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RAPID COMMUNICATION

Lens Induction by Pax-6 in *Xenopus laevis*

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Despite extensive study following the pioneering work of Spemann on lens development (Spemann, H. (1901) *Verh. Anat. Ges.* 15, 61–79) and the subsequent establishment of the concept of embryonic induction, the molecular mechanism of vertebrate lens induction remains largely unknown. Here we report that in *Xenopus* expression of Pax-6 results in lens formation in a cell autonomous manner. In animal cap experiments, Pax-6 induced expression of the lens-specific marker βB1-crystallin without inducing the general neural marker NCAM. Ectopic Pax-6 expression also resulted in the formation of ectopic lenses in whole embryos as well as in animal cap explants indicating that in vertebrates, as well as *Drosophila* (Halder, G., Callaerts, P., and Gehring, W. J. (1995) *Science* 267, 1788–1792), Pax-6 can direct the development of major components of the eye. Interestingly, ectopic lenses formed in whole embryos without association with neural tissue. Treatments giving rise to anterior neural tissue in animal cap explants resulted in the expression of both βB1-crystallin and Pax-6. Given the ability of Pax-6 to direct lens formation, we propose that the establishment of Pax-6 expression in the presumptive lens ectoderm during normal development is likely to be a critical response of lens-competent ectoderm to early lens inducers.

INTRODUCTION

A critical role for the transcription factor Pax-6 in development of the eye is indicated by the mutant phenotypes of *eyeless* in *Drosophila*, *small eye* in mouse, and *ANIRIDIA* in humans (Quiring *et al.*, 1994). More striking is the demonstration that ectopically expressed *Drosophila* Pax-6 can induce the formation of ectopic eyes in imaginal disks (Halder *et al.*, 1995). In *Xenopus*, it has recently been shown that Pax-6 is first expressed before the completion of gastrulation (stage 12) in the primordium of the neural tube and in a broad stripe that encompasses the anterior neural plate (Hirsch and Harris, 1996; Li *et al.*, 1997). By stage 12.5, two distinct patches of Pax-6 expression are visible in the presumptive lens ectoderm. Because the timing of Pax-6 expression suggests that it may be required for the early stages of lens development, we sought to investigate its role in this process by examining *Xenopus laevis* embryos in which Pax-6 was ectopically expressed.

MATERIALS AND METHODS

Cloning of Xenopus βB1-crystallin. Degenerate primers for reverse-transcriptase–polymerase chain reaction (RT–PCR) amplification and cloning of β-crystallins were upstream 5′-CGGGATCCCGGGA/GTAC/TTGA/GTANCCNCKA/GTA-3′ and downstream 5′-GGAAATCTCGAGGGNGAA/GTAC/TCCNGNTGGGA-3′. Specific primers used for routine βB1-crystallin RT–PCR were upstream 5′-TGCCTGGAGTGGAACAATGC-3′ and downstream 5′-TGTTGAACCATCCCATAGCC-3′. The sequence of the *Xenopus* βB1-crystallin cDNA obtained for this study has been submitted to GenBank.

In situ hybridization and immunostaining. Embryos were labeled according to established protocols. An antisense probes for *in situ* hybridization of βB1-crystallin was prepared using rUTP-digoxigenin (Boehringer Mannheim Biochemicals) from the *Xenopus* partial cDNA template. Antiserum to bovine β-crystallins (a gift from S. Zigler) was used at 1:1000 dilution. Neural staining was detected using the supernatant of 6F11 hybridoma cells (gift of W. A. Harris) at a 1:1 dilution in PBT + 20% goat serum. The

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neural staining seen in sections was performed on frozen sections that had been stained for β-crystallin prior to sectioning.

RT–PCR. The protocol for RT–PCR and sequence of primers employed has been described previously (Hemmati-Brivanlou and Melton, 1994).

RESULTS AND DISCUSSION

In order to study the function of Pax-6, we first examined expression of potential lens-specific markers. A survey of lens crystallins (Cvekl and Piatigorsky, 1996) revealed that while many of them are highly expressed in the lens, few are expressed exclusively in this location. β-Crystallins, however, were specific for lens in *Xenopus* as demonstrated by staining with antibodies to the bovine β-crystallin family. Labeling was restricted to lens according to both whole embryo and histological assessment (Figs. 1A–1C). Based on these observations, degenerate primers were used to amplify a number of partial β-crystallin clones from embryonic cDNA using a RT–PCR procedure. Whole mount *in situ* hybridization using a *Xenopus* βB1-crystallin probe confirmed that labeling was restricted to the maturing lens beginning at mid-neurula (stage 25, Fig. 1D). These observations indicated that βB1-crystallin is an excellent lens-specific marker.

To assess the biological activity of Pax-6 in a vertebrate embryo, we injected RNA-encoding Pax-6 into *Xenopus* at the two-cell stage and examined the consequences for the expression of a variety of tissue-specific markers including those for mesoderm, neural tissue, and lens in the context of ectodermal explants (animal caps). Expression of Pax-6 RNA resulted in the induction of βB1-crystallin in a dose-dependent fashion (Fig. 1E, lanes 1–3). Interestingly, the induction of the lens marker occurs in the absence of neural induction because the general neural marker NCAM was not induced (Fig. 1E). Muscle actin, a mesodermal marker, was also absent or present at background levels. Ectodermal explants injected with RNA-encoding Pax-6 stained positive for β-crystallin (Figs. 1G and 1H) but subsequently did not double-label with antibodies directed against the neural marker NCAM (data not shown). No staining was observed in uninjected controls (Fig. 1F). The lack of muscle actin and neural markers suggested that expression of βB1-crystallin was not secondary to mesodermal or neural induction. This, in turn, indicated that Pax-6 provided a lens induction signal for ectodermal cells that otherwise would form epidermis.

Since Pax-6 induced a lens-specific marker in embryonic explants, we tested next whether Pax-6 had the same activity in the whole embryo. RNA-encoding Pax-6-FLAG was injected into the animal pole of half the blastomeres of two- or four-cell embryos. In animal caps, this tagged version did not alter the ability of Pax-6 to induce expression of βB1-crystallin (Figs. 1E (lane 4), 1G, and 1H). Embryos were allowed to develop to tadpoles (stage 38) and stained as whole mounts for β-crystallin. There were frequently many

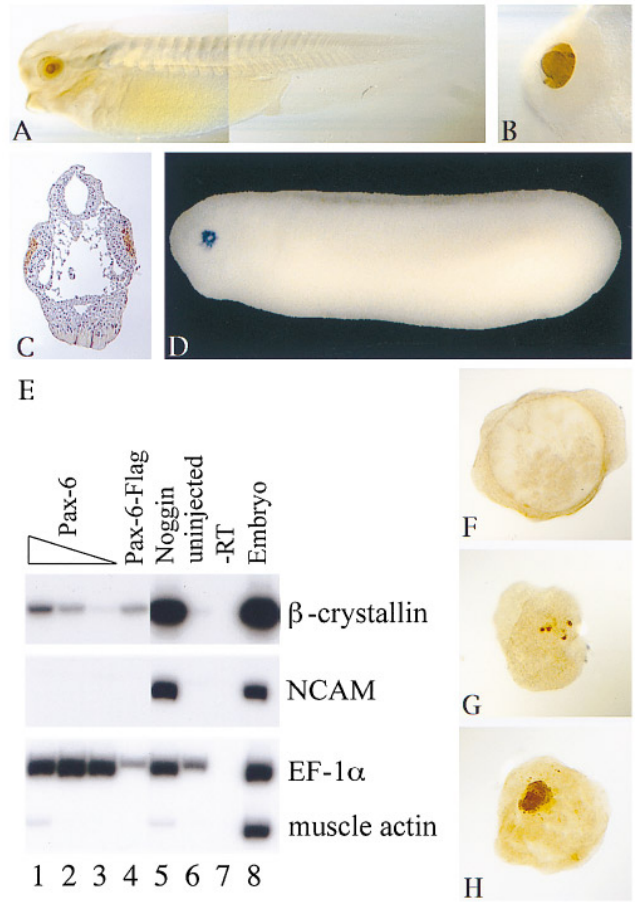

FIG. 1. Anti-bovine β-crystallin antibody labeling of tadpole stage 38 embryo (A) and close-up (B) of the normal lens from an injected embryo. (C) Transverse section of anti-bovine- β-crystallin antibody-stained tadpole, stage 28. (D) Whole mount *in situ* hybridization of a stage 28 embryo using a *Xenopus* βB1-crystallin probe. (E) Animal cap assay of Pax-6-injected *Xenopus* embryos assessed for the expression of tissue-specific markers at stage 34 using RT–PCR. The lens-specific marker βB1-crystallin was produced in a dose-dependent manner (lanes 1–4: 1.0, 0.25, 0.06, and 0.5 ng/embryo, respectively) in the absence of NCAM expression. Injection of RNA-encoding Noggin (lane 5, 2.0 ng) serves as a positive control for response of the explants and gives the expected expression of the neural marker NCAM. Muscle actin is used as a mesodermal marker and EF1α as a loading control. (F) Uninjected animal cap stained with β-crystallin antibody. (G, H) Animal caps injected with 1.4 ng Pax-6-FLAG RNA (sibling control at stage 33/34).

ectopic lenses in each embryo and these were restricted to the anterior region (Figs. 2A–2D). The proportion of embryos with one or more ectopic lenses was optimally 47% at 0.12 ng of RNA injected (see Table 1 for quantitation of two typical experiments). In embryos assayed for ectopic lenses at earlier stages, endogenous lenses could be detected, but development of significant numbers of ectopic lenses was delayed (data not shown). Embryos injected with

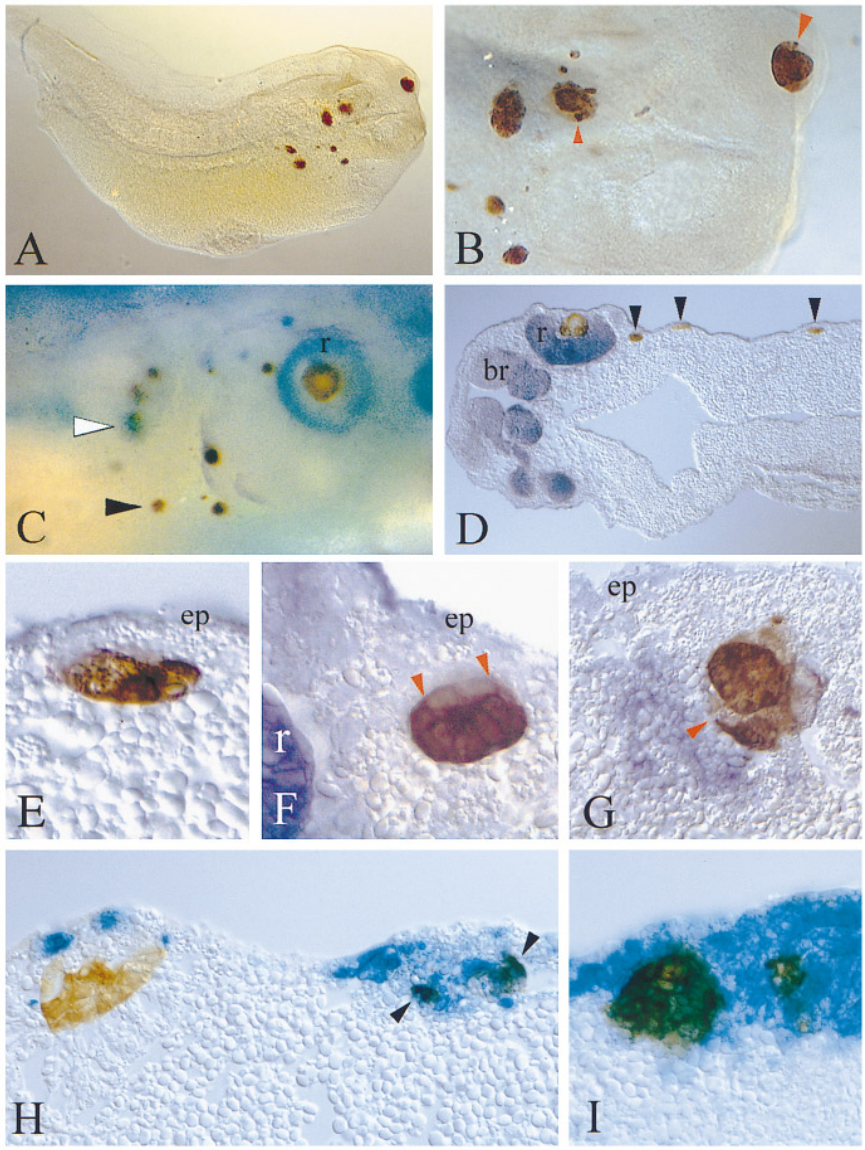

FIG. 2. Pax-6 induces ectopic lenses in whole embryos in a cell autonomous fashion. β-Crystallin staining of embryos (stage 38) injected with RNA-encoding Pax-6-FLAG (0.12–0.25 ng/embryo) revealed the formation of ectopic lenses (brown) in whole mount (A–C) and in section (D–G). Red arrowheads (B, F, and G) indicate the border between lens epithelium and fibre cells; large arrowhead in B indicates endogenous lens. The embryo in A was cleared in benzyl benzoate and is at a higher magnification in B. Embryos shown in C and D were costained (blue) with antibodies to NCAM. Some ectopic lenses were not associated with neural tissue (C, black arrowhead; D and E) while others were close to (D, far left ectopic lens; F) or tightly associated with neural tissue (C, white arrowhead; G). Sections through endogenous (H, left) and ectopic lenses (H, arrowheads, and I) in embryo coinjected with β-Gal and β-crystallin RNAs. Coincident staining for β-Gal (blue) and β-crystallin (brown) indicates the cell-autonomous function of Pax-6. Magnifications (A) $\times$50, (B, C) $\times$200, (D) $\times$100, and (E–I) $\times$400.

2.0 ng of control RNA encoding the transcription factor ETS-1 (Stiegler *et al.*, 1990) which has been reported to bind to similar DNA sequences as Pax-6 (Plaza *et al.*, 1994) did not result in the formation of ectopic lenses (data not shown).

Histological sections revealed that in all cases ectopic lenses were adjacent to the epidermis (Figs. 2D–2G) consistent with their presumed ectodermal origin. Consistent with injection into only half the blastomeres of two- or four-cell embryos, lenses were present on only one side in some examples (Fig. 2D). The distinctive appearance of mature, polarized lenses was apparent in cleared, whole em-

TABLE 1
Quantitation of Pax-6 Lens-Inducing Activity in Whole Embryos

Total RNA injected (ng)	No. of surviving embryos examined	No. of embryos with 1 ectopic lens	No. of embryos with 2 ectopic lenses	No. of embryos with >2 ectopic lenses	Percentage of embryos with ectopic lenses
0	8	0	0	0	0
0.06	35	1	1	1	8.5
0.12	55	11	5	10	47
0.25	48	8	1	8	35

Note. This table includes data from two representative experiments showing the relationship between the dose of Pax-6 RNA used and the number of ectopic lenses induced *in vivo.*

bryos for both normal and some of the ectopic lenses (Fig. 2B). This was confirmed by sectioning embryos labeled in whole mount; some, but not all ectopic lenses showed the characteristic morphological features of polarization (Figs. 2F and 2G).

To determine whether Pax-6 acted autonomously or non-autonomously in lens induction, lineage tracing experiments were performed. Anti-FLAG staining of injected embryos revealed nuclear staining consistent with expression of Pax-6-FLAG at early stages while few FLAG-positive cells remained at later stages. As an alternative strategy, RNAs for β-galactosidase and Pax-6-FLAG were coinjected and embryos were stained at the tadpole stage for both β-galactosidase (β-gal) and β-crystallin. While the injection of β-gal RNA does not induce lenses, coinjection with Pax-6 resulted in the formation of ectopic lenses at a frequency similar to that observed in experiments using Pax-6 alone. Examination of frozen sections revealed that in all cases (n = 32 ectopic lenses) β-crystallin-positive cells were also positive for β-gal, indicating that Pax-6 induction of ectopic lenses was cell autonomous (Figs. 2H and 2I).

Given the long-standing controversy as to whether the neural-derived optic cup is required for lens development (Grainger, 1996) and the results of animal cap assays indicating that βB1-crystallin was induced in the absence of NCAM (Fig. 1E), we determined whether ectopic lenses in whole embryos were associated with neural tissue. In embryos double-labeled for NCAM and β-crystallin (Figs. 2C–2G: NCAM, blue; β-crystallin, brown) some ectopic lenses were distinctly separated from neural tissue (Fig. 2C, black arrowhead), while others clearly overlaid regions of NCAM staining (Fig. 2C, white arrowhead). Labeling of histological sections from 6 different embryos revealed that 40% of ectopic lenses (6 of 15) could be found separated from neural tissue (Figs. 2D–2F). While this suggests that there is no obligatory association between ectopic lenses and neural tissue, we cannot exclude the possibility that an interaction occurs at an earlier stage. The remaining 60% of ectopic lenses in whole embryos were in direct contact with ectopic neural tissue (Fig. 2G). We are currently addressing the nature of the ectopic neural tissue using eye-specific markers.

To further examine the role of neural tissue in lens induction, we tested the activity of a series of neural inducers for both Pax-6 and β-crystallin induction in the context of animal caps. All direct neural inducers currently known induce neural tissue of anterior character (forebrain and eyes (Hemmati-Brivanlou and Melton, 1997)). Thus, it was interesting to determine if lens was part of the repertoire of markers they induce and, as might be expected, whether neural induction was upstream of Pax-6 expression. All neuralizing treatments, including expression of a type I dominant negative bone morphogenetic protein receptor, follistatin, noggin, chordin, and cell dissociation, resulted in the induction of both Pax-6 and βB1-crystallin (Fig. 3). These treatments lead directly to the formation of neural tissue without the requirement for mesoderm as an intermediate. The ability of direct neural inducers to activate the expression of Pax-6 argues that mesoderm is not a requirement for the very earliest stages of lens induction, though it cannot be excluded that it has a role at later stages, as has been implied by previous experiments (Henry and Grainger, 1990).

The observation that Pax-6 can induce ectopic lenses autonomously is of central importance to considerations of lens induction mechanism. Combined with the finding that Pax-6 can induce β-crystallin in animal caps directly, this argues that early anterior neural plate-derived signals previously implicated in lens induction (Henry and Grainger, 1990) may be necessary only to establish and perhaps maintain expression of Pax-6 in the presumptive lens ectoderm. This is supported by experiments which indicate that Pax-6 and βB1-crystallin are downstream of anterior neural fate specification (Fig. 3). We suggest that in animal caps injected with Pax-6 RNA these signals can be bypassed. This is consistent with the observation that the neural marker NCAM is not detected by either PCR or antibody staining in these animal caps. This would also be consistent with many studies showing that lenses can, in some species,

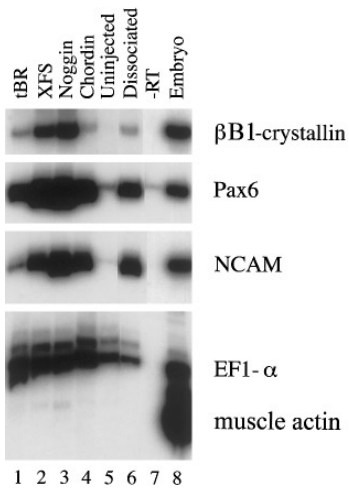

FIG. 3. Neuralizing treatments result in the expression of Pax-6 and lens-specific βB1-crystallin in animal caps. Injection of RNA-encoding dominant-negative type I BMP receptor (lane 1, 2.0 ng), follistatin (lane 2, 2.0 ng), noggin (lane 3, 2.0 ng), chordin (lane 4, 2.0 ng), dissociated animal cap cells (lane 6), but not uninjected embryos (lane 5) led to the expression of NCAM, Pax-6, and βB1-crystallin in animal caps at tailbud stage. EF-1α was amplified as a loading control and the absence of muscle actin indicated that mesoderm was not present. RNA from tailbud embryo was used as a positive control while an amplification without RT (lane 7) controlled for the absence of contaminating DNA.

form independently of the optic cup (Grainger, 1996; Mencl, 1903). The establishment of Pax-6 expression in the presumptive lens ectoderm during development is therefore likely to be an important response of lens-competent ectoderm to early lens inducers.

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