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Gene activation during early stages of lens induction in *Xenopus*

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SUMMARY

Several stages in the lens determination process have been defined, though it is not known which gene products control these events. At mid-gastrula stages in *Xenopus*, ectoderm is transiently competent to respond to lens-inducing signals. Between late gastrula and neural tube stages, the presumptive lens ectoderm acquires a lens-forming bias, becomes specified to form lens and begins differentiation. Several genes have been identified, either by expression pattern, mutant phenotype or involvement in crystallin gene regulation, that may play a role in lens bias and specification, and we focus on these roles here. Fate mapping shows that the transcriptional regulators *Otx-2*, *Pax-6* and *Sox-3* are expressed in the presumptive lens ectoderm prior to lens differentiation. *Otx-2* appears first, followed by *Pax-6*, during the stages of lens bias (late neural plate stages); expression of *Sox-3* follows neural tube closure and lens specification. We also demonstrate the expression of these genes in competent ectoderm transplanted to the lens-forming region. Expression of these genes is maintained or activated preferentially in ectoderm in response to the anterior head environment. Finally, we examined activation of these genes in response to early and late lens-inducing signals. Activation of *Otx-2*, *Pax-6* and *Sox-3* in competent ectoderm occurs in response to the early inducing tissue, the anterior neural plate. Since *Sox-3* is activated following neural tube closure, we tested its dependence on the later inducing tissue, the optic vesicle, which contacts lens ectoderm at this stage. *Sox-3* is not expressed in lens ectoderm, nor does a lens form, when the optic vesicle anlage is removed at late neural plate stages. Expression of these genes demarcates patterning events preceding differentiation and is tightly coupled to particular phases of lens induction.

Key words: Lens, Induction, *Xenopus*, *Otx-2*, *Pax-6*, *Sox-3*

INTRODUCTION

Lens induction in *Xenopus* begins during gastrulation, as animal cap ectoderm undergoes autonomous changes in competence, i.e., the ability to respond to induction, and proceeds toward determination many hours later. The lens-forming competence of animal cap ectoderm is at its peak between mid to late gastrula stages (Servetnick and Grainger, 1991a). This window of lens competence during which the ectoderm is able to begin responding to signals from the adjacent developing neural plate immediately succeeds neural competence. During neurula stages, presumptive sensory placodes of the lens, nose and ear begin to gain identity in association with the adjacent developing neural tissue (Jacobson, 1963). Between the competence period and the beginning of final differentiation at tailbud stages, the presumptive lens ectoderm (PLE) undergoes a series of tissue interactions and proceeds through distinct phases of bias and specification (Grainger, 1992, 1996). The anterior neural plate of mid-neurula embryos is a strong inducer of the lens (Henry and Grainger, 1990). It is during this period (between neural plate and neural tube stages) that the PLE receives the signals that cause it to become biased toward lens formation (Henry and Grainger, 1987; Grainger et al., 1997). Bias acquired in the head ectoderm can be demonstrated by increased lens-forming ability in tissues of the presumptive nose, lens and ear when compared to earlier stage competent ectoderm (Grainger et al., 1997). Specification of the lens, i.e., the time when the PLE can be explanted and subsequently differentiate without further tissue interactions, occurs as the neural tube closes (Henry and Grainger, 1990). At neural tube closure, the newly formed optic vesicle comes into contact with the overlying ectoderm at the site of the future lens. The influence of the optic vesicle, once thought to be sufficient for lens induction (see Saha et al., 1989), may contribute to the last stages of determination and provide late signals necessary for the positioning and differentiation of the lens. Differentiation, beginning with formation of the lens placode at tailbud stages, coincides with the onset of high levels of synthesis of crystallins, the predominant proteins in the lens (Smolich et al., 1994).

Though the properties of bias and specification suggest that new genes are being activated in the PLE, no gene activities have been clearly correlated with the early phases of the lens inductive process. Toward this end, we have studied three recently cloned genes that appear to be involved in the early stages of lens determination. Each, because of its expression pattern, mutant phenotype or biochemical analysis in other organisms, is a good candidate for a key function in lens formation. *Otx-2*, the *Xenopus* homolog of the *Drosophila orthodenticle* gene (Wieschaus et al., 1992), is expressed in

anterior neural tissue and underlying mesoderm from early gastrula stages, and is expressed at a low level in the animal cap of the embryo at these stages as well (Lamb et al., 1993; Blitz and Cho, 1995; Pannese et al., 1995). We show that it is also expressed in the presumptive nasal ectoderm (PNE) from neurula stages onward and in the PLE from mid-neurula through tailbud stages. The paired box- and homeobox-containing gene *Pax-6* shows concomitant expression in presumptive retina and lens tissue, as well as nasal epithelium, brain and spinal cord, from neural plate stages through differentiation of the lens (Hirsch and Harris, 1997). Defects in the *Pax-6* gene result in reduced and defective eyes and missing lenses in the mouse (*Small eye* mutation; Hill et al., 1991). *Pax-6* is highly conserved; for example, it is homologous to the *Drosophila eyeless* gene, which is necessary for eye development (Quiring et al., 1994; Halder et al., 1995). *Pax-6* mutation in humans results in the *Aniridia* syndrome of eye defects (Ton et al., 1991). *Sox-3* is a member of a recently defined class of genes encoding DNA-binding proteins containing the conserved HMG box (Pevny and Lovell-Badge, 1997). This gene shows two phases of expression in the PLE. Maternal, and perhaps early zygotic, *Sox-3* is expressed in animal cap ectoderm encompassing presumptive neural and placodal tissue from blastula through mid-gastrula stages; it is lost from the PLE by the end of gastrulation but is still expressed in neural tissue (R. M. G., unpublished). At later stages, we show here that *Sox-3* is expressed in PLE and PNE, as well as being expressed in midline neural tissue and several cranial ganglia derived from the dorsolateral placode from the time of lens specification through differentiation. Similar *Sox* genes in chick and mouse have been shown to bind to crystallin gene promoters and to be required for crystallin transcription (Kamachi et al., 1995).

In addition to establishing the order in which these genes appear in the PLE during lens bias and specification stages, we have evaluated their responsiveness to the lens inductive environment both in vivo and in vitro. We show that the expression of these genes is tightly linked to the pathway of responses defining the periods of bias and specification in the progressive determination of the lens.

MATERIALS AND METHODS

Embryological procedures

An albino strain of *Xenopus laevis* (Xenopus I) was used for all experiments; embryos were cultured in 1/10× Normal Amphibian Medium (NAM, Slack, 1984); dissections were performed in 3/4× NAM and tissues or embryos subsequently raised in 3/8× NAM. All embryo stages are according to Nieuwkoop and Faber (1994). Animal cap ectoderm was dissected from mid-gastrula (stage 11-11.5) or early gastrula (stage 10-10.5) embryos and used directly in transplantation experiments; late gastrula animal cap ectoderm was obtained by explantation at stage 10-10.5 and culture in vitro under glass coverslip fragments until control embryos reached stage 12-12.5. Donor ectoderm was taken from embryos injected at the 1-cell stage with fluorescein (FLDX) or rhodamine dextran amine (RLDX) as a lineage label (Molecular Probes; described by Gimlich and Braun, 1985).

Three kinds of embryological procedures were performed in this study: transplantations, tissue recombinants and tissue ablations. In transplantation experiments, ectoderm was transplanted into the PLE region or surrounding regions of ectoderm in neural plate stage (stage 14) embryos following removal of the host PLE or non-PLE tissue. To hold transplanted ectoderm in place, embryos were immobilized in clay-lined dishes and curved glass coverslip fragments were put over the transplants. Transplants were allowed to heal and cultured for either a fixed number of hours or to tailbud stages (stages 25-28). In recombination experiments, lineage-labeled ectoderm was co-cultured with stage 14 anterior neural plate tissue along with the underlying mesendoderm of the neural plate. Following dissection in a clay-lined dish, the two pieces were put together and held in place by molding small clay pieces to cover the edges of the recombinant. Recombinants were allowed to heal in place for 1 hour and cultured to stages between 20 and 24. In optic vesicle ablation experiments, stage 16 anterior neural plate (including the optic vesicle anlage) was removed on one side of the embryo, which was immobilized in clay as above. Ablated embryos were allowed to heal in place for 1 hour and cultured to stage 24. All tissues were fixed in 3.8% formaldehyde in MEM (0.1 M MOPS pH 7.4, 2 mM EGTA and 1 mM $MgSO_4$) for 1-2 hours.

Fate mapping was performed by immobilizing stage 14 embryos in clay and directly applying marks of Nile blue to the lateral surface of the anterior end of the embryo. Nile blue was prepared by spreading a 1% Nile blue sulfate, 1% agarose solution on slides, which were then allowed to dry. Small pieces were cut from the slide and the dye applied directly to the embryos. Using an eyepiece grid, initial positions of the blue marks were noted for later reference. Marked embryos were cultured to stage 23 or 35 and assayed for the location of the blue dye with respect to the developing lens ectoderm or differentiated lens.

Analysis of gene expression

In situ hybridization with *Otx-2* (probe courtesy of R. Harland), *Pax-6* (probe courtesy of N. Hirsch), *Sox-3* and *Sox-2* (R. M. G., unpublished) was carried out according to the procedure of Harland (1991) as modified by Doniach and Musci (1995). The modifications entailed washing embryos in 100 mM maleic acid buffer (pH 7.5), 150 mM NaCl (MAB) alone and with 10% BM Blocking Reagent (Boehringer-Mannheim) in MAB prior to and during antibody blocking with lamb serum. Color reaction products were obtained with BM Purple AP Substrate (Boehringer-Mannheim). Immunohistochemistry with γ-crystallin antibody was as described by Henry and Grainger (1990).

Histology and photography

Following in situ hybridization and fixation, embryos were examined as whole mounts; some were infiltrated with 30% sucrose in phosphate-buffered saline (PBS) and embedded in OCT (Tissue-Tek, Miles), frozen and sectioned at 20 μm on a Reichert Ultracut Cryostat. Embryos were photographed on a Zeiss Axioskop microscope with UV fluorescence and transmitted light (sections) or reflected light (whole mounts).

RESULTS

Expression of *Otx-2, Pax-6* and *Sox-3* in the presumptive lens ectoderm during stages of lens bias and specification

Otx-2, *Pax-6* and *Sox-3* are expressed in the developing eye through the periods of lens bias and specification, both inside the neural plate and in the placodal tissue that lies outside the neural folds. Each of the genes is expressed strongly in the lens ectoderm before differentiation commences and before the upregulation of lens crystallin. A time course of the expression of these genes with regard to one another was undertaken following precise fate-mapping studies to determine the order and place in which they appeared during the stages in which bias is acquired and the PLE specified (Fig. 1A,B). A fate map

of the PLE was made by placing Nile blue sulfate marks in the general vicinity of the PLE at stage 14 using an ocular grid to record their placement. After development had proceeded to tailbud stages, blue marks stained cells in either the lens or in nearby tissues. Using these data, a PLE fate map was generated (blue dots on wild-type embryo in Fig. 1A). The area fated to form the lens is shown circumscribed on a stage 14 embryo, which also illustrates PLE expression of *Otx-2* (Fig. 1B).

Otx-2 expression can be detected in the PLE at the open neural plate stage (stage 14-15, Fig. 1B,C), as well as in the PNE (fate maps from Eagleson and Harris, 1989; Eagleson et al., 1995; Fig. 1C) and the presumptive cement gland. It is the earliest gene that we detect in the PLE from the neural plate stage onward and expression remains at a relatively low level from its first appearance through neural tube stages. *Otx-2* expression in the lens ectoderm is still detectable at stage 23-24 (Fig. 1F,I) and disappeared around the time of lens placode thickening, by stage 26. Although *Otx-2* is the earliest gene that we detect in the PLE at stage 14, it is also expressed at a low level throughout the animal cap ectoderm (which would include the PLE) at blastula and gastrula stages (Pannese et al., 1995).

By the late neural plate stage (stage 15-16), *Pax-6* expression arises in small patches (including the PLE) adjacent to the presumptive retinal areas, as well as anteriorly in the PNE (Fig. 1D). *Pax-6* remains on strongly in both the PLE and the retina during stages after neural tube closure (Fig. 1G,J) and after the onset of differentiation of the lens (not shown).

Like *Otx-2*, *Sox-3* is expressed during the lens competence phase throughout the animal cap ectoderm (including presumptive neural and placodal regions) from blastula through gastrula stages. It becomes enriched on the dorsal side and is eventually excluded from the ventral-most ectoderm by the end of gastrulation (not shown). *Sox-3* is no longer expressed in the PLE at neural plate stages (stage 14-15), at the time when lens bias can be detected in the PLE, though it is strongly expressed in the dorsolateral placode (DLP) beginning at this time (Fig. 1E). *Sox-3* is again expressed in the PLE clearly just after neural tube closure (stage 21; Fig. 1H,K) and remains turned on very strongly during lens differentiation (not shown). *Sox-3* is expressed in the PNE beginning at late neural plate stages (stage 16-17, not shown) and continues throughout neural tube and tailbud stages. *Sox-3* expression in the lens ectoderm is particularly striking because, while *Sox-3* is expressed in most of the neural tissue, it is absent from the optic vesicle and presumptive retina as well as the floor plate (Fig. 1K). Strong expression persists in the DLP, just posterior and ventral to the PLE, a region that will contribute to the formation of the VIIth cranial ganglion (Fig. 1H).

In summary, *Otx-2* and *Pax-6* are activated in the PLE during the period of lens bias. When lens specification occurs at the time of neural tube closure, *Otx-2* and *Pax-6* are expressed in the PLE; *Sox-3* is activated just after specification.

Activation of early lens genes in transplanted ectoderm

Two predictions of the hypothesis that these genes are linked to bias and specification are that they would be activated in lens-competent ectoderm but not in non-competent ectoderm and that they would be preferentially activated in competent ectoderm transplanted to the lens-forming region but not to other regions. The first question that we addressed was whether *Otx-2*, *Pax-6* and *Sox-3* would be activated in optimally lens-competent ectoderm placed in the future lens region. The stage 14 anterior neural plate is a potent inducer of the lens (Henry and Grainger, 1990) and the stage 14 presumptive lens-forming region provides a strong in vivo environment for lens induction. The narrow window of lens-forming competence in animal cap ectoderm (Servetnick and Grainger, 1991a) between stages 11 and 12 provides a guide for predicting which responding tissue might activate early lens genes. Transplants were made by removing the stage 14 PLE and replacing it with stage 11-11.5 (lens-competent), stage 10-10.5 (pre-lens-competent) or stage 12-12.5 (post-lens-competent) animal cap ectoderm (Fig. 2A). Following transplantation and culture, embryos were assayed for expression of *Otx-2*, *Pax-6* and *Sox-3* by in situ hybridization (summarized in Fig. 2A). Host and donor marking allowed assessment of whether genes were expressed in tissues transplanted to the PLE region (*Otx-2* expression, Fig. 2B; corresponding lineage labeling, Fig. 2C; *Sox-3* expression, Fig. 2D; corresponding lineage labeling, Fig. 2E). In cases where detection of hybridization signal was difficult to score in whole mount because of transplant placement or adjacent staining, embryos were sectioned for analysis. Sections also revealed transplant staining to be appropriately localized to the inner, or sensorial, layer of ectoderm that normally gives rise to the lens (data not shown). Using lens-competent donor ectoderm, *Otx-2* is present in 92%, *Pax-6* in 82% and *Sox-3* in 94% of transplants, showing strong expression in this tissue in the PLE region. *Otx-2* is expressed at lower levels and in fewer cases in pre-lens-competent ectoderm (16% of transplants), while *Pax-6* is still activated in 71% of the cases. *Sox-3* is present in 79% of pre-lens-competent transplants. The expression of all the genes is greatly reduced in post-lens-competent ectoderm: *Otx-2* in 23%, *Pax-6* in 0% and *Sox-3* in 17% of the transplants.

We hypothesized that the expression of these genes in pre-lens-competent ectoderm might be the result of induction of neural tissue in transplanted ectoderm, since tissue at this stage has a high level of neural competence and because *Otx-2*, *Pax-6* and *Sox-3* are all expressed in neural tissue as well as PLE during lens bias and specification stages. In order to distinguish gene expression in early neural tissue from early placodal tissue, in situ hybridization with *Sox-2* was also performed on embryos receiving transplants of gastrula ectoderm. *Sox-2* is an early neural-specific gene expressed broadly in anterior neural tissues but not expressed in the PLE or the differentiating lens, in contrast to *Sox-3* which is expressed in neural tissues as well as the lens. Pre-lens competent ectoderm transplanted to the PLE region expresses *Sox-2* in 90% of the cases, while lens-competent ectoderm expresses *Sox-2* at a high level in only 14% of transplants (Fig. 2A). These data indicate that a neural response is being activated in the pre-lens-competent ectoderm. Earlier work has shown that such a response is due to homeogenetic neural induction (Servetnick and Grainger, 1991b). We suggest that the *Sox-2* results explain why *Pax-6* and *Sox-3* are activated at high levels in pre-lens-competent ectoderm.

A set of transplants of lens-competent ectoderm to different locations in the embryo was made to test whether early lens genes would be expressed exclusively in the PLE region or

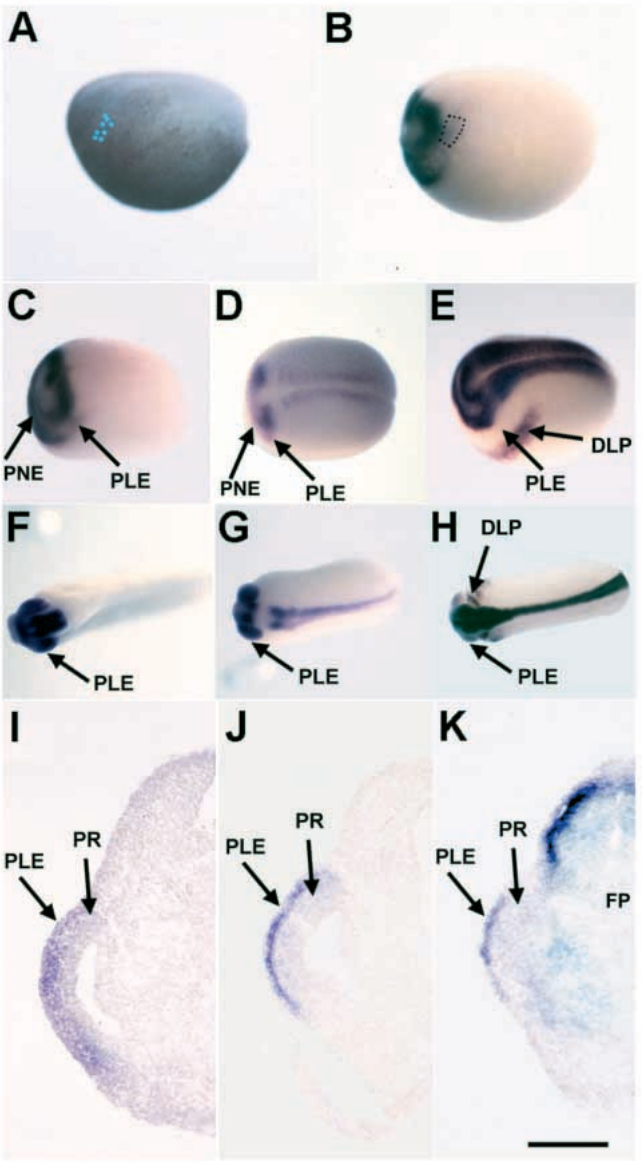


Fig. 1. Expression of genes in the *Xenopus* PLE. (A) Fate-map at stage 14-15 showing the presumptive lens region (blue dots indicate fate-mapping marks) and (B) expression of *Otx-2* in area fate-mapped to be PLE. (C-E) Whole-mount in situ hybridization of stage 14-15 embryos with *Otx-2*, *Pax-6* and *Sox-3*, showing staining in the PLE for *Otx-2* and *Pax-6* but not for *Sox-3*. The dorsolateral placode (DLP) and presumptive nasal ectoderm (PNE) are indicated. (F-H) Whole-mount in situ hybridization of stage 22-24 embryos with *Otx-2*, *Pax-6* and *Sox-3* showing staining in the PLE. (I-K) Transverse sections of embryos following in situ hybridization at stage 22-24 with *Otx-2*, *Pax-6* and *Sox-3* showing staining in the PLE, adjacent to the presumptive retina (PR). The floor plate of the neural tube (FP) is indicated. Scale bar, 160 μm.

might be activated in other placodal regions in the head, or in ventral ectoderm. Transplants were made by removing the stage 14 PLE or a piece of ectoderm anterior, posterior or ventral to the PLE and replacing it with stage 11-11.5 (lens-competent) animal cap ectoderm (Fig. 3A). Transplanted ectoderm was lineage labeled and embryos were assayed for expression of *Otx-2* and *Sox-3* by in situ hybridization. *Otx-2* expression in a transplant posterior to the PLE is illustrated in Fig. 3B. Fluorescent lineage labeling of transplanted ectoderm can be seen in Fig. 3C. *Sox-3* expression in a similar transplant is shown in Fig. 3D and corresponding lineage labeling of transplanted tissue in Fig. 3E. As described above, these genes are present in a high fraction of cases in the presumptive lens-forming region; *Otx-2* is expressed in 92% of the cases and *Sox-3* in 94% of transplants to the PLE region (Fig. 3A). They are also expressed at a very high level in ectoderm anterior to the PLE: *Otx-2* is expressed in 86% and *Sox-3* in 80% of transplants to the anterior region (Fig. 3A). Since these genes are expressed in the PNE (see Fig. 1C,D), the response in anterior transplantations may be due to an early olfactory inductive signal. Posterior transplants were made to encompass the presumptive otic ectoderm and more posterior ectoderm adjacent to the neural plate. In transplants to the posterior region, *Otx-2* is expressed in 17% and *Sox-3* in 54% of the cases (Fig. 3A). The elongated appearance of these transplants is due to convergent extension movements of the adjacent neural tissue, which cause the epidermis to extend along the anterior-posterior axis (Keller, 1975). The expression of *Sox-3* in these transplants may be attributable to signalling associated with activation of DLP properties in this more posterior tissue, since *Sox-3* is also normally expressed at high levels in this tissue (see Fig. 1E,H). These genes are not activated in most cases when transplanted to a ventral site (*Otx-2*, 0% and *Sox-3*, 4%; Fig. 3A).

These transplantation studies varying the age and placement of ectoderm further link expression of *Otx-2*, *Pax-6* and *Sox-3* to an early lens-forming response. We suggest that the appearance of these genes in tissues outside the PLE region may reflect responses associated with the other domains of their expression.

Temporal and spatial dynamics of early lens genes in transplanted ectoderm

While these experiments show that *Otx-2*, *Sox-3* and *Pax-6* are all activated in transplanted lens-competent ectoderm, we also wanted to determine if they are activated in the order seen in vivo. In addition, we wanted to establish whether their expression is localized as in normal development as further tests of how tightly coupled these responses are to the lens induction program. To do this, we examined gene expression in transplanted ectoderm during the first several hours following transplantation to compare with the data from later times described above. This analysis is complicated to some extent because *Otx-2* and *Sox-3* are expressed in animal cap tissue at the time it is transplanted to host embryos. The early expression of *Sox-3* is quite broad, encompassing the presumptive neural and non-neural ectoderm at gastrula stages, though its expression becomes restricted by early neurula stages to the neural plate (Fig. 1E and data not shown). *Otx-2* is also expressed transiently throughout the animal cap at low levels during gastrula stages (Pannese et al., 1995). To distinguish the endogenous kinetics and patterns of expression of these genes from those due to signals from the lens-inducing environment, transplants made to the PLE region were compared with transplants made to the ventral region of the embryo, where a lens-inductive response would not be activated.

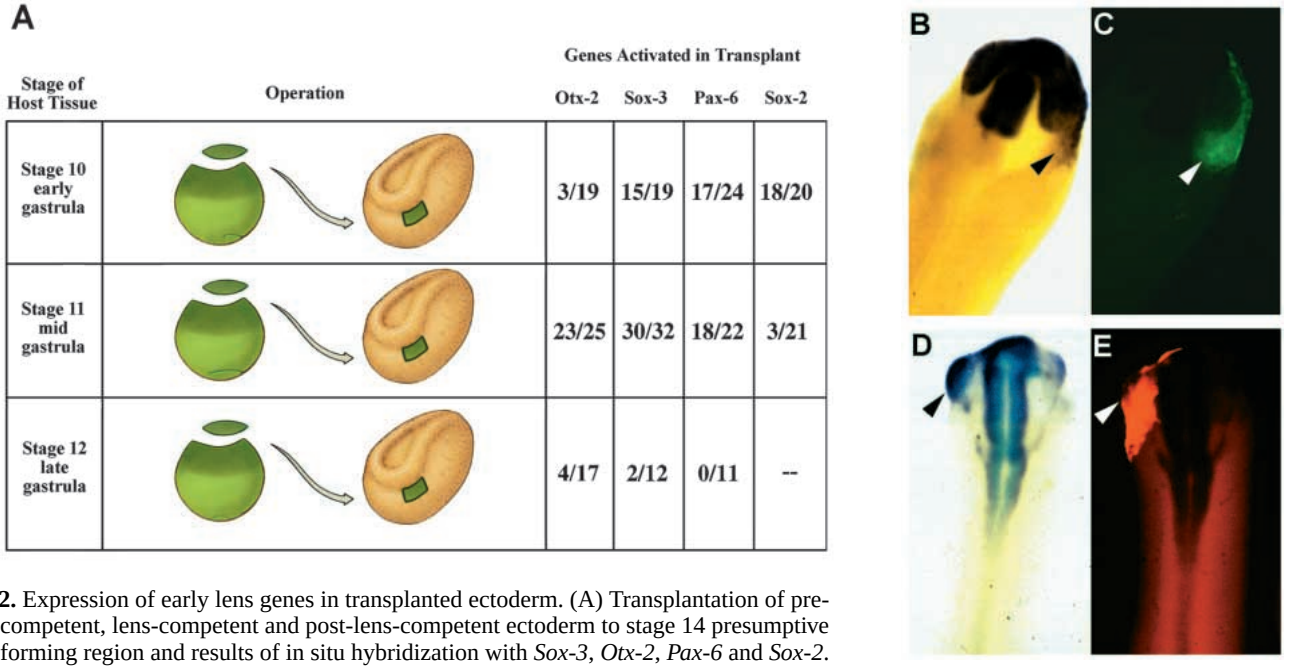
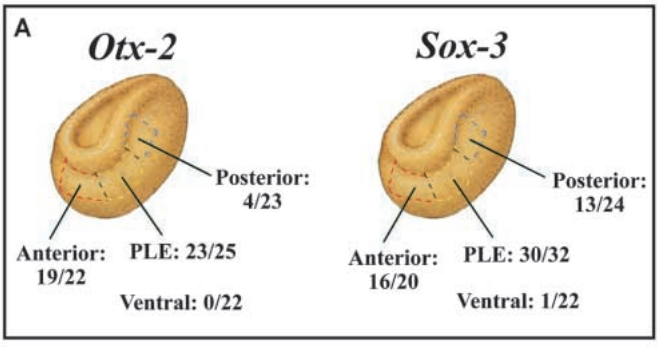


Fig. 2. Expression of early lens genes in transplanted ectoderm. (A) Transplantation of pre-lens-competent, lens-competent and post-lens-competent ectoderm to stage 14 presumptive lens-forming region and results of in situ hybridization with *Sox-3*, *Otx-2*, *Pax-6* and *Sox-2*. Transplants were cultured to stages 21-25 (*Sox-2*) 22-28 (*Sox-3* and *Otx-2*) and 24-28 (*Pax-6*). (B-E) Stage 11-11.5 lineage-labeled ectoderm transplanted to the stage 14 PLE region and cultured to stage 26. (B) In situ hybridization with *Otx-2* and (C) FLDX labeling of transplant, and (D) in situ hybridization with *Sox-3* and (E) RLDX labeling of transplant. Arrowheads indicate the position of the transplant.

Otx-2 appears to be the earliest response to lens-inductive signals. Although low levels of *Otx-2* persist in ectoderm transplanted to a ventral site at 2 hours, expression is no longer detectable at 5 hours (Fig. 4A). When transplanted to the PLE region, expression is already strong at 2 hours and persists through 6 hours (Fig. 4A). While expression is initially broad throughout the transplant, between 6 hours and 12 hours after transplantation expression disappears from the ventral and posterior portions of the transplant and narrows to the region over the optic vesicle (data not shown); expression disappears from the transplanted ectoderm with variable dynamics from one case to another. Expression is lost in the transplanted ectoderm between host stages 26 and 32 (ectodermal stages 24 to 29/30). This is consistent with the time when its expression can no longer be normally detected in PLE. The pattern of *Otx-2* expression in such transplants suggests that it is a very early



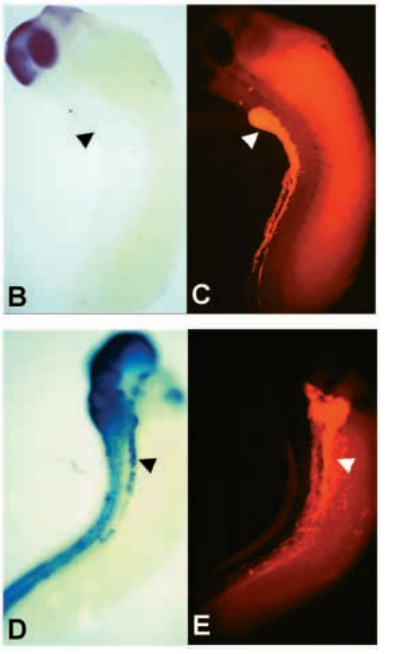


Fig. 3. Expression of early lens genes in ectoderm transplanted to different sites of neural plate stage hosts. (A) Transplantation of lens-competent ectoderm to stage 14 presumptive lens-forming region or surrounding regions, culture to stages 22-28 and in situ hybridization with *Otx-2* and *Sox-3*. *Otx-2* and *Sox-3* are activated with high frequency in transplants to the PLE area or anterior, with a reduced frequency in the posterior. *Otx-2* and *Sox-3* are not activated in transplants to the ventral region. (B-E) Stage 11-11.5 lineage-labeled ectoderm transplanted to the stage 14 posterior placodal region and cultured to stage 27. (B) In situ hybridization with *Otx-2* and (C) RLDX labeling of transplant, showing no expression. (D) In situ hybridization with *Sox-3* and (E) RLDX labeling of transplant, showing expression. Arrowheads indicate the position of the transplant.

response to the lens-inducing environment, as it appears to be in vivo.

Pax-6 is also activated shortly after transplantation. While it cannot be detected at 2 hours or 4 hours, as is seen for *Otx-2*, positive signals are detected by 6 hours (Fig. 4A). Expression of *Pax-6* appears to be more localized from its inception than that of *Otx-2* (data not shown) and the time of its activation corresponds to the stage (ectodermal stage 15) when *Pax-6* is first detected in the PLE in vivo (Fig. 1D).

Sox-3, like *Otx-2*, is expressed in ectoderm at the time of transplantation but does not appear to be upregulated further until much later stages. At 2 hours subsequent to transplantation, a strong signal is still seen in ventral ectoderm. However, expression of *Sox-3* is far weaker in ventral transplants at 5 hours while remaining strong in ectoderm transplanted to the presumptive lens region (Fig. 4A). Whether the decrease seen in transplants to the ventral site is due to an autonomous process in ectoderm, or an inhibitory influence from the ventral environment, is not known; however, ribonuclease protection analysis indicates that *Sox-3* mRNA levels drop autonomously in isolated animal cap ectoderm at similar stages (data not shown). An example of a transplant showing *Sox-3* expression and lineage label in ectoderm 8 hours after being transplanted to the presumptive lens site is shown in Fig. 4B,C. The *Sox-3* signal remains strongly expressed throughout transplanted tissue through stage 22 (not shown). Over the next 3 hours (by stage 25), however, *Sox-3* expression disappears in most of the transplanted ectoderm. At the same time, expression is upregulated specifically in the tissue overlying the optic vesicle (Fig. 4D, E) and remains turned on in the developing lens. This activation approximately corresponds to the stage at which *Sox-3* is activated in vivo (Fig. 1H).

In summary, the three genes under study appear to be activated in transplants in the order, and with the approximate time course, that they are in vivo. These observations are complicated by pre-existing *Otx-2* and *Sox-3* transcripts in the ectoderm used for transplantation; complementary transplants to a ventral site in the embryo provide a control environment, however, for assessing the significance of their expression.

Activation of early lens genes in tissue recombinants

Lens determination occurs in a number of stages over several hours and may require signals from more than a single tissue (Grainger, 1996). The early lens response genes that we describe here provide new assays for the initial stages in the induction process. While activation of crystallin synthesis serves as a definitive indication of lens determination, it is likely to be activated only in response to all of the interactions needed for lens induction. As an initial test to determine whether early markers are induced in a tissue recombinant system for studying lens induction, we examined their activation in animal cap ectoderm combined with anterior neural plate tissue. Lens induction studies discussed above (Henry and Grainger, 1990) have suggested that the anterior neural plate provides the primary lens-inductive signal. By preparing such recombinants we could determine directly whether the neural plate is sufficient as a lens inducer, testing its activity without the additional variables potentially introduced by other regions of the embryo.

Stage 14 anterior neural plates with underlying mesendoderm were recombined with lens-competent animal caps (stage 11-11.5) and subsequently analyzed for either γ-crystallin synthesis or expression of early response genes to compare the efficacy of this system in activating different lens responses. In recombinants aged to stage 40, crystallin immunoreactivity was induced in 68% of the cases (Fig. 5J-L). While γ-crystallin is present in many tissues at a low level in early development (Smolich et al., 1994), this expression is undetectable by antibody staining. To examine earlier lens responses, recombinants were cultured to between stages 20 and 24 (Fig. 5A) to assay for the expression of *Otx-2* (Fig. 5B), *Pax-6* (Fig. 5D) and *Sox-3* (Fig. 5F) by in situ hybridization. Responding ectoderm was lineage labeled with FLDX (Fig. 5C,E,G). Anterior neural plates induced a high percentage of cases with intense activation of the early genes in the responding ectoderm. *Otx-2* was expressed in the responding tissue in 92%, *Pax-6* in 45% and *Sox-3* in 100% of the recombinants (Fig. 5A). As with transplantation experiments above, in situ hybridization with the neural gene *Sox-2* was performed in some recombinants to test whether the responses in ectoderm were attributable to neural domains of expression of the early lens genes. *Sox-2* was activated in lens-competent responding tissue in only 20% of recombinants, indicating that the activation of *Otx-2*, *Pax-6* and *Sox-3* in lens-competent ectoderm was in most cases due either to an early lens response or activation of other placodal tissue expressing these genes (e.g., olfactory epithelium). Expression of *Otx-2* and *Sox-3* is very broad in the responding ectoderm; the *Pax-6* response is activated in a more narrow domain (Fig. 5B-G). Recombinants cultured for longer periods of time show activation of *Sox-3* in induced ectoderm in close association with histologically identifiable retinal tissue, suggesting that this gene is activated in lens tissue (data not shown).

These data demonstrate that the early lens genes that we have analyzed are expressed in response to signals from the anterior neural plate. Furthermore, expression of these genes in a higher percentage of cases than expression of crystallin indicates that a greater fraction of cases may begin the lens induction program under these conditions than go on to complete lens determination. Expression of the early lens genes may therefore represent a necessary, but not sufficient phase of gene expression en route to determination of the lens or may be due to a more general activation of early placodal properties.

Signalling from the optic vesicle is necessary for *Sox-3* expression following lens specification

In addition to the lens-inducing signals from the neural plate, later signals from the optic vesicle contribute to lens formation as well. Whether optic vesicle signals are required for lens determination is not certain since lens specification occurs so close to the time when the optic vesicle contacts the PLE at the time of neural tube closure (Henry and Grainger, 1990). Since *Sox-3* is activated after the PLE contacts the optic vesicle (Fig. 1H) and because members of the *Sox* family are required for crystallin activation (Kamachi et al., 1995), we designed experiments to see whether the optic vesicle was required for *Sox-3* expression. To do this, we monitored *Sox-3* after ablation of the anterior neural tissue that will form the optic vesicle at late neural plate stages (stage 16), shortly before neural tube

closure. That the PLE remains intact in these ablations has been shown in previous work (Grainger, 1992). While the unoperated side shows normal expression of *Sox-3* at stage 24 (Fig. 4G), the ablated side shows no PLE expression (0 of 34 cases; Fig. 4F). This demonstrates that the program of *Sox-3* expression in the PLE is dependent upon signals from the optic vesicle. In parallel experiments harvested at later stages, the ablated side also failed to express crystallin (0 of 16 cases; data not shown). This indicates that, under these conditions, the optic rudiment is necessary for the activation or maintenance of *Sox-3* in the PLE and to permit lens specification.

DISCUSSION

The embryologically defined periods of lens bias and specification have not previously been linked with the activity of specific genes; *Otx-2*, *Pax-6* and *Sox-3* are activated in the PLE during these time frames and were thus evaluated to assess how tightly their expression is linked to the lens determination process. We have asked whether these genes are expressed appropriately in transplanted ectoderm, and find that they respond to the presumptive lens and anterior head environment, with preferential maintenance and/or activation in lens-competent ectoderm. We have shown that the expression of *Otx-2*, *Sox-3* and *Pax-6* is activated in response to a signal from the neural plate, which has been implicated as the primary lens-inducing tissue. We have also shown that expression of *Sox-3* requires a later signal from the optic vesicle. We have thus established tight coupling between the lens-inductive responses of ectoderm and the activation of the early lens genes *Otx-2*, *Pax-6* and *Sox-3*. This discussion will address the possible roles of these genes in lens induction, as well as their potential use in elucidating as yet uncharacterized elements of the lens inductive pathway.

Comparison of gene expression in the *Xenopus* PLE with other systems

There is some corroborative evidence from other organisms to support our observations about the timing of activation of the early lens genes that we describe here, though the stages at which lens-inductive events occur (such as specification) are less well-defined than in *Xenopus*. Mouse *Otx-2* is expressed broadly in embryonic ectoderm by embryonic day (E) 7.5 (early neural plate stage), and by E8.5 (neural fold elevation) in the forebrain, midbrain, presumptive eye regions and other areas of the head (Simeone et al., 1992; Ang et al., 1994). Studies in our laboratory indicate that *Otx-2* expression becomes restricted to the PLE and PNE by E8.5 and, as in *Xenopus*, is lost from the PLE by the time placode thickening occurs (M. Fisher and R. M. G., unpublished). Early expression of *Otx-2* is unaffected in *Small eye* (*Pax-6*) mutants, suggesting that *Otx-2* be placed before *Pax-6* activation in the hierarchy of lens responses (M. Fisher and R. M. G., unpublished).

As shown here, *Pax-6* is not expressed in the *Xenopus* PLE until mid-neural plate stages, when expression is already evident in the adjacent presumptive retina. *Pax-6* expression is first seen in the mouse in the E8-8.5 developing central nervous system (Walther and Gruss, 1991) and ectoderm and mesenchyme of the head. By E8.5-9.5, *Pax-6* expression is restricted to discrete staining in the PLE and PNE and stays turned on in the lens through differentiation (M. Fisher and R. M. G., unpublished). This is in contrast to the report that *Pax-6* is expressed early in the chick PLE, by late gastrula stages (Li et al., 1994).

A striking similarity is seen between the early ectodermal expression of mouse *Sox-2* and *Xenopus Sox-3*. Mouse *Sox-2*, the mouse *Sox* family gene showing the highest degree of expression conservation with *Xenopus Sox-3*, is expressed broadly in neural tissue and anterior head ectoderm by E8.5 (Collignon et al., 1996), then turned off in head ectoderm. *Sox-2* expression then appears to arise strongly in the PLE just prior to and at the time of lens placode thickening (E 9.5, Fisher et al., submitted). These observations are consistent with our findings for *Xenopus Sox-3*, which is not expressed in the PLE between the time of lens competence and lens specification, and which, following specification, remains on strongly in the developing lens. This conservation of expression patterns implies that general mechanisms may underlie these processes in evolutionarily diverged organisms.

Comparison of dynamics of early gene expression in transplanted and recombined ectoderm with in vivo expression

The early genes whose expression we have analyzed from the neural plate stage onwards are activated in the PLE in the order: *Otx-2*, *Pax-6* and *Sox-3*. The sequence of their appearance led to the proposal that this ordered expression might be necessary for the gradual changes that take place in the PLE between the neural plate stage and lens differentiation. As an initial test of this idea, we assayed whether the expression of these genes is an obligate response to lens-inducing environments both in transplants and in tissue recombinants. In addition, we observed the order of their activation in competent ectoderm shortly after transplantation to a strong lens-inducing environment.

The order of gene activation in the latter transplantation experiments appears to be *Otx-2*, *Pax-6* and *Sox-3*, though this assessment requires taking into account initial patterns of expression in the ectoderm; *Sox-3* and *Otx-2* are expressed in the gastrula-stage ectoderm used for transplants. *Sox-3* expression in the animal cap is lost in vivo between stages 11.5 and 12.5. We have evidence (R. M. G., unpublished data) that this may be due to a putative ventral signalling center repressing *Sox* gene expression in the ectoderm during this time. Since *Sox-3* shows two phases of expression in the PLE, one in the gastrula stage and one after neural tube closure (following lens specification), the repressor of its expression is likely acting transiently at the end of gastrulation. This might explain why the early expression, which disappears in ectoderm transplanted to the ventral region of the embryo, is maintained in ectoderm transplanted to the presumptive lens site. The early broad expression of *Sox-3* is gradually lost in transplanted ectoderm and later expression of *Sox-3* is activated as it is in vivo in a localized region adjacent to the optic vesicle. *Otx-2* is also present in the animal cap ectoderm at the time of transplantation to the presumptive lens region. Its maintenance in the presumptive lens area, but not in more ventral regions of transplanted ectoderm, is apparently in response to lens-inducing signals from the anterior neural plate. The low number of positive cases of *Otx-2* expression using pre-lens-competent ectoderm (Fig. 2A) indicates that the positive cases seen in the transplants with lens-competent ectoderm represent

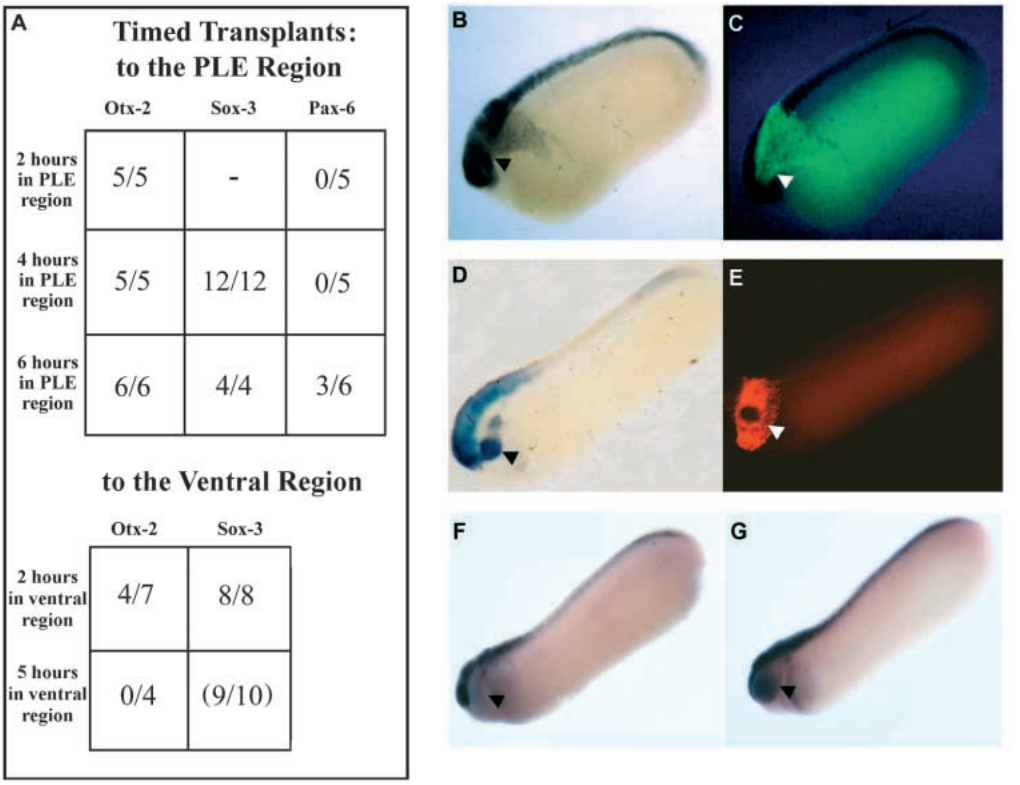


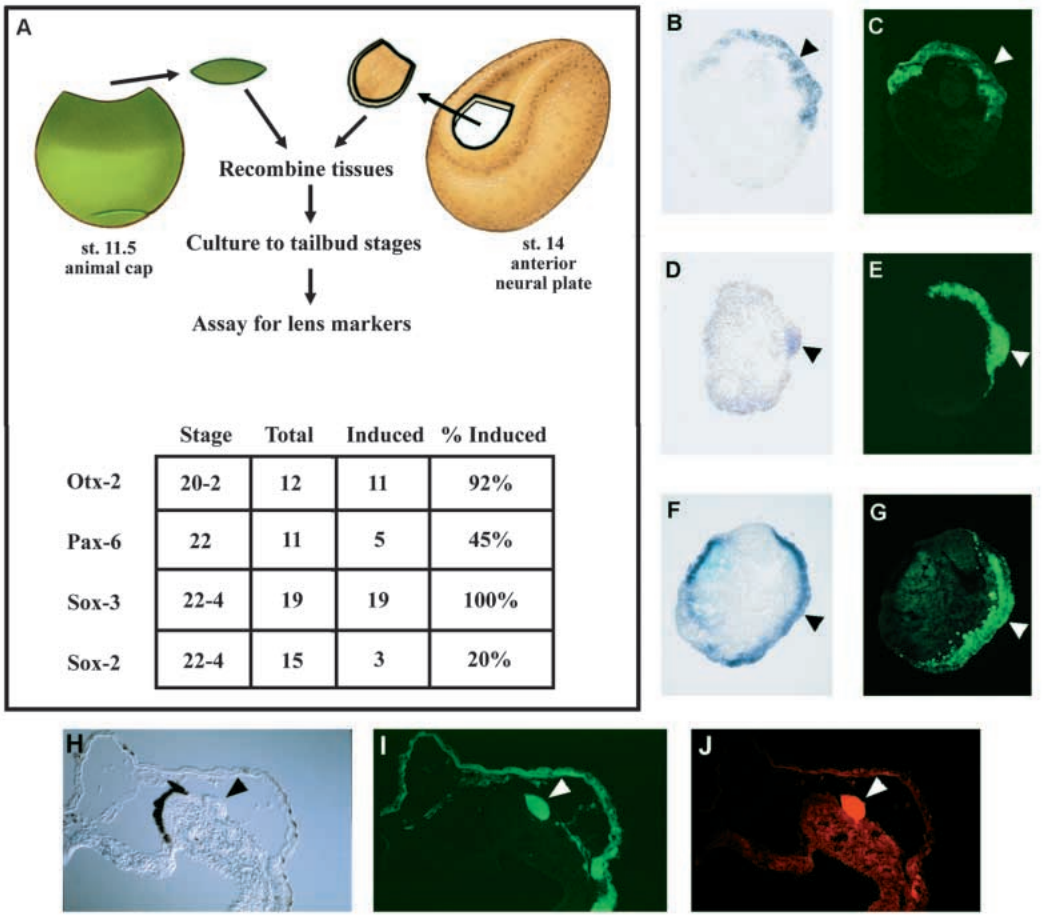
Fig. 4. Dynamics of early gene responses to lens-inducing signals. (A) Summary of transplants of lens-competent ectoderm to the stage 14 PLE area followed by incubation of embryos for 2, 4, or 6 hours and in situ hybridization for *Otx-2*, *Sox-3* and *Pax-6*. Transplants to the ventral region were incubated for 2 or 5 hours followed by hybridization with *Otx-2* and *Sox-3* probes. Expression of *Otx-2* is detected first in transplants after 2 hours, followed by *Sox-3* (4 hours) and *Pax-6* (6 hours). Number of positive cases over number of total cases is shown. (B-E) Expression of *Sox-3* in stage 11-11.5 lineage-labeled ectoderm transplanted to the stage 14 PLE area. (B-C) Transplant cultured to host stage 22 (8 hours); (B) in situ hybridization with *Sox-3* and (C) FLDX labeling of transplant. (D,E) Transplant cultured to host stage 27; (D) in situ hybridization with *Sox-3* and (E) RLDX labeling of transplant. Arrowheads indicate the position of the transplant. (F,G) Optic vesicle-dependent *Sox-3* expression in the PLE demonstrated by ablation of the stage 16 anterior neural plate on one side of the embryo. After optic vesicle removal, embryos were cultured to stage 24. (F) Operated side, showing no *Sox-3* expression in PLE; (G) control side of same embryo, inverted image. The PLE area is indicated by arrowheads.

a specific response to the lens-inducing environment. Transplantation to the PLE region appears to be insufficient to activate neural *Otx-2* expression. This suggests that the neural tissue induced in pre-lens-competent ectoderm is of a more posterior character. The variability seen in the rate of disappearance of *Otx-2* in different transplants (see Fig. 4A) may have occurred because of the way animal caps were taken in these experiments. *Otx-2* expression is less extensive in the ventral than in the dorsal animal cap (Blitz and Cho, 1995) and in our experiments animal cap tissue, including both dorsal and ventral regions, was placed in random orientations into the transplantation site.

These early genes are all strongly activated in tissue recombinants, very broadly throughout the ectoderm for *Otx-2* and *Sox-3*, and in smaller patches for *Pax-6* (Fig. 5B-G). The focused patch of expression in response to the isolated anterior neural plate is consistent with the initially small *Pax-6* pattern seen in the PLE at late neural plate stages (Fig. 1D), though at this stage *Pax-6* is also present at a low level in the PNE. One possible reason for the broad activation of *Otx-2* and *Sox-3* is that these genes are normally expressed very strongly in other regions of head ectoderm, *Sox-3* in the cranial placodes, *Otx-2* in the cement gland primordium and both in the PNE (see Fig. 1 and text).

Developmental implications of *Otx-2*, *Pax-6* and *Sox-3* expression in several neural and placodal tissues

Although *Otx-2*, *Pax-6* and *Sox-3* expression are tightly coupled to lens responses, their expression in several anterior neural regions and sensory tissues raises important questions: does the sharing of domains of expression have functional significance and how then is specificity in these different tissues generated? The expression of *Otx-2* and *Pax-6* in both retinal and lens components of the eye during determination raises the possibility that an important function involved in organizing or generating these components of the eye has been activated and preserved during evolution. The expression of *Otx-2*, *Pax-6* and *Sox-3* in both presumptive olfactory and lens ectoderm is consistent with the proposal that these tissues may share a common evolutionary origin (Gans and Northcutt, 1983) or at least that the genetic machinery used to initiate these different developmental programs is shared. It has been suggested that *Pax-6* has been co-opted for use in different sensory structures during evolution (Callaerts et al., 1997).



	Stage	Total	Induced	% Induced
Otx-2	20-2	12	11	92%
Pax-6	22	11	5	45%
Sox-3	22-4	19	19	100%
Sox-2	22-4	15	3	20%

Fig. 5. Activation of lens markers in recombinants. (A) Schematic of procedure and results of in situ hybridization with *Otx-2*, *Pax-6*, *Sox-3* and *Sox-2*. Stage 11-11.5 lineage-labeled ectoderm is co-cultured with stage 14 anterior neural plate and the mesendoderm underlying the neural plate to ectodermal stage 20-24. Induced cases indicates the number of recombinants expressing each gene in the lineage-labeled ectoderm. (B-G) Ectodermal expression of early genes in ectoderm combined with anterior neural plate. (B) In situ hybridization with *Otx-2* and (C) FLDX labeling of ectoderm. (D) In situ hybridization with *Pax-6* and (E) FLDX labeling of ectoderm. (F) In situ hybridization with *Sox-3* and (G) FLDX labeling of ectoderm. Arrowheads indicate the responding ectoderm. (H-J) Expression of γ-crystallin in ectoderm combined with anterior neural plate and cultured to stage 40. (H) Light micrograph, (I) FLDX labeling of ectoderm and (J) TRITC-conjugated antibody detection of γ-crystallin. Arrowheads indicate the induced lens.

Another possibility is that there are shared elements in the early stages of sensory development linking the developmental programs for sensory structures (Jacobson, 1966).

Diversity seen in different sensory structures clearly must be generated by activity of genes like *Otx-2*, *Pax-6* and *Sox-3* in conjunction with other genes to provide unique regulatory systems in different tissues. The *distal-less* homeobox gene family may contribute to this genetic diversity since members of this family are expressed in different sensory and neural tissues; for example, *dll-3* is expressed in the presumptive olfactory ectoderm but not in lens ectoderm (Papalopulu and Kintner, 1993). Different members of the *eyes absent* gene family are also expressed in ectodermal regions fated to form particular sensory structures and have been shown to be essential for eye development (Xu et al., 1997) and ear development (Abdelhak et al., 1997).

Defining bias and specification

Acquisition of a lens-forming bias in head ectoderm, which occurs between the time of lens competence and specification of the PLE, has been described as an intermediate step in tissue patterning (Grainger et al., 1997). How many other organ and tissue inductions require such intermediate steps is not known; however, it seems probable that the acquisition of intermediate states in the determination process and the spatial localization associated with a lens-forming bias will be seen in other tissues. For example, this is suggested in the case of neural induction where a strong predisposition of ectoderm toward neural tissue formation is acquired at gastrula stages (Gould and Grainger, 1997).

We have shown linkage between lens bias and the expression of *Otx-2* and *Pax-6*; beyond these candidates, there are likely to be other genes that contribute to establishing bias. The contribution of other genes toward generating specificity in sensory tissue formation is already discussed above. In addition, transplantation experiments indicate that a lens-forming bias already exists in the PLE by the neural plate stage (Henry and Grainger, 1987). Another point that must be

considered is that in this study we have analyzed the expression of these genes at the RNA level; since the appearance in the PLE of transcripts for *Otx-2* and *Pax-6* only occurs at neural plate stages and may precede the presence of functional protein by a significant amount of time, it is possible that these early genes are not acting until bias has already been established. Taken together, these results suggest that *Otx-2* and *Pax-6* correspond to late phases of bias, and that the initiation of bias is likely associated with genes activated in the head ectoderm which have not yet been identified.

At present, we have not identified genes that are activated at the time of lens specification, concomitant with neural tube closure. It is possible that accumulation of *Otx-2* and *Pax-6* protein after the neural plate stage contributes to specification. *Sox-3* is expressed in the lens ectoderm after specification has occurred (though before any outward signs of differentiation); our data indicate that it appears to require a signal from the optic vesicle for its activation. Whether a signal from the optic vesicle is required for lens specification remains unsettled since the time of specification so closely accompanies the time of optic vesicle contact with the PLE after neural tube closure. Although the optic rudiment is required to activate *Sox-3* expression (and for lens formation) in the experiments described here, this may be due to a repressive effect of migrating neural crest normally blocked from contact with the PLE by its contact with the optic vesicle rather than a positive effect of optic tissue.

Following specification, there is evidence that *Sox* gene products bind to γ-crystallin promoters in mouse and δ-crystallin promoters in chick and that Pax-6 binds to α and δ crystallin promoters in chick and α-crystallin in mouse (Cvekl and Piatagorsky, 1996). These factors have been shown to be necessary for lens-specific crystallin gene expression. Since Pax-6, Sox-2 and several other factors have been shown to bind crystallin promoters, it is probable that there is a combinatorial effect of multiple factors binding to effect crystallin upregulation in the lens.

Further understanding of the lens induction pathway

The embryological data defining the multiple steps leading to lens determination as well as the temporally distinct phases of gene expression in the PLE that are described here suggest that lens induction involves a sequence of signalling events. Previously it has not been possible to monitor intermediate responses in the PLE, however. The patterns of expression of the early genes characterized here are likely to provide useful assays for these intermediate responses, permitting a much more subtle evaluation of the tissue interactions required for the early stages of lens induction than has been possible to date. In addition, these genes should be very useful in the search for inducing factors involved in early patterning events, again by providing assays for early signalling events not previously possible. One caveat to this approach is the possibility that inducers of other placodal structures might be identified as well, since some early gene responses are shared by different tissues. This is not, however, necessarily a source of ambiguity: because of issues of evolutionary conservation that have already been described, these different sensory structures are likely to share common elements of genetic machinery.

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