

## A single morphogenetic field gives rise to two retina primordia under the influence of the prechordal plate

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### SUMMARY

Two bilaterally symmetric eyes arise from the anterior neural plate in vertebrate embryos. An interesting question is whether both eyes share a common developmental origin or they originate separately. We report here that the expression pattern of a new gene *ET* reveals that there is a single retina field which resolves into two separate primordia, a suggestion supported by the expression pattern of the *Xenopus Pax-6* gene. Lineage tracing experiments demonstrate that retina field resolution is not due to migration of cells in the median region to the lateral parts of the field. Removal of the prechordal mesoderm led to formation of a single retina both in chick embryos and

in *Xenopus* explants. Transplantation experiments in chick embryos indicate that the prechordal plate is able to suppress *Pax-6* expression. Our results provide direct evidence for the existence of a single retina field, indicate that the retina field is resolved by suppression of retina formation in the median region of the field, and demonstrate that the prechordal plate plays a primary signaling role in retina field resolution.

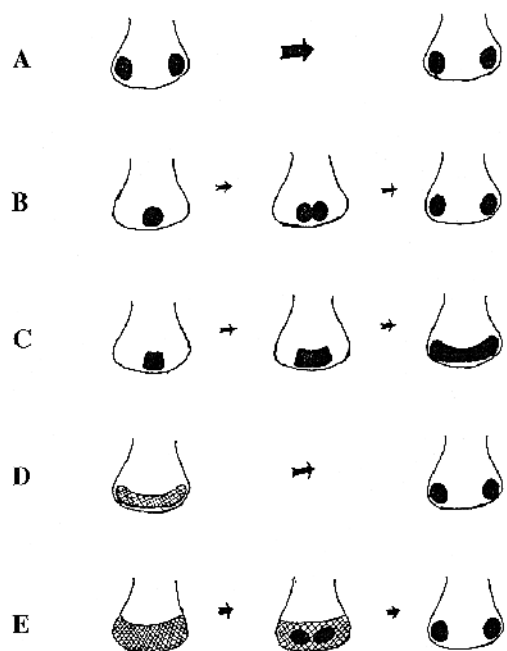
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### INTRODUCTION

Formation of bilaterally symmetric and asymmetric structures have been studied in vertebrates since the beginning of experimental embryology. It is known that several strategies are used to form these structures in the embryo. Most of the structures on both sides of an animal, such as limbs, emerge independently from separate primordia on each side of the embryo. Formation of a single heart, on the other hand, results from the fusion of two originally separate primordia (Copenhaver, 1926; Jacobs and Fraser, 1994). Here we address the question how two symmetric eyes form in the anterior neural plate.

Interest in how two eyes form in normal embryogenesis has, in part, been motivated by attempts to understand developmental origins of cyclopean eyes in abnormal situations. A single eye or two fused eyes exist in some human fetuses suffering from holoprosencephaly (Cohen, 1989; Sperber et al. 1987; Kallen et al. 1992; Muenke et al., 1994), a developmental abnormality occurring in about 1 out of 16,000 live births and 1 in 250 terminated conceptuses (Muenke et al., 1994; Cohen, 1989). Cyclopean eyes in various species have been described with reasonable accuracy since the sixteenth century. Two mechanistic explanations were offered in the last century. Speer (1819) and Meckel (1826) proposed that cyclopia resulted from the fusion of two originally separate eyes, whereas Huschke (1832) proposed that there was a single vesicle which later became two optic vesicles in normal embryos and that cyclopean eyes resulted from a failure in separating the single vesicle (reviewed by Adelman, 1936a). The debate continued

into this century. Spemann believed in the existence of two separate optic anlagen in the early neural plate (Fig. 1A; Spemann, 1938; Lewis, 1907; Stockard, 1913b; Adelman, 1936a). His view of separate eye anlagen in normal embryos led to his interpretation of cyclopean eyes as resulting from the fusion of two eye anlagen. His opinions were shared by King (1905) and Lewis (1907, 1909). In contrast, Stockard (1907a,b, 1908, 1909a,b, 1910a,b, 1913a,b, 1914), argued that there was a single eye anlage in the median region of anterior neural plate which spread laterally and separated eventually into two eyes (Fig. 1B; Stockard, 1913a,b). LePlat (1919) came to a conclusion similar to that of Stockard except that he viewed the anlage as 'optico-ocular' apparatus including both the eyes and the optic chiasma (Fig. 1C). A series of experiments by Adelman determining the developmental potential of ectodermal regions in amphibian embryos revealed that both the lateral and median portions of the anterior neural plate could give rise to eyes (Adelman, 1929a,b, 1930, 1934, 1936), suggesting the existence of an equipotential eye field (Adelman 1929a,b; Fig. 1D). However, since early embryos are known to have regulative ability, Adelman cautiously pointed out that it was not clear whether eye formation by the median region isolated from its original surroundings reflects its potential in normal development (1929b). In fact, prosencephalon areas anterior and posterior to the eye field could also form eyes when isolated from their normal neighbors (Corner, 1963 and 1966; Boterenbrood, 1970). These observations have been interpreted as revealing a forebrain field which includes the telencephalon, the eyes and other regions of the diencephalon (Fig. 1E; Corner,



**Fig. 1.** Models for retina primordium formation in vertebrate embryos. The outline illustrates the boundary of the neural plate and the filled circles represent retina primordia. (A) The two separate retina primordia model. (B) A diagram of Stockard's model of how a single primordium spreads and separates into two retinae. Some of Stockard's results were directly contradicted by later studies of Adelman (1929a,b). (C) This is essentially the same as Figs 1, 2 and 4 of LePlat (1919). (D) A schematic representation of Adelman's model. It was not determined whether midline cells could migrate laterally. (E) An illustration of the forebrain field model. Eyes are presumably the default fate of this field (Corner, 1966; Boterenbrood, 1970).

1963, 1966; Boterenbrood, 1970; Nieuwkoop et al., 1985), making it unclear whether there is a distinct eye field or that Adelman's results simply showed part of properties of the entire forebrain field. The classic studies have been largely forgotten (Hamburger, 1988, p62; Nieuwkoop et al., 1985) and the hypothesis of an eye field remains controversial.

We have been working on a new family of developmental regulators, the T domain proteins. T domain is the DNA binding domain of this new family of transcription factors (Herrmann and Kispert, 1994; Kispert et al., 1994, 1995; Kispert and Herrmann, 1993) and T domain proteins have been found in both vertebrates and invertebrates (Pflugfelder et al., 1992a,b; Kispert et al., 1994; Yasuo and Satoh, 1994; Bollag et al., 1994; Bulfone et al., 1995). The prototype of the T family, the mouse *T* locus gene and its homologs, *brachyury* (*Xbra*) in *Xenopus* and *no tail* (*ntl*) in zebrafish, are essential for posterior mesoderm and notochord formation (Chesley, 1935; Grüneberg, 1958; Bennet, 1975; Herrmann et al., 1990; Smith et al., 1991; Cunliffe and Smith, 1992; Schulte-Merker et al., 1992, 1994; Halpern et al., 1993). Injection of a mutant form of *Xbra* led to formation of the retina and the cement gland, suggesting that other T domain proteins might also exist in early *Xenopus* embryos (Rao, 1994).

We have now identified several genes encoding new *Xenopus* T domain proteins. One of them, named *ET*, was found to be expressed in the primordia for the retina and the

cement gland. Interestingly, its early expression was in a continuous band in the anterior neural plate which later resolved into two retina primordia. To confirm that this pattern reveals a retina morphogenetic field and its resolution, we isolated and characterized the expression pattern of a *Xenopus* homolog of the *Pax-6* gene, which is known to be important for eye formation in vertebrates and invertebrates. *Xenopus Pax-6* expression pattern indeed supports the location and resolution of a retina field. Fate-mapping experiments indicate that retina field resolution is achieved by suppression of retina formation in the medial region, rather than by migration of retina forming cells into the lateral regions of the retina field. Results from experiments with *Xenopus* explants and chick embryos indicate that a primary signal responsible for resolving the retina field originates from the midline region of the prechordal mesoderm, the prechordal plate.

## MATERIALS AND METHODS

### Isolation of cDNAs

The sequences of the primers for T domain protein genes are: (GGI MGI MGI ATG TTY CCI GT) coding for GRRMPFP as the upstream primer, and (TAI GCI GTI ACI GCD ATR AA) coding for FIAVTAY as the downstream primer. The conditions for polymerase chain reactions (PCR) were: 1 cycle of 94°C, 3 minutes (min.), 53°C, 1 min. and 72°C, 2 min., followed by 36 cycles of 94°C, 45 sec., 53°C, 1 min. and 72°C, 2 min. A band of about 450 bp was obtained and subcloned into pBluescript SK. About 170 individual cDNAs were isolated and more than 90 have been sequenced. 28 of them encode 7 distinct T domain containing proteins. The sequence predicted from one of the PCR fragments corresponding to the gene named *ET* is shown in Fig. 2.

The sequences of the primers for *Pax-6* are: (GIC CIC/T TIC CIG AT/CA/T G/CIA C) as the upstream primer, and (GGI AA/GA/G TCA/G/T ATC/T TTI GCI GC) as the downstream primer. The upstream primer was designed for PLPDST in the paired domain and the downstream primer was based on AAKIDL in the homeo domain (the underlined sequence were unique to *Pax-6*). Template cDNAs were made from stage 28 and 40 embryos. The PCR conditions were the same as those used for isolating genes encoding T domain proteins. A fragment of 1.2 kb was subcloned into pBluescript SK and more than 150 colonies obtained. cDNAs from 16 individual clones were analysed and 15 of them had inserts. Sequence analyses showed that 10 of them contained the same sequence (of *Pax-6*) and the other sequences were not similar to any sequences in the databank. A probe made from the *Pax-6* PCR fragment was used to screen the stage 28 cDNA library. 10 positives clones were isolated and the longest cDNA of 2.6 kb was sequenced.

### Whole-mount in situ hybridization

Whole-mount in situ hybridization of *Xenopus* embryos was performed essentially according to the method of Harland (1991). Briefly, embryos of different stages were removed from the vitelline membrane and fixed in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO<sub>4</sub>, 3.7% formaldehyde). Digoxigenin incorporated probes were prepared with appropriate templates and hydrolyzed. Embryos were hybridized with these probes followed by sequential washes and addition of alkaline phosphatase (AP) conjugated anti-digoxigenin antibodies. AP was detected by reaction with BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitro blue tetrazolium) or BM purple as substrates.

### *Xenopus* embryonic explants

Anterior neural plate explants were isolated from stage 12.5 or 13



primers based on conserved sequences in the T domain (Kispert et al., 1994). DNA fragments of the expected length were amplified. The amino acid sequence predicted from one of the PCR fragments was found to be similar to that of Optomotor blind (Omb; Fig. 2A), the product of a *Drosophila* gene essential for optic lobe development (Pflugfelder et al., 1992a,b). The gene corresponding to this PCR fragment was named *ET* because of its prominent expression in the eyes (see below). The predicted ET protein is also similar to that of Tbx2, a mouse T domain protein (Bollag et al., 1994). The published expression pattern of *Tbx2* mRNA is not similar to that of *ET* (compare Fig. 6B of Bollag et al., 1994 to data shown below), making it unclear at the present whether *Tbx2* is an ortholog of *ET*.

### Pattern of *ET* expression suggests a single retina field

The pattern of *ET* expression was examined by whole-mount in situ hybridization (Harland, 1991). *ET* is prominently expressed in the primordia of the retina and the cement gland (Fig. 3E). *ET* expression in the retina primordia begins as in a single band across the midline in the anterior neural plate at stage 12.5 (indicated by the red arrow in Fig. 3A). This band of expression persists at stage 15 (Fig. 3B). Expression in the medial region of this band decreases gradually, so that it is weaker at stage 16 (Fig. 3C) and disappears by stage 18 (Fig. 3D). In late stages, *ET* expression in the retina is clearly localized in the dorsal part of the retina, but not in the lens or the ventral half of the retina (Fig. 3H,I).

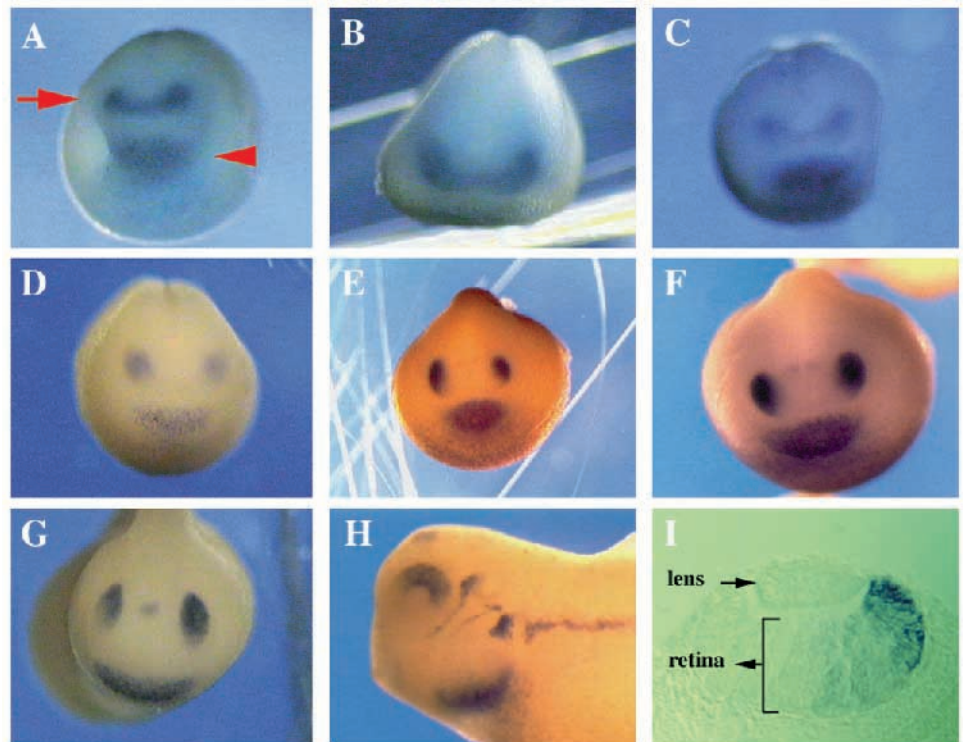
*ET* is also expressed in the cement gland, an anterior epidermal structure whose formation is often associated with neural induction (Lamb et al., 1993; Hemmati-Brivanolou et al., 1994; Rao, 1994). In contrast to its expression in the retina, *ET* expression in the cement gland does not change its pattern from the earliest time of detection at stage 12.5 (indicated by the red arrowhead in Fig. 3A) until its disappearance around stage 26 (Fig. 3A-G). Expression in the pineal gland was detected from stage 20 onwards (Fig. 3F-H). *ET* is also expressed in cephalic ganglia and the lateral line organ of stage 26 embryos (Fig. 3H).

The pattern of *ET* expression in the anterior neural plate is consistent with the possibility of a retina field in the anterior neural plate which later resolves into two retina

primordia. To ask whether *ET* expression as a band in the anterior neural plate reflects a general feature of genes expressed in retina primordia, we isolated another early marker for eye primordia, *Pax-6* and examined its expression pattern.

### Isolation of a cDNA encoding the *Xenopus Pax-6* protein

*Pax-6* is essential for eye formation in a wide range of species. In vertebrates such as the mouse, chicken, zebrafish and human, as well as in invertebrates such as *Drosophila*, *Pax-6* is expressed in the eye primordia (Walther and Gruss, 1991; Krauss et al., 1991; Püschel et al., 1992; Li et al., 1994; Glaser et al., 1994, Quiring et al., 1994). *Pax-6* mutations in mouse, human and *Drosophila* result in malformation or absence of the eyes (Hogan et al., 1988; Hill et al., 1991; Ton et al., 1991; Jordan et al., 1992; Glaser et al., 1994; Quiring et al., 1994). Ectopic expression of *Drosophila Pax-6* (*eyeless*) in imaginal discs leads to formation of supernumerary eyes, leading to the suggestion that *Pax-6* is a master regulator sufficient for initi-



**Fig. 3.** The expression pattern of *ET* in *Xenopus* embryos. A to G show anterior views and H is a lateral view. (A) A stage (st.) 12.5 embryo with a band of expression in the anterior neural plate (indicated by the arrow) and one in the primordium of the cement gland (indicated by the arrowhead). Note that the cement gland stays a single structure (A, C-G), whereas the retina band resolves into two primordia in the next few stages. (B) A st. 15 embryo showing retina field expression. The embryo was oriented to maximize visualization of retina expression and the cement gland is thus hardly visible. (C) A st. 16 embryo with *ET* expression weaker in the median region of the retina field than that in the lateral regions. (D) A st. 18 embryo with two distinct retina primordia. There is no *ET* expression in the median region. (E) A st. 19 embryo with no midline expression. (F) A st. 20 embryo. Note that weak expression of *ET* appears in the pineal gland primordium. The intensity of this signal increases in later stages (see G and H). (G) A st. 22 embryo with *ET* expression in the retina, the cement gland and the pineal gland (the dot between the two eyes). (H) A st. 26 embryo. The staining in the eye is restricted to the dorsal retina, absent from the lens and the ventral retina. Expression in the cephalic ganglia and lateral line organ also appeared. (I) A transverse section of a st. 28 embryo showing *ET* expression in the dorsal retina. *ET* is expressed in all layers of the retina, and completely absent from the lens. Dorsal is to the right.

**Fig. 4.** Comparison of Pax-6 sequence from *Xenopus* with those from other species. Alignment of the predicted sequences of full-length Pax-6 proteins from *Xenopus*, mouse and human. A dash (-) indicates identity to the *Xenopus* sequence. The paired domain (amino acid residues 3-133) and homeo domain (residues 209-269) are in bold.

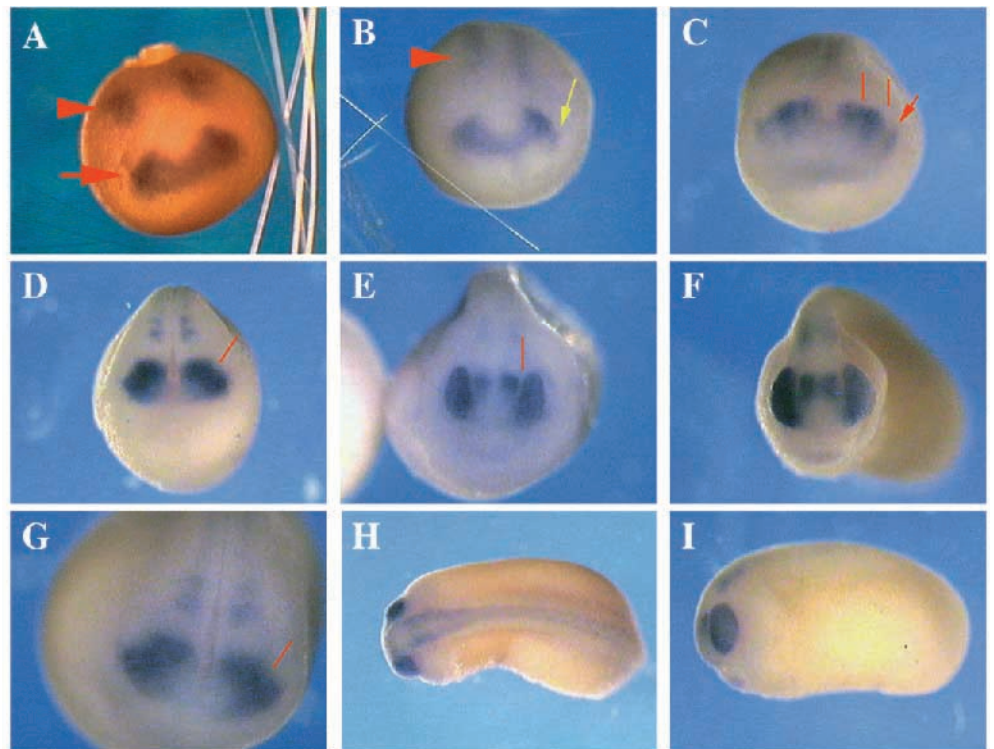
<b>Xenopus:</b>	<b>MQN SHSGVNQLGGVFNVRPLPDSTRQKIVELAHSGARPCDISRILQVSNCGVSKILGRYYETGSIRPRAIGGSKPRVATPEVNVK</b>
<b>mouse:</b>	-----S-
<b>human:</b>	-----S-
<b>Xenopus:</b>	<b>IAHYKRECP SIFAW EIRDRL LSEG VCTNDNIP SVSS INRVLRNLASDKQQMGSEGM YDKLRMLNGQTATWGSRPGWYPGTSVPGQP</b>
<b>mouse:</b>	--Q-----E----AD-----GS--T-----
<b>human:</b>	--Q-----E----AD-----GS--T-----
<b>Xenopus:</b>	<b>AQEGCQPQEGVGENTNSISSNGEDSDEAQMRLQLKRKLQRNRTSFTQEQIEALEKEFERTHYPDVFARERLAAKIDLPEARIQV</b>
<b>mouse:</b>	T-D---Q---G-----
<b>human:</b>	T-D---Q---G-----
<b>Xenopus:</b>	<b>WFSNRRAKWRREEKLRNQRRQASNTPSHIPISSFSASVYQPIQPPTPVSSFTSGSMLGRDTDALTNSYSALPPMPSFTMGNN</b>
<b>mouse:</b>	-----T-----T-----A--
<b>human:</b>	-----T-----L-----T-----A--
<b>Xenopus:</b>	<b>LPMQPPVPSQTSSYSCLPTSPSVNGRSYDITYTPPHMQTHMNSQPMGTSGLTSTGLISPGVSPVQVPGSEPDMSQYWPRLQ</b> 422
<b>mouse:</b>	----- 422
<b>human:</b>	----- 422

ating eye formation (Halder et al., 1995).

We isolated a cDNA fragment of *Xenopus Pax-6* by PCR. This fragment allowed us to isolate cDNAs encoding an apparently full-length *Xenopus Pax-6* protein (Fig. 4). Although all Pax proteins are similar within the paired and homeo domains, the primary sequences of Pax-6 proteins from all species are easily distinguishable from other Pax proteins (Quiring et al., 1994). The homeodomain of the *Xenopus Pax-6* is identical to those of other vertebrate Pax-6 while all differences in the paired domain of *Xenopus Pax-6* occur in regions where variations have been observed among the other Pax-6 sequences. The predicted full-length *Xenopus Pax-6* product is 96% identical to mouse and human Pax-6 proteins (Fig. 4).

**Pax-6 expression pattern supports formation of two retina primordia from a single field**

*Pax-6* mRNA is first expressed at stage 12 before the completion of gastrulation and the beginning of neurulation (Fig. 5A). In the trunk region, *Pax-6* is expressed in the primordium of the neural tube, beginning with two broad stripes at stage 12 (indicated by the red arrowhead in Fig. 5A). In the anterior neural plate, *Pax-6* is expressed initially in a continuous band (Fig. 5A-C). Like *ET*, *Pax-6* expression in the median region



**Fig. 5.** Distribution of *Pax-6* mRNA in *Xenopus* embryos. A-G show anterior views, H is a dorsal view with the anterior end of the embryo to the left, and I is a lateral view. (A) A st. 12 embryo; note a single band of expression continuous from one side of the embryo to the other in the anterior neural plate (indicated by the red arrow) whereas there are two broad stripes of expression in the primordium of the neural tube (indicated by the red arrowhead). (B) A st. 12.5 embryo. The stripes in the trunk region are thinner and closer to each other than those at st. 12. Note the appearance of lens primordia on each side of the embryo (indicated by the green arrow). (C) A st. 16 embryo. The two red lines demarcate the borders of the retina stripe. The arrow points to a lens primordium. *Pax-6* expression has three components: lens primordium, an outer semicircle of the retina field and an inner semicircle of a forebrain structure. The forebrain stripe later resolves into two spots posterior to the eyes shown in D and G, which are the two spots closer to the midline than the eyes in E and F. (D) A st. 18 embryo showing that *Pax-6* expression in the median region of the anterior neural plate is turned off. The red line indicates the border between the eye primordium and the forebrain structure. Note also that the lens primordia have moved into the retina primordia to form the eye primordium by this stage (compare D to C). (E) A st. 19 embryo showing distinct *Pax-6* expression in the eyes and the forebrain. (F) A stage 26 embryo. (G) Higher magnification of a st. 18 embryo. The line points to the border between the forebrain staining and the eye staining. (H) Dorsal view of a st. 24 embryo showing *Pax-6* expression in the neural tube derived from the two broad stripes in the trunk region at st. 12 (the arrowhead in A). (I) A st. 25 embryo. Compare *Pax-6* expression in the entire eye to *ET* expression in only the dorsal retina in Fig. 3H.

of this band is also turned off by stage 18 (Fig. 5D-G). The band of *Pax-6* expression in the anterior neural plate is broader than the *ET* band in the anterior/posterior dimension. On each side of the midline, *Pax-6* is expressed in the entire eye and a region in the forebrain (Fig. 5E,F). The precursor region for this forebrain structure is initially located in a stripe posterior to the retina field (Fig. 5B-D,G). This stripe also resolves into two bilaterally symmetric spots during neurulation (Fig. 5B-E).

The *Pax-6* expression pattern thus confirmed results obtained for *ET* in showing the presence of a continuous band in the anterior neural plate, supporting the suggestion that the expression patterns of these genes reveal a retina field which resolves into two retina primordia.

*Pax-6* is also expressed in the lens, which is distinguishable from its expression in the retina field from stage 12.5 onwards (Fig. 5B,C). As early as stage 12.5, lens primordia, as revealed by *Pax-6* expression, were separate on two sides of the embryo (Fig. 5B). Thus, there is so far no direct evidence for an eye field which includes both the retina and lens primordia.

#### Cells in the midline region of the retina field do not migrate into the lateral retina primordia

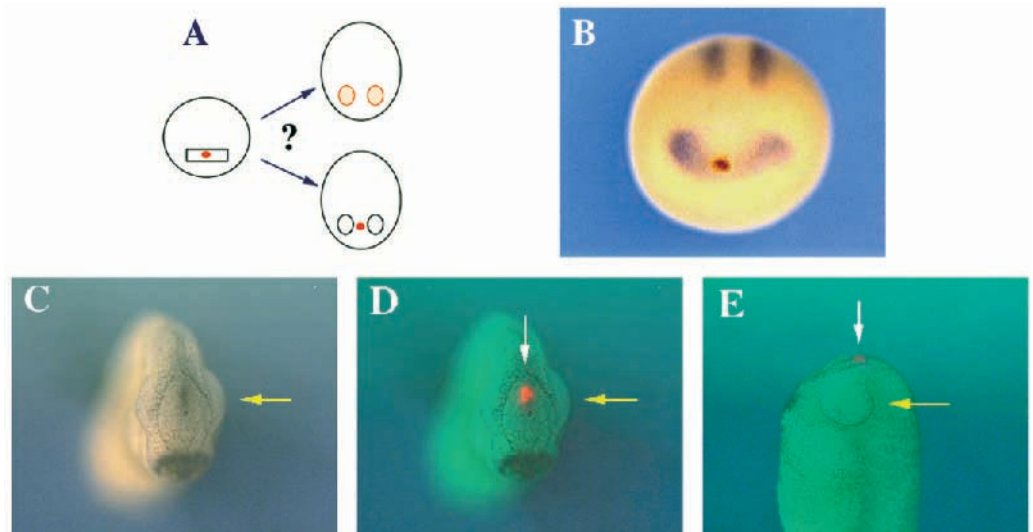
Two hypotheses can explain why retina primordia form in the lateral but not the medial regions of the retina field. One is that retina precursor cells exist in the medial region of the retina field at early stages (e.g., stages 12 and 12.5) and they migrate into the lateral regions in later stages. The alternative hypothesis is that retina formation is suppressed in the medial region of the retina field and medial cells take on fates other than that of the retina. These hypotheses can be distinguished by following the development of cells in the medial region of the retina field. The migration hypothesis predicts that some medial cells will end up in the retina, whereas the suppression hypothesis predicts that the medial cells will stay near the midline and give rise to structures other than the retina.

Previous fate mapping studies have shown that cells in the midline of the anterior neural plate of stage 15 *Xenopus* embryos will form the anterior pituitary gland and the supra chiasmatic nucleus (Eagleson and Harris, 1990; Eagleson et al., 1995). However, another study tracing the lineage of two-cell stage embryos suggested that retina cells from one side of the embryo could migrate to the other side (Jacobson and Hirose, 1978). Recent studies in zebrafish embryos suggest that midline cells may contribute to both retinae (Woo

and Fraser, 1995). A drawback in interpreting the lineage analyses of two-cell stage *Xenopus* embryos is that it cannot accurately predict the fate of midline cells since the cleavage plane at the two-cell stage is variable and independent of the midline. Results from zebrafish embryos, on the other hand, may not apply to *Xenopus* embryos because zebrafish embryos are different from *Xenopus* embryos with regard to migration of early embryonic cells (Shih and Fraser, 1995). The fate of midline cells at stages 12 and 12.5 *Xenopus* embryos thus remains unknown.

We examined the question of cell migration by labeling midline cells in the retina field with the fluorescent dye DiI at stages 12 or 12.5 and following the fate of the labeled cells (Fig. 6A). Some of the embryos were fixed shortly after labeling in order to locate the region of DiI labeling. DiI was photoconverted to give a brown signal in these embryos. *Pax-6* was used to reveal the retina field. Double labeling by DiI photoconversion and *Pax-6* in situ hybridization thus allowed determination of the spatial relationship of DiI-labeled region to the retina field. DiI labeling was indeed located in the median region of the retina field (Fig. 6B).

DiI-labeled embryos were followed to stage 25 when eyes were morphologically distinguishable. Each embryo was viewed under the microscope with epifluorescence and, immediately afterwards, with incident white light. Two images from a single embryo were thus obtained through a video camera and were individually stored. Superimposition of these two images allows precise mapping of the fluorescent cells onto the whole embryo (Fig. 6D). It is clear that DiI labeled cells do not contribute to the retinae (Fig. 6D,E). These results demon-



**Fig. 6.** Fate mapping of midline cells in the retina field of *Xenopus* embryos. A to D show anterior views. (A) A diagram showing that midline cells in the retina field were labeled by DiI at stages 12 or 12.5 and followed to stage 25 to determine whether the midline cells migrate to the lateral regions. (B) The original location of DiI-labeled cells. This embryo was labeled with DiI at stage 12 and fixed at stage 12.5. DiI was photoconverted to produce the brown spot in the middle of the retina field, which was revealed by in situ hybridization with the *Xenopus Pax-6* probe. (C) An image of a stage 25 embryo illuminated by incident light. A yellow arrow points to an eye. (D) A composite of the image shown in C and an image of the same embryo viewed under rhodamine optics to map the location of the fluorescent cells. A yellow arrow points to an eye. This embryo was previously labeled with DiI at stage 12.5. Results were similar with embryos labeled at stage 12. (E) A left side view of another DiI-labeled embryo. Note the absence of DiI labeled cells in the left retina. A white arrow points to DiI-labeled cells. A yellow arrow points to an eye.

strate that cells in the midline of the retina field do not migrate into the more lateral regions to form retina precursor cells in *Xenopus* embryos.

### Prechordal mesoderm is required for the formation of two retinae in *Xenopus* embryonic explants

To investigate a role for the prechordal mesoderm in the formation of two retinae (Adelmann, 1936b), we examined retina formation in explants with or without the prechordal mesoderm. Explants of the anterior neural plate were isolated from stage 12.5 or stage 13 *Xenopus* embryos. To ensure inclusion of the retina field in the explants, we fixed some explants immediately after isolation. They were examined by in situ hybridization with the *Pax-6* probe and the retina field was found to be present in the explants (data not shown).

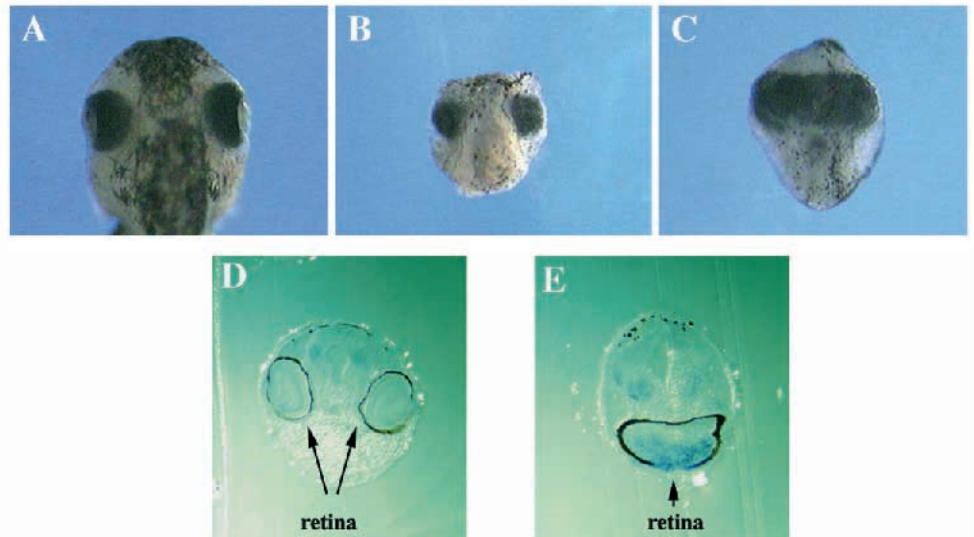
We then examined retina formation by allowing the explants to develop to stages 35 and 42 when pigmented retina is visible (Fig. 7A). When the anterior neural plate together with its underlying prechordal mesoderm was isolated, two retinae formed (Fig. 7B,D). In contrast, only one retina formed in explants from which the prechordal mesoderm was removed (Fig. 7C,E). Thus, the prechordal mesoderm is essential for the formation of two retinae.

### Prechordal mesoderm is required for resolving the retina field

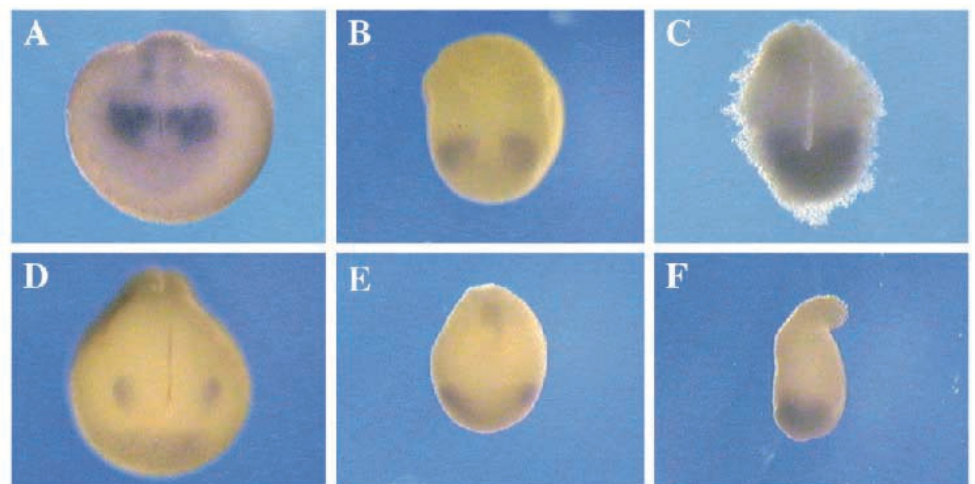
The formation of a single retina in anterior neural plate explants lacking the mesoderm could have resulted from one of the following: (1) a constant requirement of a retina inducing factor from the mesoderm for the formation of two retinae; (2) fusion of two retina primordia; or (3) failure of the single retina field to resolve. Our ability to examine retina primordia at early stages made it possible to distinguish among these different models. If the first model is true, the retina field would shrink, or in the second scenario, it would become two primordia, whereas the retina field would remain as a continuous band if the third hypothesis is correct.

We therefore examined directly the retina field with regard to the role of the prechordal mesoderm.

This was carried out by hybridizing the explants with *Pax-6* and *ET* probes. In the presence of the prechordal mesoderm, the retina field became two primordia by stage 18 (Fig. 8B,E). In the absence of the prechordal mesoderm, however, a single unresolved retina field remained (Fig. 8C,F). These results indicated that formation of a single retina in explants without the prechordal mesoderm was due to the failure of the retina field to resolve, demonstrating directly a role for the prechordal mesoderm in retina field resolution.



**Fig. 7.** Effects of removing the prechordal mesoderm on retina formation in *Xenopus* embryonic explants. (A) A wild-type embryo at st. 35. (B) A st. 35 control explant of the anterior neural plate with the underlying prechordal mesoderm. (C) An anterior neural plate explant without prechordal mesoderm. The explants were isolated at st. 12.5 and cultured until st. 35. (D) A transverse section of a st. 42 control explant after in situ hybridization with a *Pax-6* probe. (E) A transverse section of a st. 42 explant without prechordal mesoderm at st. 12.5 and fixed for in situ hybridization with *Pax-6* at st. 42. Note the presence of *Pax-6* expressing neural retina as well as the retina pigment epithelium.



**Fig. 8.** Effects of prechordal mesoderm removal on retina field resolution in *Xenopus* embryonic explants. (A) A control embryo hybridized with *Pax-6* at st. 18. (B) *Pax-6* expression in a control anterior neural plate explant with the underlying mesoderm, showing two separate primordia by st. 18. (C) *Pax-6* expression in a st. 18 explant of the anterior neural plate without the underlying mesoderm. Note the retina field did not resolve into two separate primordia. (D) A control embryo hybridized with *ET* at st. 18. (E) *ET* expression in a st. 18 control explant with the prechordal mesoderm. (F) *ET* expression in a st. 18 explant without prechordal mesoderm.

**Table 1. Effects of prechordal plate removal on retina formation in chick embryos**

Treatment	Number of embryos tested	Number of embryos with	
		2 eyes	1 eye
a. PP removal	51	37	14 (27.4%)
PP removal followed by transplantation of:			
b. Prechordal plate	39	39	0
c. Lateral prechordal mesoderm	37	26	11 (29.7%)
d. Lateral posterior mesoderm	48	37	11 (22.9%)

PP is the abbreviation of the prechordal plate. The area where the prechordal plate was removed from is shown in Fig. 9B.

The origins of tissues added back to the PP removed embryos (lateral prechordal mesoderm and lateral posterior mesoderm) were illustrated in Fig. 10A as green bars.

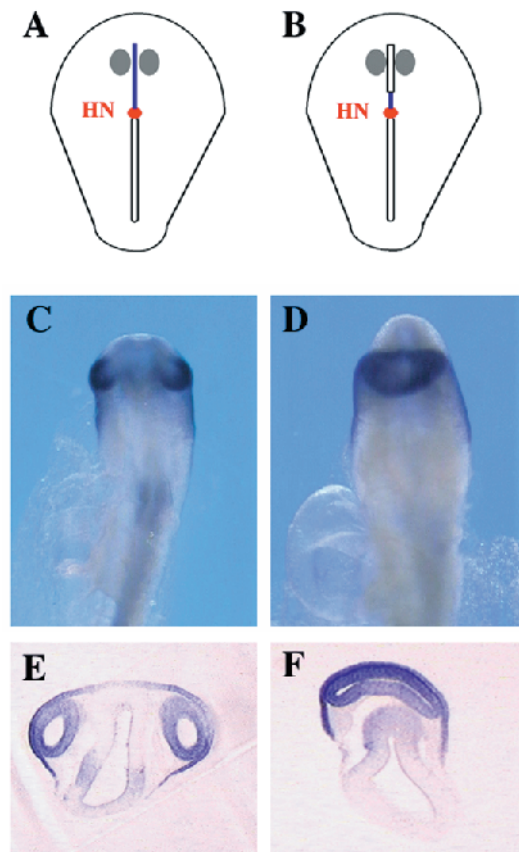
$\chi^2$  analyses indicate that the difference between a and b is statistically significant ( $P < 0.01$ ) whereas c and d are not significantly different from a ( $P > 0.5$  in both cases).

### Prechordal plate is required for the formation of two retinae in chick embryos

Explant experiments with *Xenopus* embryos have shown that the prechordal mesoderm is required for retina field resolution. We have carried out further experiments in chick embryos for three reasons. (1) Results discussed so far were all obtained from amphibian embryos. Their general significance remains to be tested by extending these studies to other vertebrate species. (2) Results from the explant experiments need to be confirmed by examining retina development in whole embryos. (3) The *Xenopus* experiments indicate retina field resolution requires the prechordal mesoderm, which is a rather large region. It is important to test the possibility that a smaller region in the prechordal mesoderm is involved in retina field resolution.

We therefore studied the consequence of removing the median region of the prechordal mesoderm, i.e., the prechordal plate, on retina formation in chick embryos. Chick embryos were operated on and cultured in albumen agar plates. The prechordal plate was removed from embryos at stage 5 (Fig. 9B) and they were cultured to stage 13 and fixed. Retinae were revealed by in situ hybridization with a chicken *Pax-6* probe (Li et al., 1994). In control embryos, two retinae formed (Fig. 9C,E). In approximately 27% of embryos without the prechordal plate, a single retina, continuous from one side of the embryo to the other, formed (Fig. 9D,F). That only a fraction of embryos had cyclopia phenotype was most likely due to the difficulty in complete removal of the prechordal plate. These results thus extended the conclusions from amphibian to avian embryos and further localized the requirement for the prechordal mesoderm to its median region, the prechordal plate.

The prechordal plate could provide either a non-specific physical support or a specific signal for the formation of two retinae. To distinguish between these possibilities, mesodermal tissues from different regions were tested for their ability to rescue the cyclopia phenotype in embryos in which the prechordal plate had been removed (see Fig. 10A for a diagram of the sources of the donor tissues). The prechordal plate was able to rescue the cyclopia phenotype (Table 1), whereas meso-



**Fig. 9.** Effects of prechordal plate removal on retina formation in chick embryos. A-D are ventral views. (A) A diagram of a st. 5 chick embryo showing the location of presumptive retina primordia (indicated by two gray circles) relative to the prechordal plate (marked as a blue line) (Li et al., 1994). The red dot symbolizes Hensen's node. (B) A diagram of the region removed from the prechordal mesoderm (indicated by a box superimposed on the blue line). (C) A st. 13 chick embryo showing *Pax-6* expression in the eyes. (D) *Pax-6* expression in a st. 13 chick embryo from which the prechordal plate was removed at st. 5. (E) A transverse section of a st. 13 control chick embryo after in situ hybridization with the *Pax-6* probe. Ventral is up. (F) A transverse section of a st. 13 embryo which lacked the prechordal plate. The level of section is similar to that of the control embryo shown in E. Ventral is up.

dermal tissues taken either from the lateral parts of the prechordal region or from the lateral posterior region could not substitute for the prechordal plate (Table 1). These results indicate that the prechordal plate plays a specific role in the formation of two retinae.

### Prechordal plate suppresses *Pax-6* expression in chick embryos

Our results from *Xenopus* explants and chick embryos suggest that the prechordal plate sends a signal to the medial region of the retina field to suppress retina formation in the medial region of the anterior neural plate. To further test this hypothesis, we carried out transplantation experiments in chick embryos to examine whether the prechordal plate could suppress the expression of *Pax-6* in the retina, which should serve as a reasonable indicator for retina development because of the known role of *Pax-6* in retina formation.



A slit was made in the lateral prechordal mesoderm underlying the retina primordium on the left side of a stage 5 host embryo (Li et al., 1994). A piece of the prechordal plate or mesodermal tissues from other embryonic regions was isolated from a donor embryo of the same stage, and placed into the slit in the host embryo (see Fig. 10A and E for diagrams). Embryos were then cultured to stage 13 and fixed for *in situ* hybridization with *Pax-6*.

In experiments with prechordal plate transplants, some embryos were fixed shortly after the transplantation and examined for the expression of *sonic hedgehog* (*shh*), a marker for the prechordal plate (Marti et al., 1995; Shimamura et al., 1995). Presence of a transplanted prechordal plate was confirmed by *shh* *in situ* hybridization (Fig. 10F).

In approximately 75% of embryos with transplanted prechordal plates, *Pax-6*-expressing areas were smaller on the side receiving the transplants (Fig. 10G, H), indicating that *Pax-6* expression was suppressed. The suppression is partial in most embryos (Fig. 10G), perhaps because contact between the transplanted prechordal plate and the anterior neural plate was not as tight as that in the wild-type situation, or because the width of the region in the prechordal plate with *Pax-6* suppressing activity is not large enough to cover the entire retina primordium on the left side of the chick embryo.

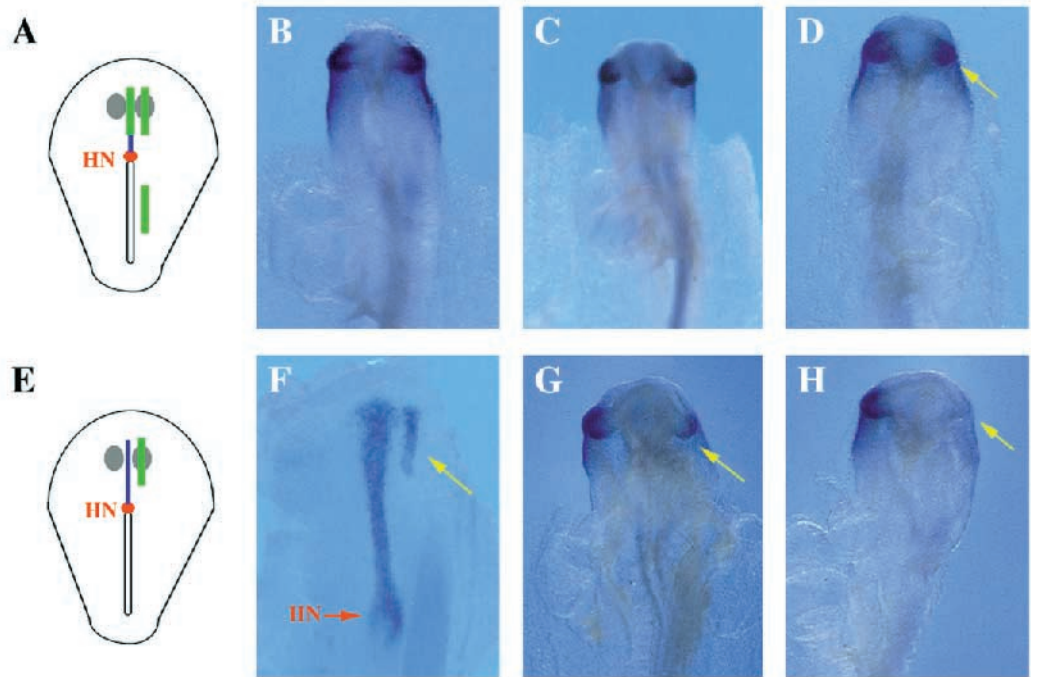
Neither the transplantation of a piece of lateral prechordal mesoderm, nor the making of a slit alone, affected the size of the retina on the experimental side (Fig. 10C and Table 2). Transplantation of a piece of mesoderm from the lateral posterior region of the embryo sometimes reduced the size of the retina (Fig. 10D), perhaps due to interference of retina induction after such transplantations.

The reduction of retina in embryos transplanted with the prechordal plate was more severe than that in embryos transplanted with lateral posterior mesoderm (compare Fig. 10G and D). Strong suppression like that shown in Fig. 9H was only observed after prechordal plate transplantation, but not after lateral posterior mesoderm transplantation (Table 2). Furthermore, the number of embryos with retina size reduction in those

transplanted with the prechordal plate was significantly higher than that in embryos transplanted with lateral posterior mesoderm (Table 2). Taken together, these results are consistent with the suggestion that the prechordal plate can inhibit *Pax-6* expression.

## DISCUSSION

Our major conclusions are that there is a single retina morphogenetic field which resolves into two retina primordia, that retina field is resolved by suppression of retina formation in the median region of the field, and that the primary signal for retina field resolution comes from the prechordal plate. Mechanisms responsible for bilateral asymmetry have been investigated in recent work on the left-right axis (Brown and Wolpert, 1990; Yost, 1990, 1991, 1992, 1995; Klar, 1994; Danos and Yost, 1995; Levin et al., 1995). Our experiments have studied formation of the eyes, a model for bilaterally symmetric struc-



**Fig. 10.** Suppression of *Pax-6* expression by the prechordal plate in chick embryos. All panels show ventral views. (A) A diagram of a st. 5 chick donor embryo with the green stripes indicating the origins of the mesodermal tissues used in transplantation. The piece in the midline of the prechordal mesoderm is the prechordal plate and that lateral to the prechordal plate is the lateral prechordal mesoderm. The green stripe in the trunk region shows the origin of the lateral posterior mesodermal transplants. (B) *Pax-6* expression in a wild-type st. 13 embryo. Note that *Pax-6* expression is equivalent in the two eyes. (C) *Pax-6* expression in a st. 13 embryo in which a piece of lateral prechordal mesoderm was inserted into the prechordal mesoderm underlying the left retina primordium at st. 5. The two eyes are equivalent. (D) *Pax-6* expression is reduced in a small fraction of embryos receiving a transplanted piece of posterior lateral mesoderm (see also Table 1). This is a typical result from such transplantations with reduced *Pax-6* expression in the left eye (indicated by the green arrow). (E) A diagram of the position where a transplant is placed in a host embryo (indicated by the green stripe on the left side of the embryo). (F) *Shh* expression in a st. 5 embryo with a transplanted piece of prechordal plate on the left side (indicated by a green arrow). A red arrow points to Hensen's node. (G) *Pax-6* expression is clearly reduced in the left eye (indicated by the green arrow) of a st. 13 embryo in which a piece of the prechordal plate was previously transplanted into a slit in the mesoderm underlying the left retina primordium at st. 5. (H) In this embryo, *Pax-6* expression was more severely suppressed in the left eye (indicated by the green arrow). Strong *Pax-6* suppression was observed only in embryos with prechordal plate transplants (see Table 2 legend).

**Table 2. Effects of mesodermal transplantation on *Pax-6* expression in chick embryos**

Transplanted tissue	Number of embryos examined	Number of embryos with <i>Pax-6</i> expression reduced on the operated side
a. Prechordal plate	37	28 (75.7%)
b. Lateral prechordal mesoderm	13	0
c. Lateral posterior mesoderm	34	9 (26.5%)
d. Lateral slit without any transplant	33	0

The origins of the donor tissue are shown as green bars in Fig. 10A, and the position where the transplants were placed into the host embryo is illustrated in Fig. 10E.

A typical result from prechordal plate transplantation is shown in Fig. 10G. There were 5 embryos in this group of 28 embryos with strong suppression of *Pax-6* similar to that shown in Fig. 10H.

A typical result after lateral prechordal mesoderm transplantation is shown in Fig. 10C.

A typical result after lateral posterior mesoderm transplantation is shown in Fig. 10D. No complete suppression like that shown in Fig. 9H was observed after such transplantations.

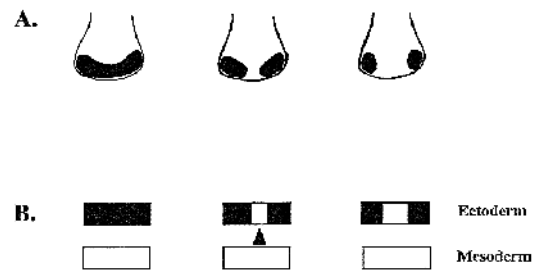
$\chi^2$  analyses indicate that a is significantly different from b, c and d ( $P < 0.01$  in all three pair-wise comparisons).

tures, revealing mechanisms underlying the establishment of bilateral symmetry in the forebrain.

### Retina morphogenetic field and retina primordia

Our model of retina primordium formation is outlined in Fig. 11. Thus, there is a single retina field in the anterior neural plate of vertebrate embryos which becomes two primordia (Fig. 11A). This model is similar to one suggested by Adelmann who based his suggestion on the findings of retina formation in the median portion as well as the lateral portions of the anterior neural plate (Adelmann, 1929a,b). Our observations of *ET* and *Pax-6* expression patterns in the anterior neural plate provide direct support for the existence of a single retina field in the early embryo. These results strengthened the conclusion of Adelmann in showing that the median as well as the lateral regions were indeed endowed with the potential for retina formation. Availability of the molecular markers makes it possible to locate the retina field precisely and to follow the process of its resolution into two primordia. Support for equipotentiality in the retina field also came from the experiments studying the role of the prechordal mesoderm on retina field development. Our finding of the formation of a single retina primordium in the entire field in the absence of the prechordal mesoderm (Figs 7 and 8) indicates that the entire retina field is indeed capable of forming retina. Our experiments with chick embryos further extend conclusions from studies of amphibians to other vertebrate species.

Results from our fate mapping experiments rule out the possibility that the medial region of the retina field contains retina precursor cells which later migrate into the lateral retina primordia (Fig. 6). Results from these experiments in *Xenopus* and ectopic transplantation of the prechordal plate in chick embryos support the suggestion that retina field resolution is achieved by suppression of retina forming potential in the



**Fig. 11.** Retina primordium formation in vertebrate embryos. (A) Our model of retina field development. Dorsal views of the anterior neural plate are illustrated here. There is initially a single retina morphogenetic field in the anterior neural plate of vertebrate embryos. Retina formation is suppressed in its median region, resulting in the resolution of the retina field into two retina primordia. (B) The prechordal plate provides a primary signal for retina field resolution. These are transverse section views of the anterior neural plate and its underlying prechordal mesoderm. Experiments reported here demonstrate a role for the prechordal plate while those performed in zebrafish embryos suggest the midline of the neural plate also plays a role (Hatta et al., 1991, 1994; Hatta, 1992; Macdonald et al., 1995; Ekker et al., 1995b).

median region and maintenance of retina development in the lateral regions of the retina field.

### Sources of signals for retina field resolution: the prechordal plate and the midline cells of the anterior neural plate

Our experiments have addressed the role of the prechordal mesoderm in influencing the bilaterality of the retina primordia. It is shown that removal of the prechordal mesoderm could result in the formation of a single retina in the retina field. Direct examination of the retina field shows that it stays as a single field after the removal of the prechordal mesoderm (Fig. 8). Prechordal plate removal and transplantation experiments in chick embryos indicate that the prechordal plate plays a specific signaling role in inhibiting retina formation in the ectoderm (Figs 9, 10). Taken together, these results suggest that the prechordal plate provides an essential signal to resolve the retina field (Fig. 11B).

The phenotype of chick embryos removed of the prechordal plate is very similar to that of zebrafish mutants defective in the *cyclops* gene. Thus, fusion of *Pax-6* expressing domains (Hatta et al., 1994; Macdonald et al., 1995) and formation of a single eye were observed in zebrafish *cyclops* mutants (Hatta et al., 1991). Cell transplantation experiments have shown that midline cells in the anterior neural plate as well as floor plate cells in the neural tube are defective in *cyclops* mutant embryos (Hatta et al., 1991, 1994; Hatta, 1992). Failure to form separate eyes in *cyclops* mutant embryos was shown to be secondary to defects of these midline cells in the anterior neural plate, suggesting that the midline cells in the ectoderm is important for the separation of the two eyes (Hatta et al., 1994).

Thus, results from amphibian and chick embryos indicate that the prechordal plate is crucial for retina field resolution while results from zebrafish embryos suggest a role for the midline cells in the anterior neural plate. This scenario is quite similar to the roles of the notochord and the floor plate in inducing ventral cell types and suppressing dorsal cell types in

the neural tube (Jessell et al., 1988; van Straaten et al., 1988; Placzek et al., 1990, 1993; Yamada et al., 1991, 1993; Basler et al., 1993; Liem et al., 1995; Goulding et al., 1993).

### Molecular nature of the signal for retina field resolution

Similarity of retina field resolution to dorsal/ventral patterning in the neural tube suggests that molecules responsible for neural tube patterning may also be involved in resolving the retina field. Thus, sonic hedgehog (*shh*), a molecule known to be important for neural tube patterning (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994, 1995), is a candidate signal for retina field resolution. Injection of *shh* mRNA into zebrafish embryos leads to overall decrease of *Pax-6* expression, reduction of eyes and increased expression of *Pax-2*, a marker for the optic stalk (Macdonald et al. 1995; Ekker et al., 1995b). These observations, together with the finding of *shh* expression in the midline of the anterior neural plate (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994, 1995; Ekker et al., 1995a; Ericson et al., 1995), have led to the suggestion of *shh* as the signal in the midline of the neural plate for patterning the retina and optic stalk (Macdonald et al. 1995; Ekker et al., 1995b). Studies involving activating and inhibiting the signal transduction pathway of *shh* lend further support to this suggestion (Hammerschmidt et al., 1996). Observation of cyclopia in mice lacking *shh* proves that *shh* is required for the formation of two eyes. Since expression of *shh* is also detected in the prechordal mesoderm (Marti et al., 1995; Shimamura et al., 1995), *shh* may act as the prechordal plate signal for resolving the retina field. Curious findings of human fetuses with both holoprosencephaly and digit anomalies (Young and Madders, 1987; Moerman and Frys, 1988; Shiota and Tanimura, 1988; Atkin, 1988; Cohen, 1989) could be best explained by defects in signaling pathways used both in patterning the limb bud (Riddle et al., 1993) and in determining bilaterality in the anterior neural plate.

### Retina field vs. lens field

Classic literature has called the morphogenetic field in the anterior neural plate, the eye field (Adelmann, 1936b). Our examination of *Pax-6* expression in *Xenopus* embryos shows that formation of the lens primordia is different from the retina primordia: there are two separate lens primordia as early as stage 12.5 when the retina field is still a single field (Fig. 5B). Thus, either there is no lens field or the lens field behaves differently from the retina field. In the former case, two lenses are determined from separate primordia. In the latter scenario, it is possible that at stage 12 (Fig. 5A), the lens field could not be distinguished from the retina field by examining *Pax-6* expression. If this is true, then the lens field must resolve into two lens primordia by stage 12.5 (Fig. 5B). Since the retina field remains one field through stages 14, 15, 16, separation of this hypothetical lens field is accomplished earlier than separation of the retina field. Thus, whether or not a lens field exists, the mechanism underlying retina primordium formation appears to be different from that for lens primordium formation.

### Bilateral symmetry in the forebrain

The *Pax-6* expression pattern suggests that the bilaterality of forebrain regions other than the retina is established in a similar manner to that of the retina. Thus, a forebrain structure express-

ing *Pax-6* was also found to be a continuous band just posterior to the retina field and this band became two spots over the same time course as the retina field resolution (Fig. 5), suggesting a mechanistic similarity in the formation of the bilaterally symmetric retina and forebrain structures. This suggestion could explain why, in cyclopean embryos, not only eyes but also other forebrain structures remain unseparated (e.g. Adelmann, 1934; Hatta et al., 1991).

Regulative changes of the forebrain primordial region observed by Corner and Boterenbrood have led to the proposal of a forebrain field (Corner, 1963, 1966; Boterenbrood, 1970; Nieuwkoop et al., 1985). This hypothesis suggests that an area larger than the retina field is equivalent in developmental potential (Corner, 1963, 1966; Boterenbrood, 1970; Nieuwkoop et al., 1985). It is not clear how the retina forms in this field, although the retina fate is supposed to be the default in this field (Corner, 1963, 1966; Boterenbrood, 1970; Nieuwkoop et al., 1985). In contrast to our data supporting the existence of a retina field, there is so far no evidence that complements the results of Corner (1963, 1966) and Boterenbrood (1970) in directly demonstrating a forebrain field.

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### Note added in proof

Two papers indicating involvement of abnormality in human *shh* functioning as the underlying cause of a subset of holoprosencephaly have now been published.

Belloni, E., Muenke, M., Roessler, E., Traverso, G., Siegel-Bartelt, J., Frumkin, A., Mitchell, H. F., Donis-Keller, H., Helms, C., Hing, A. V., Heng, H. H. Q., Koop, B., Martindale, D., Rommens, J. M., Tsui, L.-C. and Scherer, S. W. (1996). Identification of Sonic hedgehog as a candidate gene responsible for holoprosencephaly. *Nature Genet.* **14**, 353-356.  
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