fluorescence stereomicroscope. For explants, PLE tissue was removed from the right side of stage 19 transgenic donors into 1× MBS+gent. Explants, and the donors from which they were taken, were cultured in pairs in 24-well dishes. GFP3 expression was assayed as described above.

RESULTS

Modifications to the *Xenopus* REMI transgenic technique

To improve survival to late stages and increase the proportion of embryos with normal axial morphology, as required for generating transgenic adult animals, the original REMI protocol (Amaya and Kroll, 1999; Kroll and Amaya, 1996) was modified in both *X. laevis* and *X. tropicalis*. After re-evaluating several aspects of the published protocol, we found that embryo losses occurred primarily at two steps: just following injection (early cleavage stages) and at gastrulation. Losses following injection were due primarily to poor egg quality and use of cysteine in dejellying, while the losses following gastrulation (after stage 10) were due primarily to treatment with the high-speed egg extract (Table 1 and data not shown). The issue of egg quality is always a key component of experimental techniques in *X. laevis* but, with the REMI technique, it is imperative that the animals used be well rested since their last induction and in good health. Embryos generated by the REMI procedure from suboptimal

Table 1. Optimization of the REMI transgenic protocol

Extract[1]	Dejellying reagent	Injection buffer	Number injected	Number of embryos surviving at stage				Transgenic at stage 40
				Cleaved	Stage 10	Stage 20	Stage 30	
12 μl	Cysteine[2]	SDB[4]	350-400[6]	64	16	12	0	
12 μl	DTT[3]	SDB	350-400	88	43	16	0	0
12 μl	Cysteine	MOH[5]	350-400	66	20	12	0	0
12 μl	DTT	MOH	350-400	184	151	NS	9	2
6 μl	DTT	MOH	350-400	134	77	NS	6	3
2 μl	DTT	MOH	350-400	138	107	NS	50	35[7]

[1]High-speed egg extract added per 19 μl reaction volume.
[2]2% cysteine in 1× MMR pH 7.9.
[3]3 mM dithiothreitol in 1× MMR pH 8.9.
[4]Sperm dilution buffer as previously described by Amaya and Kroll (1999).
[5]Buffer mimicking normal intracellular physiological salts supplemented with spermine and spermidine.
[6]Estimated based on the volume of the injection dish that was filled.
[7]This varied with egg quality with a range of 5-10% of the number of eggs injected.

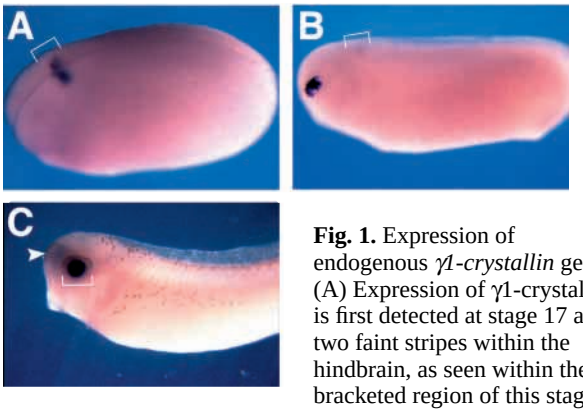

Fig. 1. Expression of endogenous *γ1-crystallin* gene. (A) Expression of γ1-crystallin is first detected at stage 17 as two faint stripes within the hindbrain, as seen within the bracketed region of this stage 18 embryo. (B) This expression strengthens through stage 22 or 23 and then gradually diminishes until it is barely detectable at stage 25, as seen in B, demarcated by the bracket. At this time, the earliest expression within the presumptive lens ectoderm is seen beginning at first in single cells and gradually spreading throughout the PLE by stage 26. (C) By stage 30, the γ1-crystallin expression is restricted to the lens vesicle (bracketed region) where high levels are seen. Faint diffuse staining was also seen within the forebrain (as indicated by the arrowhead) at these stages, but this was also seen with control embryos hybridized with crystallin sense probes.

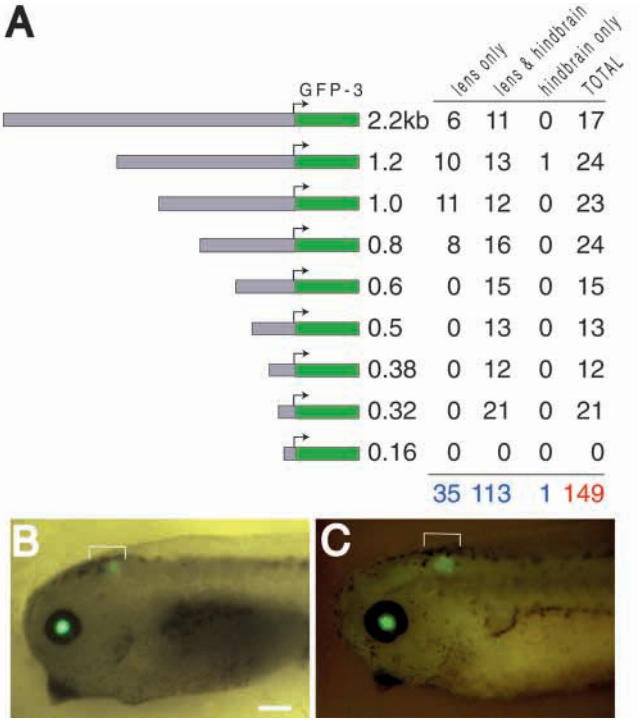

Fig. 2. Analysis of the γ1-crystallin promoter. (A) Deletion constructs were analyzed in transgenic *X. laevis* and *X. tropicalis* using the modified transgenic procedure described in the text. Embryos with normal heads and eyes were scored between stage 30 and 36 for the presence of GFP3 fluorescence (typically >50% showed expression). With the exception of the final 160γ1-crys/GFP3 construct, expression driven by these reporter constructs mimics the endogenous expression pattern at this stage. From a total of 149 transgenic embryos, 113 showed the normal expression pattern. Those with lens-only expression were generally cases with weaker expression overall, and because hindbrain expression is much less intense than that in the lens, there may have been expression in these embryos within the hindbrain that was below the level of detection. (B) *X. tropicalis* embryos which are transgenic for the full-length construct mimic the endogenous pattern at this stage and (C) the same pattern is seen with the 320γ1-crys/GFP3 construct (scale bar = 180 μm for both).

females typically do not survive beyond early cleavages. While our observations on the use of DTT versus cysteine (Table 1) for dejellying indicate there is a slight increase in survival using DTT, more critical than this is the understanding that, prior to fertilization, the eggs are very fragile and easily damaged during the dejellying procedure. Recently we have observed that partially dejellying is also beneficial. In addition, a slight increase in percent survival was seen using lower injection volume/egg (data not shown) and when the MOH injection buffer was used rather than SDB (Table 1). The MOH buffer was based on normal intracellular ion concentrations with some components from the SDB buffer (e.g. spermidine and spermine) that aid in nuclear stability. It should be noted that, although the use of DTT and MOH buffer do result in increased survival prior to gastrulation, the number of embryos surviving beyond gastrulation was unaffected, suggesting that other factors influence survival at these later stages.

A number of factors were examined for their contribution to gastrulation defects. Although the contributions to the mortality of later stage embryos by the restriction enzyme and the DNA construct were evaluated, these did not prove to be major factors within the dose ranges from the original protocol. The most important variable was found to be the amount of high-speed egg extract. If the amount of extract is reduced to one-sixth of that used in the original protocol, survival is greatly increased and 50-90% of the embryos are still transgenic (see Table 1). It is thought that this mortality may be due to increased fragility of the decondensed nuclei in a high concentration of egg extract resulting in the endogenous DNA becoming damaged during subsequent manipulations. These changes have allowed us to evaluate expression patterns in tadpole stage embryos that are predominantly normal morphologically, reducing the fraction with the axial defects common with the original protocol.

Transgenic analysis of the γ1-crystallin promoter

The γ1-crystallin mRNA is predominantly lens-specific, but for unknown reasons is seen first at stage 18 as a stripe within the hindbrain (Fig. 1A), which fades gradually during development (first observed by M. Saha and R. M. G., unpublished data). Expression within the PLE is first seen at stage 25 and strengthens through tadpole stages (Fig. 1B,C). To map the γ1-crystallin promoter/enhancer elements, a 2.2 kb region extending 5′ from the initiator AUG of the *γ1-crystallin* gene was generated by PCR and fused to a modified GFP reporter, GFP3 (see Materials and Methods). Based on the mapping of the promoter sequences of other crystallin family members (Goring et al., 1987; Lok et al., 1984, 1985), it was thought that the regulatory sequences for γ1-crystallin would be contained within this region. A nested set of 5′ deletions was produced from the full-length construct ranging in size from 1.2 kb to 160 bp using *Sau*3A partial digestion techniques and *Exonuclease III* treatment (see Fig. 2A; Materials and Methods). These and the full-length construct were analyzed in both transgenic *X. laevis* and *X. tropicalis* using the modified transgenic protocol.

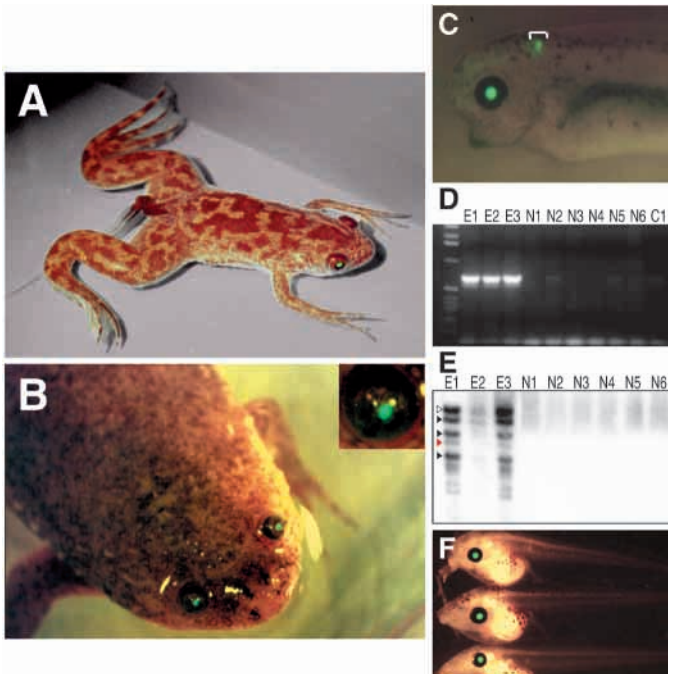

Fig. 3. Germline transmission of the 2.2γ1-crys/GFP3 transgene. (A,B) γ1-crystallin promoter/GFP3 transgenic animals have been raised through metamorphosis in both *X. laevis* and *X. tropicalis*. (A) A recently metamorphosed *X. laevis* showing bright GFP3 fluorescence seen here with only white light. (B) Similar strong expression in an adult *X. tropicalis* F_0 animal. (C) Of the five *X. tropicalis* transgenic animals that have been bred so far, four have transmitted the transgene to their offspring, as shown by this stage 36 tadpole showing the predicted hindbrain and lens expression. In all cases, ~50% of the offspring were expressing the transgene, indicative of a single active locus. (D) F_1 animals from the first founder to transmit were analyzed by PCR using GFP3 specific primers. Lanes E1-E3 represent samples from three tadpoles expressing the transgene, all of which show a strong band of the expected size. Lanes N1-N6 represent samples from non-expressing tadpoles and are negative indicating that the founder is carrying a single locus that is driving normal expression. Lane C1 shows the response from wild-type *X. tropicalis* DNA and C2 is a water control. (E) These same DNA samples were digested with *Bam*HI, which cuts once within the 2.2γ1crys/GFP3 construct, and analyzed by Southern blot (sample C is wild-type *X. tropicalis* DNA). The black arrowheads indicate the 7.6, 6.0 and 4.4 kb (top to bottom) fragments that are expected to be released from the concatamer. The band indicated by the open arrowhead results from incomplete digestion. The red arrowhead indicates the putative single copy end fragment that was used to estimate the copy number of the transgene (see Materials and Methods). (F) Three F_1 tadpoles from outcrosses of the second founder show strong expression within the lens that persists into adult stages.

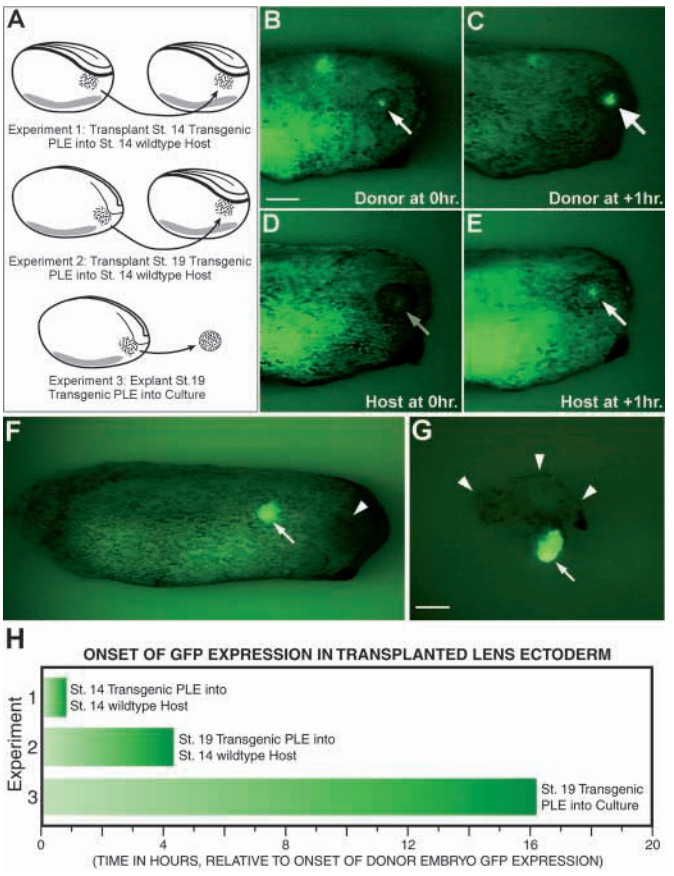

Fig. 4. Environmental signals affect the timing of lens differentiation. (A) The experimental designs for all three transplant/explant experiments discussed in the text are diagrammed. The hatched box represents the region of lens ectoderm that is transplanted from one embryo to another or explanted into culture. (B,C) A typical donor embryo is shown from experiment 1. The two panels show fluorescence images taken one hour apart, from the first moment GFP3 is detected in the PLE (designated 0 hour – panel B) until the expression has strengthened significantly (designated +1 hour – panel C). White arrows indicate GFP3 expression in the PLE while the width of the arrowhead is used to indicate the strength of expression, wider is stronger. The scale bar in B represents 200 μm and the scale is identical for panels C-F. (D,E) A typical host embryo from the same experiment is illustrated. The two panels show fluorescence images taken one hour apart, with the timing identical to B and C. The gray arrow in D points to the PLE but indicates that no GFP3 expression is yet visible. The white arrow in E indicates GFP3 expression with a level similar to that of the donor embryo one hour earlier. (F) When transplants were located more posteriorly, the transplanted PLE still expresses GFP3 indicating the environmental influences are not localized to the region of the eye. (G) A typical, positive PLE explant from experiment 3 is shown. Arrowheads mark the extent of the explanted tissue while the arrow shows the region expressing the transgene. Scale bar, 50 μm. (H) A graphical representation of the data from six independent experiments (two stage 14 to 14 transplants, two stage 19 to 14 transplants and two stage 19 explants) is shown, indicating the mean delay for each experiment. All bars indicate the delay, in hours, of host GFP3 expression relative to the onset of GFP3 expression in the unoperated donor PLE.

Expression within the presumptive lens can be seen as early as stage 25 in transgenic animals with high expression levels (data not shown) but, due to the diffuse nature of the early staining and the autofluorescence from the yolk, this was very difficult to see in more weakly expressing animals. For this reason, the presence of lens expression was scored after stage 30 when the expression was easily detected in all cases. For the first three deletion constructs, a few examples of ectopic expression were seen primarily in animals with slight axial defects. For this reason, in subsequent analyses only completely normal embryos were scored. A few examples of faint expression in the developing ear were also seen (data not shown).

GFP3 expression driven by the 2.2γ1-crys/GFP3 construct is seen in the hindbrain and lens of both *X. laevis* (data not shown) and *X. tropicalis* (Fig. 2B) transgenic tadpoles (stage 29-34). Although the endogenous hindbrain expression of γ1-crystallin is almost completely extinguished at these stages (see Fig. 1B), the hindbrain expression of the GFP3 protein (stable up to 24 hours; Bokman and Ward, 1981) is still visible.

Transgenic analysis of the 5′ deletion constructs reveals that all replicate the same pattern and timing of expression as the full-length construct, with the exception of the 160γ1-crys/GFP3 construct (Fig. 2A). This indicates that elements necessary and sufficient for the normal expression of the *γ1-crystallin* gene are located within the proximal 320 bp (e.g. see Fig. 2C). It should be pointed out that the use of the GFP3 reporter allows only a qualitative analysis of these constructs and does not distinguish subtle differences in expression levels. In the transgenics derived from the more proximal constructs, there does appear to be some reduction in the GFP3 signal within the hindbrain (data not shown). This could indicate the presence of more distal elements that contribute to the normal levels of γ1-crystallin expression but are not necessary for the normal patterning of its expression.

GFP3 expression can also be seen with the full-length construct as early as stage 19 in the hindbrain, consistent with the normal pattern, as well as ectopic expression in the forebrain (data not shown). The forebrain misexpression is greatly diminished by stage 30 and therefore was not seen at these later stages. The forebrain expression was not seen in any of the shorter constructs, which showed the normal hindbrain expression, with exception of the 160γ1-crys/GFP3 construct. This again points to the proximal 320 bp as containing the sequences necessary for the correct temporal and spatial patterning.

The required sequences have been analyzed for the presence of putative binding sites for Pax-6 (Sharon-Friling et al., 1998), L-Maf (Ogino and Yasuda, 1998), Retinoic Acid Receptor (RAR; Tini et al., 1993) and Sox (Nishiguchi et al., 1998), all of which have been implicated in the regulation of other crystallin genes. For Pax6, there were no potential binding sites found within the 5′ 160 bp region of the promoter that was shown to be necessary for γ1-crystallin expression. However, there are several potential sites for L-Maf, RARE and Sox binding within this region. Further mutational and biochemical analysis is necessary to determine whether in fact these sites play a role in γ1-crystallin regulation.

Germline transmission of transgenes in *Xenopus tropicalis*

The 2.2γ1-crys/GFP3 transgenic animals have been raised through metamorphosis in both *X. laevis* and *X. tropicalis* (Fig. 3A and B, respectively). As seen in these figures, γ1-crystallin-driven GFP3 expression persists in the lens of adult animals. Due to the shorter lifecycle, several *X. tropicalis* lines have reached sexual maturity and these were bred to wild-type animals. In 4 of 5 transgenic founders tested to date, the normal transgene expression pattern was transmitted to ~50% of their offspring; an expressing embryo is shown in Fig. 3C. This frequency of expression is indicative of a single expressing locus for each of these lines. To determine whether silent loci were also present, DNA samples from late stage tadpole tail snips were analyzed by PCR with GFP3-specific primers. As shown in Fig. 3D, for the three expressing animals, a strong signal of the predicted size is obtained. GFP3 sequences were not detected, however, in samples from non-expressing tadpoles. Southern blot analysis of the remaining sample digested with *BamHI*, which cuts once within the construct, indicates that the insertion contains approximately 13 copies of the transgene (see Fig. 3E and Materials and Methods). This is consistent with the initial reports concerning the REMI technique (Amaya and Kroll, 1999). Further, the 2.2γ1-crys/GFP3 F_1 and F_2 animals have also been raised to sexual maturity and all have transmitted the normal transgenic expression to ~50% of their offspring when bred to wild-type animals.

Analysis of the timing of differentiation using 2.2γ1-crys/GFP3 lines

The use of these transgenic lines has allowed us to study the timing of lens differentiation with a precision not attainable previously. Although earlier experiments conclusively showed that explants of specified ectoderm, taken from stage 19 embryos, were able to activate crystallin expression when assayed after three days of development (control stage 41), the time course of this expression was not determined (Henry and Grainger, 1990). It had been assumed that the time course of crystallin expression in explants and transplants paralleled that of unoperated control embryos. We find, however, that this is not the case: environmental signals significantly affect the timing of differentiation.

Neural plate (stage 14) hosts were chosen because previous work (Henry and Grainger, 1987) had shown that the lens-inducing environment is strongest at this stage, when signaling from the anterior neural plate acts on the lens ectoderm. Because the lens ectoderm at this stage is biased toward lens formation, but has not received all of the signals needed for specification (Grainger, 1996), embryos of this stage were used as hosts in the following timing experiments. Conversely, by the time of neural tube closure (stage 19), the inductive effects of the environment are weaker, although the PLE itself has become specified. Lens ectoderm taken from this stage donor is thought to have already received the inductive signals required to allow differentiation when cultured in isolation (Grainger, 1996).

In our first experiment investigating the onset of differentiation (Fig. 4A, experiment 1), neural-plate-stage PLE tissue expressing the 2.2γ1-crys/GFP3 transgene was transplanted to the PLE region of an identically staged, wild-type host. This experiment controls for any surgically induced delay in the onset of γ1-crystallin transgene expression in the transplanted tissue. Transplants were first assayed for transgene expression when unoperated donor embryos reached stage 28, the earliest time that lens-specific GFP3 expression can be detected. A typical result from this experiment is shown in Fig. 4B-E, in which stage 29/30 donor and host embryos are assayed every hour for GFP3 fluorescence. There is an average delay of 0.8 hours (s.e.m. ± 0.4 hours) in GFP3 expression (seen in 5 of 12 cases) when a transgenic stage 14 PLE is transplanted homotopically into a wild-type stage 14 host. This delay is likely a surgical artifact and is minor when compared with the delays in differentiation seen in subsequent experiments. With respect to the seven negative cases, it should be noted that similar transplant experiments have consistently

failed to show 100% induction in transplanted lens ectoderm (Henry and Grainger, 1987). It is also possible that, in this case, an insufficiently robust lens response produced levels of GFP expression that were undetectable against the autofluorescence of *Xenopus* yolk. Any case in which GFP fluorescence was not clearly distinguishable from background was scored as negative. Alternatively, non-responsive PLEs may have been transplanted with some of the underlying mesoderm attached and, in some cases, this mesoderm may have blocked inductive signals from reaching the ectoderm.

When a specified stage 19 PLE is transplanted into a stage 14 host (Fig. 4A, experiment 2) a significant delay of 4.4 hours (s.e.m. ± 0.2 hours) is seen in the onset of transgene expression. This delay is relative to the onset of transgene expression in the stage 19 control donor PLE and is detected in all positive cases (5 of 12 transplants). This suggests that the younger host environment is retarding the development of the older, transplanted tissue. This retardation is even seen in transplanted PLEs that were inadvertently placed posterior to the eye (Fig. 4F, 2 of the 5 positive cases), suggesting that the host effect is not confined to the optic region.

If specified neural-tube-stage (stage 19) ectoderm is explanted and grown in culture, there is an extremely long delay in the activation of γ1-crystallin expression. Henry and Grainger (1990) had previously shown that explanted tissue from this stage embryo went on to express crystallin to some degree in 75% of cases when assayed by antibody staining approximately 72 hours later, although most were small, weakly expressing aggregates of cells. Our results using transgenic stage 19 donors (Fig. 4A, experiment 3) show that the delay in the onset of γ1-crystallin expression in explants is remarkably long, averaging 16.3 hours (s.e.m. ± 2.1 hours) in positive cases (8 out of 23) relative to the control PLE in the donor animals from which explants were taken (Fig. 4G). Again, the percentage of negative cases is not out of line with that seen in previous experiments. A summary of the relative delays in transgene expression observed in the three previous experiments is displayed in graphic form (Fig. 4H).

DISCUSSION

The data presented here introduces new strategies for performing embryonic manipulations in *Xenopus* and uses them to address a long-standing question: how is the timing of cell differentiation initiated? Using a *Xenopus tropicalis* transgenic reporter line for assaying lens differentiation in vivo, we have used transplantation and explantation experiments to show that, during lens formation, the cues for the onset of differentiation come from the tissues surrounding the presumptive lens ectoderm. This result leads us to reevaluate the general issue of developmental timing, which we review and discuss below. We also discuss here the utility of the *X. tropicalis* system for future embryological and genetic studies.

Extrinsic signals initiate lens differentiation

Using the γ1-crystallin-driven GFP3 reporter lines for tissue transplant and explant studies, we have shown that the timing of lens differentiation can be altered by environmental cues, suggesting the presence of at most a weak internal timer. These cues are not unique to the region of the eye, as one might have expected, but are present even in more posterior regions. These results contrast the conclusion of Gurdon et al. (1985) and Steinbach et al. (1998), examining muscle differentiation, and Palmeirim et al. (1997), studying somitogenesis, that the timing of onset is controlled by a tissue-intrinsic mechanism. Particularly in the case of Gurdon (1985), however, a contribution by an extrinsic signal is not entirely ruled out, since the use of large amounts of tissue in his animal/vegetal recombinants may have inadvertently included extrinsically signaling tissues. During organogenesis, as noted in the Introduction, there is often a requirement for tissue interactions to initiate differentiation, suggesting that an extrinsic timing mechanism may be involved in many of these systems. These studies do not, however, test this proposal explicitly, perhaps because of the difficulty associated with such onset assays. During formation of the pancreas and liver, for example, an early ‘instructive’ signal specifies the fate of the endoderm, although the differentiation of the tissue requires diffusible non-instructive inputs from the mesodermal mesenchyme (LeDouarin, 1975; Wessells and Cohen, 1967).

It is interesting to note that Wessells and Cohen (1967) found that mesenchyme from any region tested could be substituted and still direct normal differentiation. This observation may be related to our finding that specified ectoderm can be placed posterior to the eye region and yet still differentiate normally, as indicated by γ1-crystallin-driven GFP3 expression. These ‘non-instructive’ signals from various tissues, which these embryological manipulations reveal, may in fact function normally as a means of coordinating the timing of organogenesis throughout the embryo. Wessells and Cohen (1967) report one result related directly to the issue at hand: they show that a minimum mass of pancreatic endoderm is required to trigger differentiation. This ‘mass effect’ may be related to our finding that explanted PLE’s differentiate significantly later than intact controls, if the signal coordinating differentiation is present, as it must be to some extent in the PLE, but is greatly augmented by extrinsic cues. In none of the studies on organogenesis in other systems do we find, however, data about what sets the time of differentiation in vivo.

The evidence presented here is consistent with the hypothesis that a timer governing the onset of organogenesis is largely controlled by extrinsic cues. In the absence of external cues from the embryo, as in the stage 19 explants, differentiation occurs but is substantially delayed. Extrinsic signals are also important for the normal temporal onset of lens differentiation in vivo. The 4.4-hour delay seen in the stage 19 to 14 transplants is almost exactly the same length of time required, during normal development, for the embryo to proceed from stage 14 to 19. This is consistent with what one would predict if specified ectoderm were dependent on signals from the host environment. The origin of these signals is currently unknown, although there is evidence that retinal-derived growth factors such as FGF play a critical role in the differentiation of lens cells (McAvoy et al., 1991). Though our findings represent only the first piece of a larger puzzle, it is a necessary first step towards identifying the molecular signals which are responsible for regulating these timing events.

Identification of γ1-crystallin promoter elements

Although the mapping of promoter elements presented here

only roughly defines the sequences required for tissue-specific gene expression, it illustrates the successful adaptation of the transgenic method, developed originally in *X. laevis* (Amaya and Kroll, 1999), to *X. tropicalis*. In addition, this study highlights the efficiency of the *Xenopus* system for promoter analysis, where one can assay several promoter constructs in hundreds of embryos in a single day. Using both *X. laevis* and *X. tropicalis*, we have identified a 160 bp region of the γ1-crystallin promoter that is essential for mimicking the endogenous expression pattern. Analysis of these sequences has indicated the presence of several possible binding sites for RAR, SOX and Maf family members, which are known to play important roles in crystallin gene regulation (Tini et al., 1994; Nishiguchi et al., 1998; Ogino and Yasuda, 1998). The *Xenopus* system should provide an efficient means for more refined analysis of the crystallin promoter, defining the roles of these and other transcriptional regulators.

Transgenic lines provide powerful new tools for the *Xenopus* system

We have obtained normal healthy adult transgenic animals using the transgenic technique in both *X. laevis* and *X. tropicalis* and have demonstrated germline transmission from 4 of 5 2.2γ1-crys/GFP3 *X. tropicalis* founder animals. This was not a foregone conclusion because achieving germline transmission has proven to be difficult in other vertebrate developmental systems (Amsterdam et al., 1995; Higashijima et al., 1997). The ability to achieve germline transmission and subsequent expression of transgenes has also been seen with reporter constructs expressing GFP3 from *Pax-6* (K. Hartley, N. Papalopulu, E. Amaya and R. M. G., unpublished data) and *Rx* (L. Zimmerman, J. Gray and R. M. G., unpublished data) promoter elements, suggesting that this is likely to be a generally useful approach. Germline transmission has also been demonstrated in *X. laevis* (Bronchain et al., 1999; Marsh-Armstrong et al., 1999).

The ability to make stable lines will have a significant impact on experimental designs in the *X. tropicalis* system. The utility of such lines for achieving rapid, consistent and sensitive detection of temporally regulated events is illustrated by the experiments presented here. The ability to observe domains of gene expression revealed by such reporter constructs permits surgical manipulations that would have been inconceivable in the past. For example, the expression of genes like *Rx*, which is important in early eye determination (Mathers et al., 1997), can be seen at stages when determination is occurring (L. Zimmerman, J. Gray and R. M. G., unpublished data), permitting one to test whether these observable domains correspond to functionally determined regions. With the recent identification of a number of fluorescent proteins in addition to GFP (Matz et al., 1999), the ability to make multiple-reporter lines having different color fluorescent proteins to mark expression of different genes offers yet another level of sophistication in embryological analyses.

This technology provides a way in which to drive the expression of developmentally important genes within restricted domains of the embryo. Strategies involving crossing of multiple lines for activation of genes in particular tissues also become feasible, especially in the short-generation *X. tropicalis*. For example, the GAL4-UAS transcription activation system, so widely used in *Drosophila* developmental genetics (Phelps and Brand, 1998), now becomes a possibility. Finally, the generation of stable transgenic lines in a rapidly developing amphibian implies that it will be feasible to perform genetic manipulations using strategies that require this new approach. For example, the use of gene trap approaches as a tool to induce mutagenesis becomes possible (Amaya et al., 1998). Recent results indicate that the REMI technique for transgenesis can be successfully used to generate gene trap insertions in *X. laevis* (Bronchain et al., 1999) and that this methodology is transferable to *X. tropicalis* (O. Bronchain, L. Zimmerman, E. Amaya and R. M. G., unpublished data).

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