

Pax6 induces ectopic eyes in a vertebrate

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SUMMARY

We report here that misexpression of the transcription factor Pax6 in the vertebrate *Xenopus laevis* leads to the formation of differentiated ectopic eyes. Multiple molecular markers indicated the presence of mature lens fiber cells, ganglion cells, Müller cells, photoreceptors and retinal pigment epithelial cells in a spatial arrangement similar to that of endogenous eyes. Lineage tracing experiments showed that lens, retina and retinal pigment epithelium arose as a consequence of the cell-autonomous function of Pax6. These experiments also reveal that the cell autonomous activity of misexpressed Pax6 causes the ectopic expression of a number of genes including

Rx, *Otx2*, *Six3* and endogenous *Pax6*, each of which has been implicated in eye development. The formation of ectopic and endogenous eyes could be suppressed by coexpression of a dominant-negative form of Pax6. These data show that in vertebrates, as in the invertebrate *Drosophila melanogaster*, Pax6 is both necessary and sufficient to trigger the cascade of events required for eye formation.

Key words: Pax6, Eye, *Xenopus laevis*, Ectopic organ, Induction, Vertebrate

INTRODUCTION

Experimental evidence suggests that vertebrate eye development is dependent upon coordinated inductive interactions between presumptive neural retina and the surface ectoderm that will give rise to the lens (Spemann, 1938; Jacobson, 1958; Grainger, 1996). The first morphological signs of eye development are observed in neurulating embryos with the evagination of the future forebrain to give the optic pit (Kaufman, 1992). As the evaginating neural tube approaches the surface ectoderm and future lens, it becomes more extended in shape and forms the optic vesicle. Close contact between the optic vesicle and surface ectoderm is followed by a coordinated invagination of these tissue layers and results in the formation of the lens vesicle within the optic cup (Kaufman, 1992). The anterior deficiencies and anophthalmia present in mouse embryos carrying null mutations in *Pax6* (Hill et al., 1991), *Rx* (Mathers et al., 1997) and *Otx2* (Acampora et al., 1995; Matsuo et al., 1995) indicate a role for these transcription factors at this early stage of eye development. Embryological manipulations support the idea that eye formation also requires reciprocal signaling between the neurally derived optic cup and the ectodermally derived lens (Jacobson, 1958; Cuny and Malacinski, 1986; Grainger, 1996).

The paired-class transcription factor Pax6 is known to be critical for eye development (Callaerts et al., 1997). This

conclusion is drawn from analyses showing that mutations in the *Pax6* gene cause the *ANIRIDA* syndrome in humans (Glaser et al., 1992; Jordan et al., 1992), the *Small eye* phenotype in mouse (Hill et al., 1991) and the *eyeless* phenotype in flies (Quiring et al., 1994). As would be expected, *Pax6* is expressed in presumptive eye tissues with a pattern implying a role in early development of both lens and retina (Walther and Gruss, 1991). Both the semi-dominant pattern of inheritance of *Pax6* mutant phenotypes (Hill et al., 1991; Jordan et al., 1992) and experimental manipulations using transgenes based on the *Pax6* locus (Schedl et al., 1996) indicate that achieving the correct level of Pax6 expression is important for development of a normal eye. Pax6 misexpression studies have also proved revealing. In *Drosophila*, expression of mouse or *Drosophila eyeless* (*ey*, the *Drosophila* equivalent of *Pax6*) in imaginal discs resulted in the formation of ectopic eyes (Halder et al., 1995) while the misexpression of Pax6 in a vertebrate resulted in the formation of ectopic lenses (Altmann et al., 1997). Combined, these observations suggested that at least some of the functions of Pax6 in eye development were conserved from invertebrates to vertebrates (Quiring et al., 1994; Gehring, 1996).

In the present study, we describe experimental conditions in which Pax6 can induce small but fully differentiated ectopic eyes in the frog *Xenopus laevis*. Based on this, we suggest that in the context of the vertebrate embryo, Pax6 is sufficient to initiate the regulatory cascade for lens and eye development.

MATERIALS AND METHODS

Microinjection and lineage tracing

Xenopus embryos were injected with RNA at the 16-cell stage according to established protocols (Smith and Harland, 1991) using DNA templates CS2-XPax6-Flag (Altmann et al., 1997), nuclear localized β -galactosidase (Nuc-lacZ; Smith and Harland, 1991), CS2-follistatin-HA (encoding a follistatin-haemagglutinin fusion protein) and CS2-*Pax6* Δ CT. The latter encoded a dominant-negative mutant of *Pax6* (Singh et al., 1998) and was constructed by cleaving the open reading frame of CS2-XPax6 at a unique *AccI* site separating the homeodomain and PST domain of *Pax6*. The modified open reading frame was predicted to terminate at a fortuitous stop codon within the multiple cloning sequences of the CS2 vector. The concentration of *Pax6* RNA that was optimal for a phenotypic response varied from experiment to experiment and was in the range of 100–200 pg. Lineage tracing was performed as previously described (Smith and Harland, 1991) with the exception that the RedGal substrate (Research Organics, Inc.) was used.

In situ hybridization and immunostaining

In situ hybridization (Smith and Harland, 1991) and immunostaining (Maniatis et al., 1982) were performed according to established protocols. The following templates were used; *Otx2* (Lamb and Harland, 1995), *Pax6* 3' UTR (Hirsch and Harris, 1997) *Rx* (Mathers et al., 1997) and *Six3* (this paper). Antibodies were used at the following dilutions: rabbit anti-bovine β -crystallin (Zigler and Sidbury, 1976) 1:1000; mouse anti-rhodopsin (Hicks and Molday, 1985) 1:100; rabbit anti-Islet-1 (gift from T. Jessell) 1:500; mouse anti-glutamine synthetase (Sigma) 1:500; mouse monoclonal XAR-1 (Sakaguchi et al., 1997) 1:250. Secondary antibodies used were rhodamine-conjugated goat anti-rabbit (Molecular Probes) and Alexa 488-conjugated goat anti-mouse (Molecular Probes) at 1:100. An alkaline phosphatase-conjugated mouse anti-digoxigenin antibody (Boehringer Mannheim Biochemicals) was used for whole-mount in situ hybridizations at a dilution of 1:2000. For sections that were stained multiple times, immunofluorescence was performed twice on the same slide. The photographed fluorescence image from the first round of staining was converted in Adobe Photoshop to another color and subsequently superimposed onto the image obtained from the second round of staining.

Cloning of *Xenopus laevis* *Six3*

Xenopus stage 28 head cDNA library (Hemmati-Brivanlou et al., 1991) generated in the vector λ ZAPII was screened using a radiolabeled probe generated from a murine *Six3* cDNA fragment (Oliver et al., 1995) according to established protocols (Maniatis et al., 1982). Sequence alignments of a partial clone obtained (GenBank accession AF175342) revealed highest homology at both nucleotide and amino acid level to chicken, human and mouse *Six3*.

RT-PCR

The protocol for RT-PCR has been described previously (Smith and Harland, 1991). The primers used for RT-PCR analysis are either in the cited references or as listed; β B1-crystallin (Altmann et al., 1997); cardiac actin, NCAM and EF-1 α (Hemmati-Brivanlou and Melton, 1994); rhodopsin, 5' ttacttagcagagccatggc, 3' caaagatgaagtaccgtgc.

RESULTS

Pax6 misexpression has multiple effects on eye development

We modified an established protocol (Altmann et al., 1997) for injection of *Pax6* RNA to determine whether we could unveil additional embryological activities. A series of injection sites, RNA concentrations and times were tested and the phenotypic consequences examined in whole *Xenopus* embryos. It was found that when embryos were injected in one blastomere at the 16-cell stage or in two blastomeres at the 32-cell stage they displayed numerous eye-related phenotypes at a frequency of up to 50% when examined as tadpoles (stage 48; Nieuwkoop and Faber, 1967; Fig. 1, Tables 1 and 3). The phenotypes were concentration-dependent and included (i) the formation of isolated ectopic lenses (Fig. 1A–C), (ii) defects in the eye region proximal to the neural tube (Fig. 1D), (iii) the appearance of ectopic retinal pigment epithelium (RPE; Fig. 1D, arrow, 1E, open arrowhead), and (iv) ectopic eyes (Fig. 1E). In tadpoles, ectopic lenses (Altmann et al., 1997) were observed as pearl-like objects adjacent to the ectoderm (Fig. 1A, arrows). In section, these lenses showed a lens epithelial layer (Fig. 1B) and labeled in the fiber cell region with antibodies to the lens-specific β -crystallins (Zigler and Sidbury, 1976; Piatigorsky et al., 1984; Altmann et al., 1997; Fig. 1C).

Proximal eye defects

The most prevalent phenotype observed in embryos

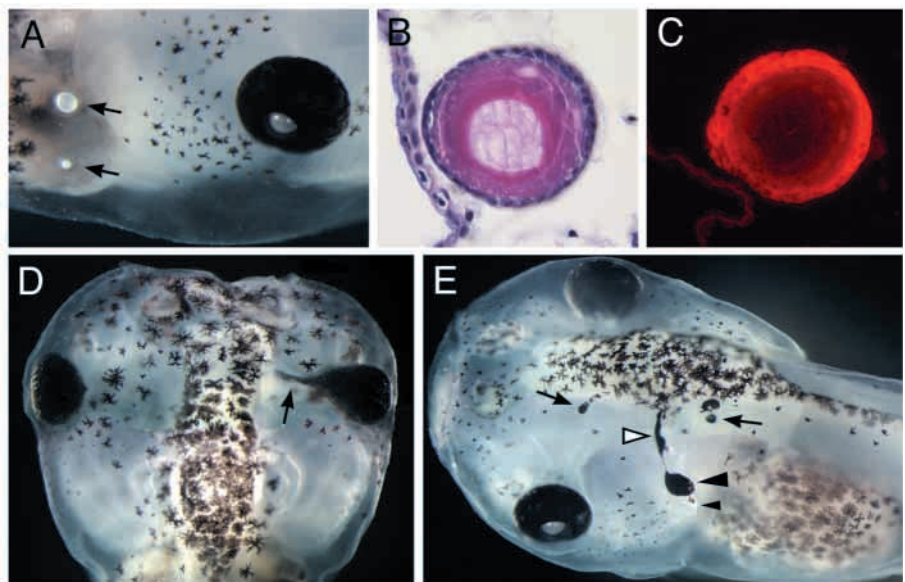


Fig. 1. *Pax6* misexpression results in eye-related phenotypes. See Tables 1 and 3 for summary of results. *Xenopus* embryos were injected with 160 pg *Pax6* RNA in one animal pole blastomere at the 16-cell stage and fixed at stage 48. (A) Ectopic lenses (arrows) in the absence of retinal tissue. (B) Hematoxylin and eosin stained section showing an isolated ectopic lens adjacent to surface ectoderm. The lens fiber cell mass is surrounded by hematoxylin stained nuclei of the lens epithelial layer. (C) β -crystallin immunolabeling of the ectopic lens in B. (D) Proximal eye defect in right eye displaying an extension of RPE (arrow) towards the midline. (E) Ectopic eye displaying a lens-like structure (small arrowhead) in association with an optic cup-like structure (large arrowhead), extension of RPE towards the midline (white arrowhead) and additional ectopic RPE-like structures (arrows).

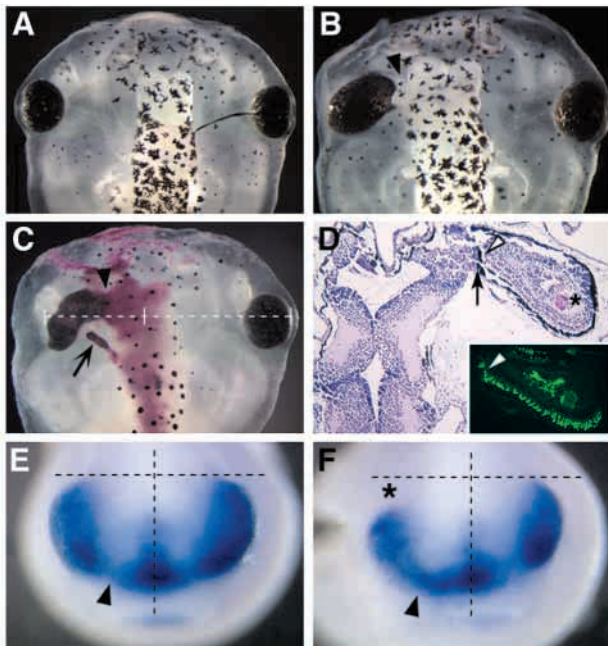


Fig. 2. Proximal eye defects in embryos misexpressing Pax6. *Xenopus* embryos were injected with 160 pg Pax6 RNA in one animal pole blastomere at the 16-cell stage and fixed at stage 48 (A,B) and stage 41 (C). (A) Extension of RPE in right eye toward the midline. (B) Expansion of RPE and positioning of eye cup adjacent to forebrain region (arrowhead). (C) RedGal labeling in left eye of embryo coinjected with 400 pg of Nuc-lacZ RNA shows an RPE expansion adjacent to the forebrain region (arrowhead) and ectopic RPE-like structure (arrow). The dotted white line indicates the reduced distance from the midline to the distal boundary of the eye on the affected side. (D) Section through embryo displaying proximal eye phenotype in which the expanded RPE is adjacent to the forebrain (arrow); the arrowhead indicates photoreceptor cells adjacent to the brain region; (inset) rhodopsin staining in the eye region of the same section in D showing rod photoreceptor cells adjacent to the brain (arrowhead). (E) Uninjected embryo showing the normal *Six3* expression pattern in the late neurula (stage 20). (F) Pax6-injected embryo showing an expansion of *Six3* expression towards the midline (arrowhead) compared with that of E (arrowhead) and diminished *Six3* expression distally (asterisk). Vertical dotted line indicates the midline, and the horizontal line the dorsal limit of *Six3* expression.

misexpressing Pax6 was a developmental defect in the proximal regions of the endogenous eye (defined as the region between the optic cup and optic tectum). This affected between 20% ($n=80$; Table 3) and 39% ($n=120$; Table 1) of injected embryos depending on the experiment performed. This defect appeared as an extension of the endogenous RPE towards the midline (Figs 1D, 2A), sometimes as a failure of the ventral eye fissure to close (data not shown), and in extreme cases,

resulted in a fusion of the brain to the optic cup (Fig. 2B,C, arrowheads – for discussion of red labeling, see below). In some cases, the RPE extension appeared as a fine track of pigmented cells apparently associated with the optic nerve (Fig. 2A) while in others a broad band of RPE extended medially (Fig. 2B,C). In histological sections, it was observed that retinal laminae and RPE extended through the region normally occupied by the optic nerve (Fig. 2D). The presence of mature photoreceptor cells throughout was confirmed with anti-rhodopsin immunofluorescence (Hicks and Molday, 1985; Fig. 2D, inset). Some aspects of this phenotype were similar

Fig. 3. Ectopic eyes induced by Pax6 misexpression resemble normal eyes morphologically and histologically. *Xenopus* embryos were injected with 160 pg of Pax6 RNA in one animal pole blastomere at the 16-cell stage and fixed at stage 48. (A-C,E,F) Ectopic eyes from different embryos displaying eye cup (white arrowhead) and lens (black arrowhead, top). (B,F) Side view; dorsal, top; anterior, right. (C) Dorsal view; anterior, left. (E) Dorsal view; anterior, right; RPE-like extension from eye cup (C,E, arrow). (D) Dorsal view of C showing that this particular ectopic eye is positioned posterior to the otic vesicle (OV). (G-I) Hematoxylin and eosin staining of coronal sections through (G) normal eye, and (H,I) ectopic eyes. Section in H taken from ectopic eye in E; section in I taken from ectopic eye in F. In I note reverse orientation of lens (L) which is separated from the epidermis (bottom) by ectopic retina and therefore not visible in F. P, retinal pigmented epithelial layer; O, outer nuclear layer; I, inner nuclear layer; G, ganglion cell layer; L, lens; arrows indicate ciliary margin zone in normal eye and region with similar morphology in ectopic eyes.

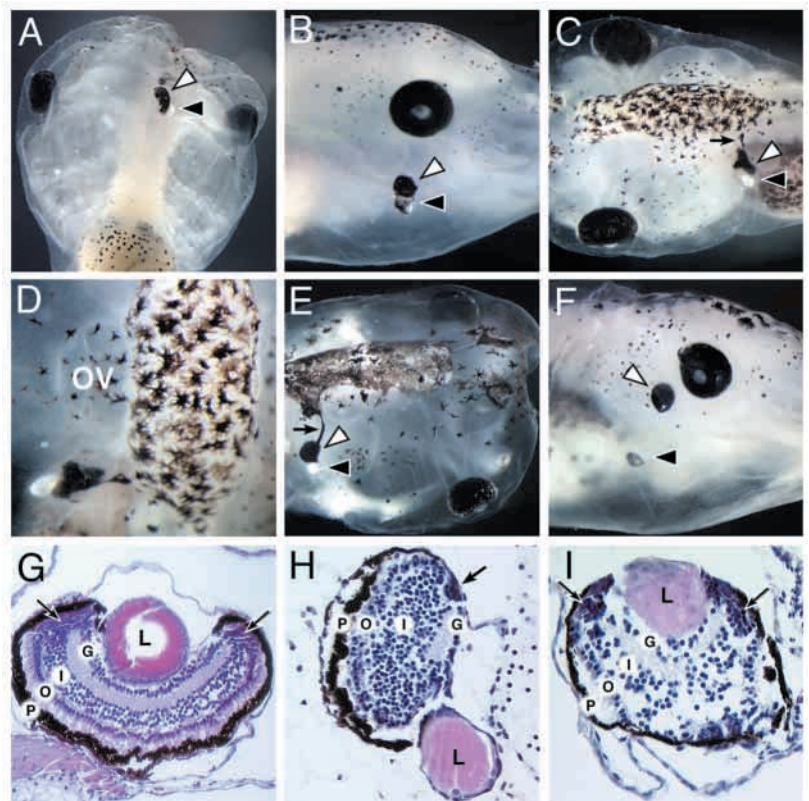


Table 1. Phenotype of embryos misexpressing Pax6*.

	No. embryos	%
Total injected	120	100
Normal and no eye phenotype	57	48
Abnormal†/no eye phenotype	10	8
Dead	2	1.6
Proximal eye defect‡	39	33
Isolated ectopic eye§	7	5.8
Ectopic lens only	5	4
Total phenotypes observed	51	43

Embryos were injected with 160 pg of *Pax6* RNA in one animal blastomere at the 16-cell stage and allowed to develop to stage 48.

*Data from representative experiment.

†Embryos with defects similar to those published (Hirsch and Harris, 1997).

‡Embryos having an extension of the RPE from the endogenous eye, the juxtaposition of the endogenous eye cup and brain, a failure of the ventral fissures of the eye to close or a combination of all phenotypes (see text).

§As defined in text.

to that observed with gain-of-function expression of *Rx* in *Xenopus* embryos (Mathers et al., 1997).

Cell fate changes are brought about by modified gene expression patterns. We therefore performed whole-mount in situ hybridization to detect the mRNAs for *Six3* (Oliver et al., 1995) and *Rx* (Mathers et al., 1997) two molecular markers with overlapping expression in the eye. In situ hybridization using a *Xenopus Six3* probe revealed the expected normal pattern of *Six3* expression (Oliver et al., 1995) in a crescent-shaped band at the anterior neural plate boundary of the early neurula (stage 14; data not shown) and in the optic vesicle of the late neurula (stage 20; Fig. 2E). Embryos injected in one of 16 blastomeres with RNA encoding an epitope-tagged version of Pax6 (Altmann et al., 1997) showed changes in the expression domain of *Six3* that correlated with the appearance of proximal eye defects. The *Six3* expression domain was more pronounced proximally and diminished distally (compare Fig. 2E and F). Only very subtle changes were observed in the expression domain of *Rx* in response to misexpressed Pax6 (data not shown). These observations demonstrate that misexpression of Pax6 results in a shift of *Six3* expression towards the midline of the embryo. This modified gene expression pattern may reflect the cell-fate changes observed in tadpoles with proximal eye defects.

Ectopic eye formation

Misexpression of Pax6 resulted in ectopic eye formation. Ectopic eyes were scored positive only when four criteria were met. These included (i) the expression of molecular markers for one or more mature retinal cell types, (ii) the presence of β -crystallin-positive cells with lens fiber cell morphology, (iii) the presence of an RPE-like cell layer, and (iv) the close association of these three elements in a near-normal arrangement. Ectopic eyes occurred at a frequency of up to 10% of injected embryos with a total of 35 ectopic eyes generated in six independent experiments (see Tables 1 and 3 for representative examples). Ectopic eyes could be identified readily in tadpoles (stages 43-48) as they were associated with ectopic pigment (Figs 1E, arrowheads, 3A-F, white arrowheads) and with the dense spherical structure characteristic of ectopic lenses (Figs 1E, small arrowhead, 3A-E, black arrowheads). Ectopic eyes were located

anywhere in the region anterior to the spinal cord-hindbrain junction (Fig. 3A-F) and were more frequently located dorsally. In many, but not all cases of ectopic eyes, an RPE-like layer was observed to extend from the central region of the ectopic eye to the brain (Figs 1E, white arrowhead, 4C,E, arrows) in a similar manner to that observed for proximal eye defects (Fig. 1D, arrow, 2A).

Ectopic eyes induced by Pax6 misexpression (Fig. 3H,I) displayed a similar morphology and organization to that of endogenous eyes (Fig. 3G). Histological sections through ectopic eyes of embryos at stage 48 revealed presumptive retinal ganglion cell, inner nuclear and outer nuclear layers separated by inner and outer plexiform layers (the ectopic eyes in Figs 3E and F are shown in section in Figs 3H and I, respectively). This suggested the presence of well differentiated neural retinas in ectopic eyes. As is the case in normal eyes (Fig. 3G), a pigmented RPE-like layer was observed in close association with the outer (photoreceptor) cell layer of ectopic eyes (Fig. 3H,I). In some cases, the RPE-like layer surrounded the entire eye cup (Fig. 3I) while in others it surrounded only the retinal elements (Fig. 3H). Interestingly, a group of cells located at the distal tips of apparent neural retinas in ectopic eyes (Fig. 3H,I, arrows) often displayed a tightly clustered arrangement reminiscent of that found in the ciliary margin zone of normal eyes (3G, arrows). The ciliary margin zone is a region at the distal tip of normal, fully differentiated retinas that is made up of progenitor cells that can give rise to all retinal cell types (Wetts and Fraser, 1988). Combined, these data provide morphological evidence that Pax6 can induce ectopic eyes that contain all of the major cell layers found in normal eyes.

To extend this argument, we investigated whether molecular markers would indicate the presence of differentiated cells in ectopic eyes. Immunofluorescence experiments were conducted using cell-specific antibodies against rhodopsin (rod photoreceptor cells), islet-1 (retinal ganglion cells and amacrine cells), glutamine synthetase (Müller glial cells; Vardimon et al., 1991), β -crystallin (lens) and a monoclonal antibody that identifies RPE (Sakaguchi et al., 1997). Frozen sections through ectopic eyes of embryos fixed as tadpoles (stage 48) revealed that like endogenous eyes (Fig. 4A,B), ectopic eyes labeled for all of the markers examined and in a manner that reflected appropriate positioning of the various cell types within the eye (Fig. 4C,D,F-H).

In both endogenous (Fig. 4A,B) and ectopic eyes (Fig. 4C-F) the outer layer of the eye cup was pigmented. In examples

Table 2. Ectopic gene expression in embryos misexpressing Pax6

In situ probe	% of embryos with ectopic gene expression
<i>Rx</i>	47 (n=34)
<i>Six3</i>	21 (n=31)
<i>Otx2</i>	32 (n=34)
Pax6-UTR	11 (n=37)

160 pg *Pax6* and 400 pg Nuc-lacZ RNAs were injected into one animal pole blastomere of 16-cell stage albino embryos. Embryos were fixed at neural stages and processed for whole-mount in situ hybridization (see Materials and Methods). Control embryos injected with 400 pg Nuc-lacZ alone did not display any ectopic expression (data not shown).

Table 3. Effect of RNA concentration, site of injection and coinjection of a truncated form of Pax6 on phenotype of embryos misexpressing Pax6*

RNA	Site of injection	No. embryos	Proximal eye defect	Ectopic RPE	Ectopic eye	Ectopic lens	Total with phenotype
80 pg <i>Pax6</i>	dorsal	80	0	0	0	0	0
120 pg <i>Pax6</i>	dorsal	80	17	4	2	2	25
160 pg‡ <i>Pax6</i>	dorsal	80	0	0	0	0	0
120 pg <i>Pax6</i> +50 pg <i>Pax6</i> ΔCT	dorsal	40	0	0	0	0	0
120 pg <i>Pax6</i>	ventral	80	0	0	0	0	0

Embryos were injected in one animal blastomere at the 16-cell stage and allowed to develop to stage 48. Embryos were fixed in MEMFA and scored for phenotypes as described in the text.

*Data from representative experiment.

‡The RNA used for this experiment was a different batch from that used elsewhere and displayed a different activity optimum (compare with Table 1).

of ectopic eyes where we attempted to label this layer with the anti-RPE antibody, a positive signal was obtained confirming that it represented bona fide RPE (Fig. 5C,D). In endogenous (Fig. 4A,B) and ectopic eyes (Fig. 4C,D,F-H), the cells immediately adjacent to the RPE were rhodopsin-positive photoreceptors. Located adjacent to photoreceptors but on the opposite side from the RPE were layers of interspersed cells positive for either glutamine synthetase or Islet-1. These markers identify Müller glia and either ganglion or amacrine cells respectively and indicate that at least two additional differentiated cell types characteristic of endogenous retina (Fig. 4B,E) can be found in ectopic retinae (Fig. 4C,D,F). Though the location of the lenses associated with ectopic eyes was variable, in many cases, they were within the eye cup in a normal location (Fig. 3I) or associated with the eye cup at the edge of the RPE (Figs 3H, 4C,D,G,H). In all cases, β -crystallin positive lens cells could be detected (Figs 4D,H, 5E,F). Immunostaining also revealed that rod photoreceptor cells of ectopic eyes had an elongated outer segment morphology (Fig. 4H, inset) similar to that found in normal eyes. These results demonstrate that Pax6 induces complete ectopic eyes that resemble normal eyes both at the morphological and molecular levels.

Ectopic eye induction by Pax6 is autonomous

To determine whether ectopic eyes were formed cell-autonomously or cell non-autonomously with respect to Pax6 function, we co-injected a lineage tracer RNA encoding nuclear β -galactosidase (Nuc-lacZ) with that encoding Pax6. Embryos were injected in one dorsal blastomere at the 16 cell stage, allowed to develop into tadpoles (stage 48) and then labeled in whole-mount for β -gal activity with the RedGal substrate. RedGal staining in injected embryos was distributed widely, but in all cases, staining was detected at the location of proximal eye defects (Fig. 2E). Furthermore, RedGal labeling was also found within all ectopic eyes (Fig. 5A,B, arrows). A histological section through the ectopic eye shown in Fig. 5B confirmed that RedGal labeling was found within the cells of the RPE layer (Fig. 5C, arrows) as well as in adjacent tissue (Fig. 5C). Immunofluorescence labeling with cell-specific antibodies revealed that this region contained retinal ganglion cells, rod photoreceptors and RPE laminated in an order identical to that of a normal retina (Fig. 5D, red, green and purple labeling respectively). The ectopic lens associated with this ectopic eye arose in a different plane of section but was also positive for the β -gal substrate RedGal in

the epithelial layer and peripheral fiber cells (Fig. 5E) all of which were positive for the mature lens marker β -crystallin (Fig. 5F). The lack of labeling in the central fiber cells is a reproducible observation for ectopic lenses and is a consequence of the inability of β -gal substrates to penetrate the fiber cell mass of a whole-mount mature lens completely (S. Williams and R. A. L., unpublished observations). We conclude that in directing the formation of all components of ectopic eyes, Pax6 functioned cell autonomously.

Pax6 misexpression induces markers of eye development ectopically

Given that Pax6 misexpression led to the formation of ectopic eyes cell autonomously, we analyzed embryos to determine whether Pax6 induced ectopic expression of genes implicated in early eye development. These included *Otx2*, a gene normally expressed in midbrain, forebrain and eye in the neurula (Pannese et al., 1995) as well as the previously mentioned *Six3* and *Rx* genes. We also examined expression of the endogenous *Pax6* gene using a probe to 3' untranslated sequences not present in the *Pax6* construct to ask if Pax6 could induce its own expression. Embryos were injected with a mixture of *Pax6* and Nuc-lacZ RNAs and subsequently examined for ectopic gene expression in the late neurula. Late neurula stage embryos displayed ectopic expression of *Otx2*, *Six3*, *Rx* and endogenous *Pax6* (Fig. 6A-D, Table 2). In addition, *Rx* expression could be detected in neural plate stage embryos (stage 13; Fig. 6E,F). Coinjection of Pax6 and Nuc-lacZ RNAs indicated that *Rx* expression was an autonomous response to Pax6 (Fig. 6G). The ectopic expression of additional eye markers supports the conclusion from morphological and immunolabeling analyses that Pax6 misexpression induces ectopic eyes.

Inhibition of eye fates by dominant-negative Pax6

The evidence presented suggests that in the context of the *Xenopus* embryo, Pax6 is sufficient for formation of the eye. That Pax6 is necessary for eye formation in vertebrates has been documented in the *Small eye* mouse (Glaser et al., 1990; Hill et al., 1991) as well as with the human *ANIRIDIA* mutation (Glaser et al., 1992; Jordan et al., 1992). We nevertheless wanted to address both the issue of necessity as well as specificity of Pax6 function in the frog. To this end, we employed a recently identified dominant-negative Pax6 protein (Singh et al., 1998). This mutant form (Pax6 Δ CT) is deleted for the C-terminal proline-serine-threonine rich (PST) region that

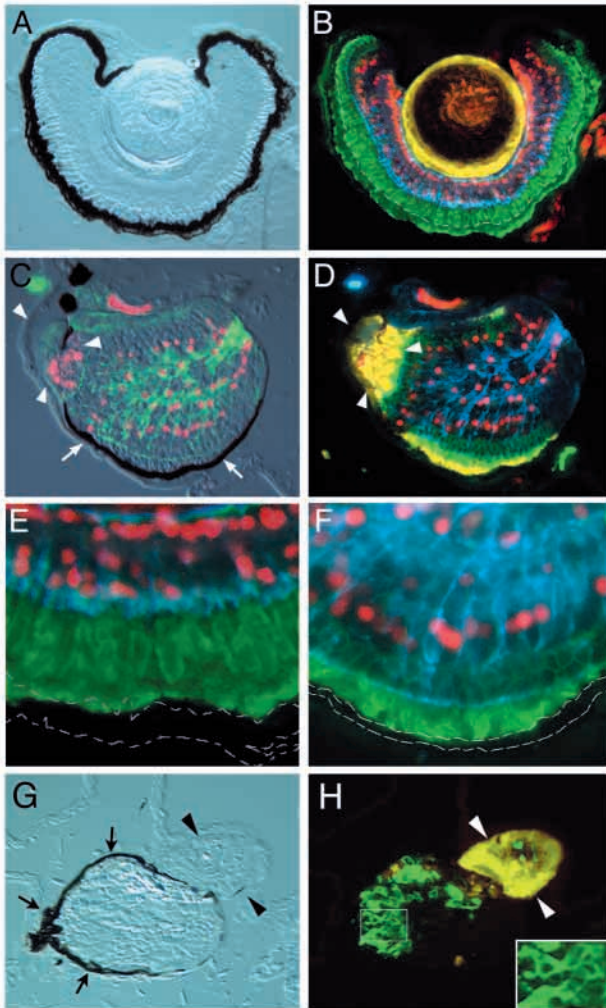


Fig. 4. Ectopic eyes express markers of differentiated retinal cell types and lens. Sections of endogenous normal eye (A,B,E), and ectopic eyes (C,D,F-H). DIC illumination is shown in A, C and G and fluorescence illumination in B,D,F and H. Wild-type (A,B) and ectopic (C,D,G,H) eyes contain lenses or lens-like structures (C,D,G,H, arrowheads) which show immunoreactivity with antibodies to β -crystallin (yellow labeling). The presence of photoreceptors is demonstrated with rhodopsin immunoreactivity (green labeling) in normal (B,E) and ectopic (D,F,H) eyes. The location of ganglion and amacrine cells in the inner layers of the retina is indicated by labeling with an antibody to Islet-1 (red nuclear labeling) in both normal (B and E) and ectopic (C, D and F) eyes. Müller cells, indicated by blue labeling (B,D-F) or green labeling (C) for glutamine synthetase, are closely associated with islet-1-positive ganglion and amacrine cells in the inner retinal layers of both normal (B,E) and ectopic (D,F) eyes. High magnification section through retina in examples of endogenous (E) and ectopic (F) eyes indicate that retinal lamination follows the expected pattern. The location of the pigmented cell layer in (E,F) is indicated by the dashed lines. In all cases, ectopic eyes were associated with a layer of pigmented RPE-like cells (C,G, arrows). The inset in H shows higher magnification of rhodopsin stained cells of ectopic eyes with a similar morphology to that of endogenous rods.

has been implicated in transcriptional activation (Tang et al., 1998) and can effectively compete with wild-type Pax6 in a cotransfection reporter assay (Singh et al., 1998). We reasoned that if the response to misexpressed Pax6 reflected events

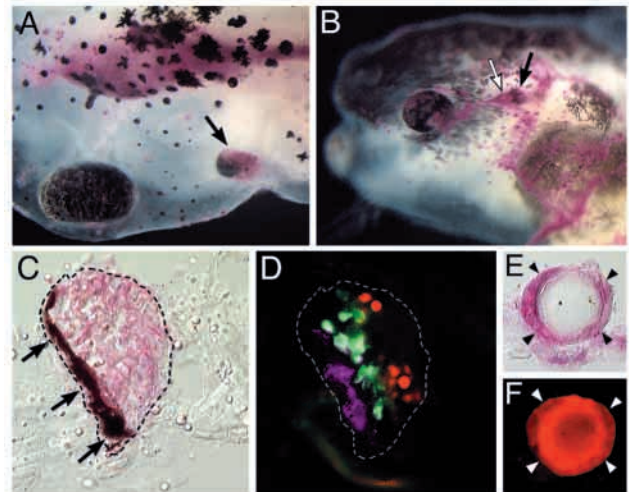


Fig. 5. Ectopic eye formation by Pax6 is autonomous. Embryos were coinjected with 160 pg *Pax6* and 400 pg of Nuc-lacZ RNA and stained with the RedGal substrate. (A,B) Embryos displaying ectopic eyes in whole-mount (black arrows); lens is indicated by white arrow in B. (C) Section through ectopic eye of embryo in B showing RedGal labeling in the eye cup and surrounding RPE (arrows); dotted line in C and D indicates boundary of RedGal labeling. (D) Immunofluorescence labeling of section shown in C; XAR (RPE; purple), rhodopsin (rod photoreceptors; green) and Islet-1 (ganglion cells; red). (E,F) Adjacent section through the ectopic eye in C and D showing (E) RedGal labeling in lens epithelium surrounding lens fiber cells and (F) β -crystallin labeling; arrowheads in E and F indicate boundary of the lens.

occurring during normal eye development, Pax6 Δ CT should be able to inhibit the formation of both endogenous and ectopic eyes. Since Pax6 and Pax6 Δ CT bind the same DNA target sequences, experiments employing Pax6 Δ CT should also provide a control for the specificity of the responses observed.

Confirmation that Pax6 Δ CT was functioning in the expected manner came from experiments examining normal eye development. To target anterior neural tissue, 8- to 16-cell embryos were injected in one dorsal animal pole blastomere with 60 pg of RNA encoding Pax6 Δ CT and allowed to develop to tadpoles. Injected embryos showed a reduced eye size on one side (Fig. 7A, left panel) and occasionally, eye development was blocked completely (Fig. 7A, middle panel). When Nuc-lacZ RNA was coinjected with that for Pax6 Δ CT and the tadpoles stained with the β -gal substrate RedGal, 55% ($n=20$) of those embryos with RNA distributed in the eye region had diminished eye size. No effect was observed when Nuc-lacZ RNA was injected alone (data not shown). This indicated that, as would be anticipated, Pax6 Δ CT could inhibit normal eye development.

To ask whether Pax6 Δ CT could suppress the molecular markers induced by Pax6, we performed experiments where the two proteins were coexpressed in animal cap explants (Vize et al., 1991). Unfortunately, the combined expression of RNAs encoding Pax6 and Pax6 Δ CT at the required concentrations precluded explant survival. As an alternative approach we decided to determine whether Pax6 Δ CT could suppress the activation of eye-associated genes in response to the neuralizing agent follistatin (Hemmati-Brivanlou et al., 1994). This factor is known to promote anterior neural fate in animal

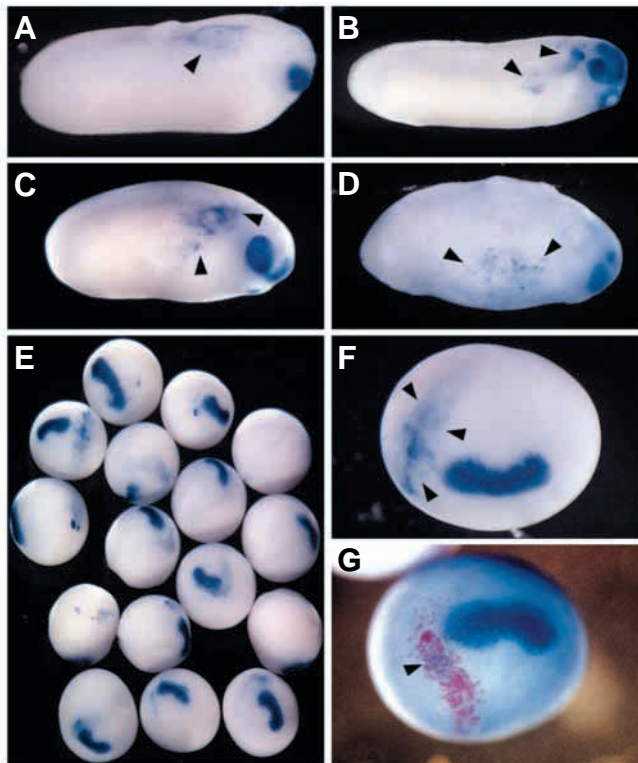


Fig. 6. Ectopic gene expression in embryos misexpressing Pax6. See Table 2 for summary of results. In situ hybridization was performed on embryos injected with 160 pg *Pax6* and 400 pg Nuc-lacZ RNAs. Embryos at neurula stages showing ectopic expression (blue labeling and arrowheads) of (A) *Rx* (embryo tilted to show dorsal aspect), (B) *Otx2*, (C) *Six3* and (D) endogenous *Pax6*. (E-G) Embryos at the early neurula displaying ectopic *Rx* expression (arrowheads). (G) Ectopic *Rx* staining (arrowhead) localized within a region of RedGal labeling (red).

caps in the absence of mesoderm (Hemmati-Brivanlou et al., 1994) and can stimulate the expression of eye markers (Hemmati-Brivanlou et al., 1994; Altmann et al., 1997).

Animal cap experiments were performed in which RNA encoding Pax6 Δ CT was titrated against a constant level of that encoding follistatin. Interestingly, Pax6 Δ CT inhibited rhodopsin and β B1-crystallin expression in the tadpole (stage 41; Fig. 7B) in a dose-dependent manner. The observation that Pax6 Δ CT could block eye markers but did not (relative to the loading control EF-1 α) affect expression of the neural cell marker (NCAM) supports the idea that Pax6 functions downstream of neural induction in the formation of eyes (Altmann et al., 1997). Consistent with this suggestion is the observation that misexpression of *Pax6* RNA resulted in eye phenotypes in whole embryos only when injections were performed into the dorsal animal blastomeres that give rise to anterior neural and ectodermal structures (Keller, 1976; Table 3).

To test whether Pax6 Δ CT could suppress the phenotypic consequences of Pax6 activity in the whole embryo, we misexpressed Pax6 alone or coinjected RNAs encoding both proteins. Embryos were injected in one animal blastomere at the 16 cell stage and allowed to develop to tadpoles (stage 48). The response to Pax6 alone was dose-dependent as previously

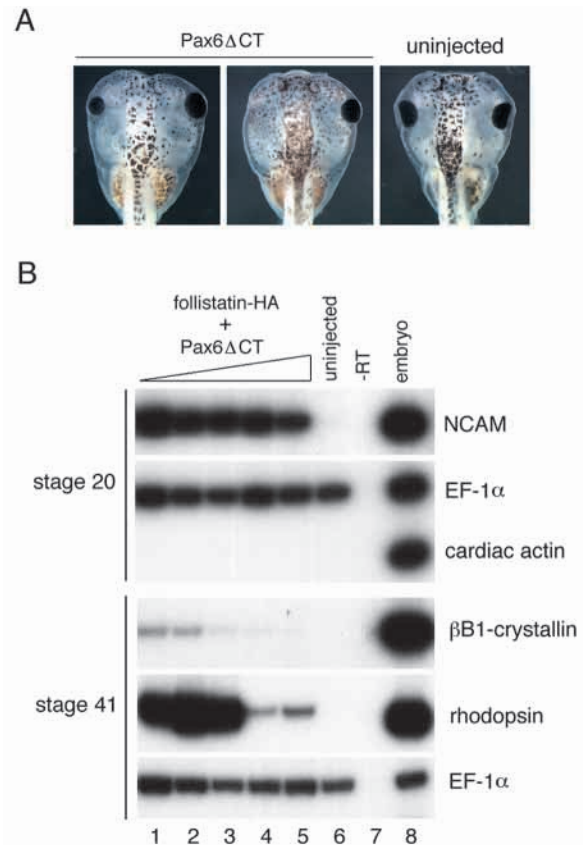


Fig. 7. Inhibition of eye formation in whole embryos and animal caps by a truncated version of Pax6. (A) Embryos were injected with 60 pg Pax6 Δ CT RNA in one dorsal animal blastomere at the 8-cell stage and fixed at stage 45. Embryos displayed phenotypes ranging from a reduction in eye size (left panel) to the complete loss of eye structures (middle panel). (B) Embryos were injected in both blastomeres of 2-cell embryos with a fixed amount of RNA for the neuralizing factor follistatin (1 ng) and challenged with increasing levels of Pax6 Δ CT (lanes 1-5 show 6, 12, 25, 50, 100 pg of Pax6 Δ CT RNA respectively). Animal cap ectoderm was explanted at mid-blastula (stage 8.5) and processed for RT-PCR at stages 20 and 41 as indicated on the left side of the panel. An elongation factor-1 α (EF-1 α) cDNA product was amplified as an internal loading control for animal caps at both stages. Lane 6: uninjected animal cap control. Lane 7: embryo minus reverse transcriptase negative control. Lane 8: whole embryo positive control. NCAM, neural cell adhesion molecule.

reported (Altmann et al., 1997) with a response apparent for this batch of RNA only at 120 pg (Table 3) and resulted in 31% ($n=80$) of the embryos showing either proximal eye defects or ectopic eye structures. When this same concentration of Pax6 RNA was challenged with 50 pg of RNA encoding Pax6 Δ CT, eye defects were eliminated (Table 3). Combined with animal cap experiments (Fig. 7B), these data show that Pax6 is required for the formation of both endogenous and ectopic eyes.

DISCUSSION

In the present study, we asked whether the paired class

transcription factor Pax6 (Walther and Gruss, 1991; Quiring et al., 1994) is sufficient for eye formation in a vertebrate system. We have demonstrated that Pax6 misexpression in the frog *Xenopus laevis* results in the formation of ectopic eyes that contain at least five different mature cell types and have a morphology that is characteristic of normal endogenous eyes. We document that the response to Pax6 misexpression is cell-autonomous and involves the activation of expression of several genes normally associated with eye development, including *Six3* (Oliver et al., 1996; Loosli et al., 1998, 1999) and *Rx* (Mathers et al., 1997). Finally, we show that development of both endogenous and ectopic eyes can be suppressed by a dominant negative mutant of the Pax6 protein. Combined, these data show that in the context of the *Xenopus* embryo, Pax6 is necessary and sufficient for eye formation. These observations extend previous work performed in invertebrates (Halder et al., 1995; Czerny et al., 1999) to show that Pax6 can direct eye formation in a vertebrate. This argues strongly for the evolutionary conservation of the molecular mechanism involved in the genesis of the visual system in eyes as different as the compound eye of the insect and the camera eye of vertebrates and suggests a monophyletic origin for this important sensory organ.

Proximal eye defects

Embryos misexpressing Pax6 displayed numerous defects affecting the endogenous eye ranging from an extension of the RPE towards the midline to the juxtaposition of an expanded eye cup adjacent to the brain. Morphological and molecular data support the idea that these defects arose as a conversion of proximal eye fates to more distal fates characteristic of the eye cup. The demonstration that Pax6 functions cell autonomously in these phenotypes highlights our conclusion that Pax6 can direct ectopic eye formation in vertebrates and also sheds light on the role of Pax6 in defining the eye fields during normal eye development.

Classical embryology has provided strong evidence for the existence of a single morphogenetic eye field which spans the midline of neural plate stage embryos (Mangold, 1928; Adelman, 1929, 1936). These and more recent studies (Ekker et al., 1995; Macdonald et al., 1995; Li et al., 1997) have shown that signals derived from the underlying prechordal mesoderm and ventral forebrain resolve this single eye field into two. These studies have also shown that suppression of *Pax6* expression in the midline occurs in the absence of cell migration and is under the control of the prechordal mesoderm signals (Li et al., 1997). Interestingly, *Pax6* is ectopically expressed in the proximal eye regions of cyclopic mutants in which this midline signaling is deficient (Ekker et al., 1995; Macdonald et al., 1995). These observations, in combination with the presented gain-of-function data suggest that the regulation of *Pax6* plays a critical role in defining the eye fields.

Interestingly, eyes of mice having a null mutation in *Pax2* bear a striking resemblance to the proximal eye defects seen in *Xenopus* embryos misexpressing Pax6. In these mice the RPE extends towards the midline and the ventral choroidal fissure fails to close (Torres et al., 1996). Both these phenotypes are observed in *Xenopus* embryos misexpressing Pax6 and suggest that Pax2 may function in part by inhibiting the Pax6-directed development of distal eye fates in the region of the optic stalk. Pax6 expressed from an injected RNA may bypass the normal

function of Pax2 in specifying proximal eye fates in the optic stalk region.

The proximal eye defects caused by Pax6 misexpression also resemble those in *Xenopus* embryos misexpressing *Rx* where extensions of RPE towards the midline are observed (Mathers et al., 1997; Andreazzoli et al., 1999). Like *Pax6*, *Rx* is initially expressed in the anterior neural plate as a single band that resolves with time to the distal eye regions destined to become the optic vesicles (Casarosa et al., 1997; Mathers et al., 1997). These data imply that *Rx*, like Pax6, can direct distal cell fate in the optic cup. The similarity in the phenotypic responses to *Rx* and Pax6 misexpression, combined with data showing that Pax6 can activate *Rx* expression argues that during development of optic cup-derived eye structures, *Rx* may function downstream of Pax6. This does not preclude the possibility that *Rx* may also have a function in regulating *Pax6* in some cell types. Indeed, it has been shown recently that ectopic Pax6 expression is induced in embryos misexpressing *Rx* (Andreazzoli et al., 1999).

Ectopic eyes

Our data show that in the frog *Xenopus laevis*, as in the fly *Drosophila melanogaster* (Halder et al., 1995), Pax6 is sufficient for the formation of all the major components of an eye. Ectopic eyes contain fully differentiated cells for lens as well as the RPE, photoreceptor, Müller, ganglion and amacrine cells characteristic of retinal layers. Furthermore, the morphological arrangement of these cell types is consistent with that of normal eyes and argues that bona fide ectopic eyes have been generated. The demonstration that a dominant-negative acting form of Pax6 (Singh et al., 1998) can suppress both endogenous and ectopic eye development argues that misexpressed Pax6 initiates the normal transcriptional program for the eye ectopically. The ability of Pax6 to direct eye formation in flies (Halder et al., 1995) and in *Xenopus* underscores the conservation in the hierarchy and regulation of the genetic machinery controlling eye development and provides further support for the monophyletic evolution of light sensing organs.

The data presented suggest that there is a cell-autonomous requirement for Pax6 in the development of both the lens and retinal components of ectopic eyes. This is consistent with results from analysis of chimeric mice (Quinn et al., 1996), explant experiments (Fujiwara et al., 1994) and our previous demonstration that Pax6-induced ectopic lens formation requires autonomous Pax6 (Altmann et al., 1997). Combined with previous data indicating that eye formation lies downstream of neural induction (Altmann et al., 1997) it is also reasonable to suggest that ectopic eyes may be formed through respecification of regions of the neural tube. In this vein, however, it is interesting to note that the *Pax6* mutant mouse *Small eye* homozygote forms optic vesicle-like structures in the absence of functional *Pax6* product. Although this indicates that the initiation of optic vesicle formation occurs in a Pax6-independent manner, the maintenance and continued development of these structures may be Pax6 dependent. Pax6 misexpression may recapitulate the genetic program responsible for optic vesicle formation. The lack of complete structure in ectopic eyes may therefore reflect the existence of eye-competent tissue that can respond to misexpressed Pax6 in the absence of normal optic vesicle morphology.

Our data also suggest that eye development is sensitive to the level of Pax6 expression. Support for this idea comes from previous experiments where the level of Pax6 expression has been manipulated (Schedl et al., 1996) and from the semi-dominant inheritance pattern of Pax6 defects in mice (Hill et al., 1991) and humans (Jordan et al., 1992). In the heterozygous state in Pax6 loss-of-function mutants, the only severe defects observed are in the development of the eye (*ANIRIDIA* in humans; Jordan et al., 1992) while homozygotes display, in addition to a loss of eye structures, defects in other regions such as the forebrain, nose, hindbrain and pancreas (Hogan et al., 1986; Hill et al., 1991; St-Onge et al., 1997). In contrast, mice carrying more than two copies of the Pax6 locus show specific developmental abnormalities of the eye, but not of other tissues (Schedl et al., 1996). This sensitivity of eye development to the level of Pax6 could explain why ectopic eye formation is highly concentration dependent.

It may be something of an oversimplification to conclude from the presented data that Pax6 functions in isolation to direct normal eye development. The observation that ectopic eyes arise only in the head region argues that other factors are required and perhaps that only certain embryonic tissues are competent for eye formation. In *Drosophila*, misexpression of other genes such as *sine oculis* and *eyes absent* (Pignoni et al., 1997) or *dachshund* (Chen et al., 1997) can also lead to ectopic eye formation. It has been proposed that these genes, along with *ey/Pax6*, form part of a self-regulating network of genes whose combinatorial activity specifies eye formation. Specifically, it has been shown that both *eyes absent* (*eya*) and *sine oculis* (*so*) are target genes of *ey*, but that in turn, *ey* expression is up-regulated by *eya* and *so* (Chen et al., 1997; Pignoni et al., 1997; Halder et al., 1998). The observation that expression of these genes in *Drosophila* is co-dependent suggests that ectopic eye formation may occur because the de novo misexpression of these genes can initiate eye development.

We show that *Xenopus* embryos misexpressing Pax6 ectopically express several genes implicated in vertebrate eye development. Since exogenous Pax6 is no longer detectable in the late neurula (Altmann et al., 1997) but the ectopic expression of *Rx*, *Six3*, *Otx2* and endogenous Pax6 continues, we can suggest that misexpressed Pax6 initiates the cascade of gene expression required for eye development. Once initiated however, there may be no further requirement for exogenous Pax6 as endogenous genes may self-regulate and perpetuate an eye development pathway. Recent studies performed in the fish *Medaka* have shown that *Six3* misexpression can induce ectopic expression of Pax6, *Rx* and endogenous *Six3* (Loosli et al., 1999) further illustrating the notion that there will likely be a complex set of regulatory interactions between the genes involved in eye development. Future studies are aimed at understanding these regulatory interactions in detail.

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