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Midline signalling is required for Pax gene regulation and patterning of the eyes

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SUMMARY

Pax6 and Pax2 are members of the Pax family of transcription factors that are both expressed in the developing visual system of zebrafish embryos. Pax6 protein is present in all cells that form the neural retina and pigment epithelium, whereas Pax2 is located primarily in cells that will give rise to the optic stalk. In this study, we have addressed the role of midline signalling in the regulation of Pax2 and Pax6 distributions and in the subsequent morphogenesis of the eyes. Midline signalling is severely perturbed in *cyclops* mutant embryos resulting in an absence of ventral midline CNS tissue and fusion of the eyes. Mutant embryos ectopically express Pax6 in a bridge of tissue around the anterior pole of the neural keel in the position normally occupied by cells that form the optic stalks. In contrast, Pax2 protein is almost completely absent from this region in mutant embryos. Concommitant with the changes in Pax protein distribution, cells in the position of the optic stalks differentiate as retina.

These results suggest that a signal emanating from the midline, which is absent in *cyclops* mutant embryos, may be required to promote Pax2 and inhibit Pax6 expression in cells destined to form the optic stalks. Sonic hedgehog (Shh also known as Vhh-1 and Hhg-1) is a midline signalling molecule that is absent from the neuroepithelium of *cyclops* mutant embryos at early developmental stages. To test the possibility that Shh might be able to regulate the spatial expression of Pax6 and Pax2 in the optic primordia, it was overexpressed in the developing CNS. The number of cells containing Pax2 was increased following *shh* overexpression and embryos developed hypertrophied optic stalk-like structures. Complimentary to the changes in Pax2 distribution, there were fewer Pax6-containing cells and pigment epithelium and neural retina were reduced.

Our results suggest that Shh or a closely related signalling molecule emanating from midline tissue in the ventral forebrain either directly or indirectly induces the expression of Pax2 and inhibits the expression of Pax6 and thus may regulate the partitioning of the optic primordia into optic stalks and retinal tissue.

Key words: zebrafish, paired box, pax, eye, cyclops, *hedgehog*

INTRODUCTION

The development of the vertebrate eye involves a hierarchy of inductive interactions by which the retina, optic stalk and lens progressively become specified within the developing forebrain (review: Saha et al., 1992). Inductive events during gastrulation establish the rostrocaudal and dorsoventral axes of the neural plate (Doniach, 1993; Ruiz i Altaba, 1994) and by the end of gastrulation, further specification results in the localisation of the presumptive retinal fields to lateral regions of the rostral neural plate (Saha et al., 1992). During neurulation, the optic vesicles evaginate from the forebrain and make contact with the overlying surface ectoderm, remaining attached to the rostral diencephalon via the optic stalks. The optic vesicles subsequently invaginate to form optic cups with their characteristic double layer of neural retina and pigment epithelium (Grant et al., 1980) and the surface ectoderm which contacts the optic cups gives rise to the lens placodes (Grainger, 1992). The optic stalks progressively decrease in size (Schmidtt and Dowling, 1994) such that eventually only the optic nerve connects the eyes to the forebrain.

The identity of molecules involved in the early inductive events which determine the eye fields are largely unknown, although a number of potential regulatory genes are expressed in optic tissue during the formation of the eye (reviewed by Beebe, 1994). These include two members of the paired box (Pax) gene family, *pax6* and *pax2*. In zebrafish (Krauss et al., 1991b,c; Püschel et al., 1992a; Macdonald and Wilson, unpublished results) and mice (Püschel et al., 1992; Walther and Gruss, 1991), *pax6* is expressed throughout the optic vesicles in all cells of the prospective retina, pigment epithelium and lens epithelium. Mutations in *Pax6* result in severe visual defects in *Small eye* mice (Hill et al., 1991; Hogan et al., 1988; Schmahl et al., 1993), rats (Fujiwara et al., 1994; Matsuo et al., 1993) and in humans with aniridia syndrome (Glaser et al., 1994; Jordan et al., 1992; Ton et al., 1991) demonstrating that this gene is crucial for normal eye development. Recently, it has been shown that the *Drosophila* homologue of *pax6*,

eyeless, is also required for eye development (Quiring et al., 1994) and ectopic eye structures can be induced by misexpression of *eyeless* in various imaginal disc primordia (Halder et al., 1995). Although Pax6 is required for eye development, its precise role and mechanism of action are not understood. In contrast to Pax6, Pax2 is restricted to the optic stalks and retinal cells around the choroid fissure (Krauss et al., 1991a; Püschel et al., 1992b). The function of Pax2 during development of the visual system is not known although it has recently been shown that mutations in the *PAX2* gene can cause optic nerve colobomas in humans (Sanyanusin et al., 1995).

Signals derived from axial midline mesoderm and ventral midline CNS induce the differentiation of ventral cell types in the spinal cord (Placzek et al., 1993; Yamada et al., 1993). The possibility that similar signals may be involved in patterning the CNS in more rostral regions is suggested by the phenotype of zebrafish embryos homozygous for the *cyclops* mutation in which midline signalling is disturbed and ventral forebrain development is abnormal (Hatta et al., 1991, 1994; Macdonald et al., 1994). Mutant embryos exhibit fusion of the eyes, reduction in size of the diencephalon and loss of the floorplate in more caudal regions. Cell transplantation experiments have suggested that the *cyclops* gene product is involved in a signalling pathway between mesoderm and ventral neuroectoderm (Hatta et al., 1991, 1994). Supporting this hypothesis, there is no expression of the midline signalling molecule *shh* within the neuroectoderm of *cyclops* mutant embryos during early stages of development (Krauss et al., 1993; Barth and Wilson, 1995). *shh* is a vertebrate homologue of the *Drosophila hedgehog* gene which encodes a secreted protein involved in a variety of signalling events including floorplate induction and anteroposterior patterning of the limb bud (Fietz et al., 1994). Shh and other Hedgehog (Hh) family proteins undergo autoproteolysis to generate two smaller peptides and it appears that all signalling activity resides in the amino-terminal cleavage product (Lee et al., 1994; Bumcrot et al., 1995; Fan et al., 1995; Fietz et al., 1995; Marti et al., 1995; Porter et al., 1995; Roelink et al., 1995).

The absense of *shh* from the neurectoderm of *cyclops* mutant embryos together with the defects in eye development raises the possibility that Shh may regulate some aspects of eye development. We have tested this hypothesis by examining the role of Shh in the regulation of Pax6 and Pax2 protein distributions and in the morphogenesis of the eyes. We find that in *cyclops* mutant embryos, cells in the normal position of the optic stalks differentiate as retina and not as optic stalks resulting in fusion of the eyes. At the molecular level, this is reflected by the near absence of Pax2 and the presence of Pax6 in the region that forms retina in place of optic stalk. Conversely, overexpression of *shh* results in increased numbers of Pax2-containing cells and hypertrophied optic stalk-like tissue together with decreased numbers of Pax6 containing cells and reduced pigment epithelia and retinae. We suggest that Shh is involved in regulating the spatial distribution of Pax2 and Pax6 which in turn may regulate the subdivision of the optic primordia into optic stalks and retinae.

MATERIALS AND METHODS

Maintenance of fish

Breeding fish were maintained at 28.5˚C on a 14 hours light/10 hours dark cycle. Embryos were collected from the colony by natural spawning and raised in 10% Hank's saline. Embryos up to 24 hours (30 somites) were staged according to 'The Zebrafish Book' (Westerfield, 1993). *Cyclops*^{b16} mutant embryos were obtained from a stock originally provided by C. Kimmel and C. Nüsslein-Volhard. The *cyclops* mutation has a slightly variable phenotype in the head – the majority of the mutant embryos from our stock exhibit partial fusion of the two eye fields (synopthalmia) and many such embryos retain two lenses.

Immunohistochemistry and in situ hybridisation

Standard procedures were used for antibody labelling (Wilson et al., 1990), frozen sectioning (Westerfield, 1993) and in situ hybridisation (Xu et al. 1994). For primary incubations, anti-Pax6 antibody (Macdonald et al., 1994) was diluted 1:400, anti-Pax2 antibody (Mikkola et al., 1992) was diluted 1:3000 and HNK1 was diluted 1:20. Control and experimental embryos were usually processed in the same tubes to ensure identical labelling conditions.

Observations of living embryos

Living embryos were viewed in their chorions under differential interference contrast optics as described by Xu et al. (1994).

RNA injections

shh RNA for injections was transcribed from the pSP64T-shh plasmid (kindly provided by J.-P. Concordet and P. Ingham; see Krauss et al., 1993). Methods for RNA preparation and injection are described by Barth and Wilson (1995). The cytoplasm of individual blastomeres of embryos at the 1-4 cell stage were injected with several picoliters at a concentration of 0.1 mg/ml which results in widespread although mosaic distribution of the injected RNA (see Barth and Wilson, 1995). For control injections, RNA encoding β-galactosidase was injected at the same or higher concentration as *shh* RNA.

RESULTS

Eye development in *cyclops* mutant embryos

Cyclops mutant embryos exhibit abnormal specification of the ventral CNS and fusion of the eyes. These defects are likely to arise because of deficiencies in axial mesoderm and in the signalling pathway between dorsal midline mesoderm and ventral CNS (Hatta et al., 1991, 1994; Thisse et al., 1994). Supporting this hypothesis, *shh* expression initially fails to be induced within the ventral CNS of *cyclops* mutant embryos (Krauss et al., 1993). The large deficencies in ventral forebrain tissue of *cyclops* embryos have raised the possibility that the cyclopic eye might arise by fusion of the two eye primordia, ventral to the diencephalon (Hatta et al. 1994). However, we find that the initial fusion of the two eye primordia is around the rostral pole of the neural keel in the position normally occupied by the optic stalks and rostral hypothalamus and that the fused eye only later lies ventral to the brain due to subsequent rotation of the eye. Below, we describe the morphogenesis of the fused eye of *cyclops* mutant embryos as an understanding of this process is critical to the interpretation of changes in Pax protein distributions seen in these embryos.

Comparison of eye development in living wild-type (Fig. 1A,B) and *cyclops* mutant embryos (Fig. 1C,D) shows that the prospective retinae are fused around the rostral pole of the neural keel in mutant embryos (Fig. 1C,D). However, at early stages, more caudal/distal regions of the eyes remain in their normal positions lateral to the forebrain and are not fused ventral to the diencephalon (compare Fig. 1C,D with 1A,B) despite the large deficiency of CNS tissue that is present ventral to the mid- and

caudal diencephalic neuroepithelium (Fig. 1E,F). The bridge of presumptive retinal tissue lies ventral to the telencephalon (Fig. 1E,F) and occupies the position of cells that normally form the optic stalks and ventral/anterior hypothalamus. Although invagination of the retina is most prominent in the lateral regions of the fused eye vesicle, some invagination usually occurs all the way around the rostral pole of the forebrain, thus forming a horseshoe shaped retina continuous from the lateral parts of the retinal primordia around the rostral pole of the brain (Fig. 1D,F). Histological analysis (see below) indicates that the rostral bridge of tissue linking the lateral retinal primordia forms retina rather than optic stalk and rostral hypothalamus which normally occupy this region in wild-type embryos. Indeed, in *cyclops* mutant embryos, the rostral hypothalamus is absent (Fig. 1C) and we see no identifiable optic stalks such that the fused retina is in direct contact with the forebrain neuroepithelium (Fig. 2F and data not shown).

Although the initial fusion of the cyclopic eye is around the rostral pole of the brain, the fused eye eventually becomes positioned ventral to the forebrain (Hatta et al., 1994 and see Fig. 2F). The primary reason for this repositioning is the rotation of the eye in relation to the main body axis during the second day of development, such that the anterior region of the optic primordia becomes positioned ventrally (Ross et al., 1992; Schmitt and Dowling, 1994). The reduction of mid-diencephalic neuroepithelium in the brains of *cyclops* mutant embryos exacerbates the repositioning of the fused eye such that the temporal retina eventually comes to lie adjacent to the midbrain tegmentum (Fig. 2F).

Altered distribution of Pax6 and Pax2 in *cyclops* mutant embryos correlates with abnormal optic stalk and retinal development

In wild-type embryos, Pax6 is present within prospective eye tissue from the end of gastrulation, and continues to be expressed in all cells of the developing pigment epithelium and neural retina throughout the first day of development (Macdonald and Wilson, unpublished results and see Fig. 2A). In contrast to wild-type embryos (Fig. 2A), the Pax6 expression domain of *cyclops* mutant embryos extends from the lateral retinal primordia around the rostral pole of the neural keel including the medial regions of the optic primordia which normally consist of presumptive optic stalk cells (Fig. 2B-E; see also Hatta et al., 1994). The bridge of prospective retinal tissue linking the retinal primordia of *cyclops* mutant embryos colocalises with this region of ectopic Pax6 expression (compare Fig. 2B,C with 1C,D). In accordance with morphological observations, the bridge of Pax6-containing tissue linking the two eyes is restricted to the anterior/ventral neural keel and there is no retinal fusion ventral to the mid- or caudal diencephalon (compare Fig. 2D with Fig. 1F).

Despite the abnormal appearance of the fused eye, immunohistochemical analysis with antibodies that reveal lamination and neuronal differentiation indicated that retinal differentiation did occur in the fused neural retina of *cyclops* mutant embryos (data not shown). However, within the bridge of retinal tissue across the midline, the pigment epithelium was incomplete, there was some disorganisation of photoreceptors and the fused neural retina was in direct contact with forebrain neuroepithelium.

The results presented above suggest that cells within medial regions of the optic primordia of *cyclops* mutant embryos contain ectopic Pax6 and may be respecified to forming retina in place of optic stalk tissue. If optic stalk tissue is replaced by retina, it might be expected that genes normally expressed in the optic stalks would fail to do so in *cyclops* embryos. In wild-type embryos, Pax2 protein is detected at low levels in a small number of cells at the anterior/medial region of the optic vesicle from the 6-8s (somite) stage and continues to be expressed at higher levels within the presumptive optic stalks and some cells of the ventral/anterior retina throughout the first day of development (see for example Figs 2G and 7A). Thus the restriction of Pax2 protein to the medial part of the optic primordium is almost complementary to the distribution of Pax6 within more distal cells (Fig. 7A and compare Fig. 2G with 2A; Krauss et al. 1991a; Mikkola et al., 1992; Püschel et al., 1992b). As predicted, *cyclops* mutant embryos exhibit an almost complete absence of Pax2 protein in the optic primordia (Fig. 2H) while the midbrain expression of Pax2 appears unaffected (Fig. 2H). This reduction in Pax2 is more dramatic than has previously been observed (Hatta et al., 1994). While we are not certain of the reason for this difference, it could be that the study by Hatta et al. used *cyclops* mutant embryos which exhibited a less severe phenotype than the embryos from our own stock of *cyclops* carrier fish.

Gene expression in distal/posterior retinal tissue is unaffected by the *cyclops* mutation

Our results indicate that the initial site of retinal fusion in *cyclops* mutant embryos is around the rostral pole of the neural keel, linking the anterior/medial retina on both sides of the brain. If this is correct, then the *cyclops* mutation might not affect the development of posterior parts of the retina. To determine if this is indeed the case, we examined the expression of two genes, *rtk2* and *msxC,* normally transcribed in restricted domains of the developing retina. Rtk2 is a member of the Eph family of receptor tyrosine kinases expressed throughout the temporal half of the retina from the posterior groove dorsally to the choroid fissure ventrally (Fig. 3A; Macdonald et al., 1994; Xu et al., 1994). *msxC* (Ekker et al., 1992) is a homeobox-containing gene that is expressed in the caudal part of the retina, around the posterior groove.

The boundary of *rtk2* expression between nasal and temporal retina at the posterior groove was unaffected by the *cyclops* mutation (compare Fig. 3A and B). However, in support of our interpretation of the *cyclops* retina, *rtk2* expression in the anterior/ventral region of the temporal retina was continuous around the rostral pole of the neural keel (Fig. 3C). Similarly, *msxC* expression around the posterior groove of the retina was unaffected in *cyclops* mutant embryos (Fig. 3F).

Eye development following overexpression of *shh*

The results presented above suggest that a signal emanating from the midline and absent in *cyclops* mutant embryos may be required to regulate the distribution of Pax2 and Pax6 within the developing eye primordia. Shh is a signalling molecule (Johnson and Tabin, 1995) that is expressed along the ventral midline of the CNS (Fietz et al., 1994) including cells at the base of the optic stalks (Fig. 7A; Krauss et al., 1993; Barth and Wilson, 1995). In *cyclops* mutant embryos, *shh* transcripts are absent from the neuroepithelium at early developmental stages (Krauss et al., 1993). To test whether Shh is able to regulate the spatial distribution of Pax6 and Pax2 in the optic primordia, it was overexpressed in the developing CNS. 380 embryos injected with *shh* RNA were

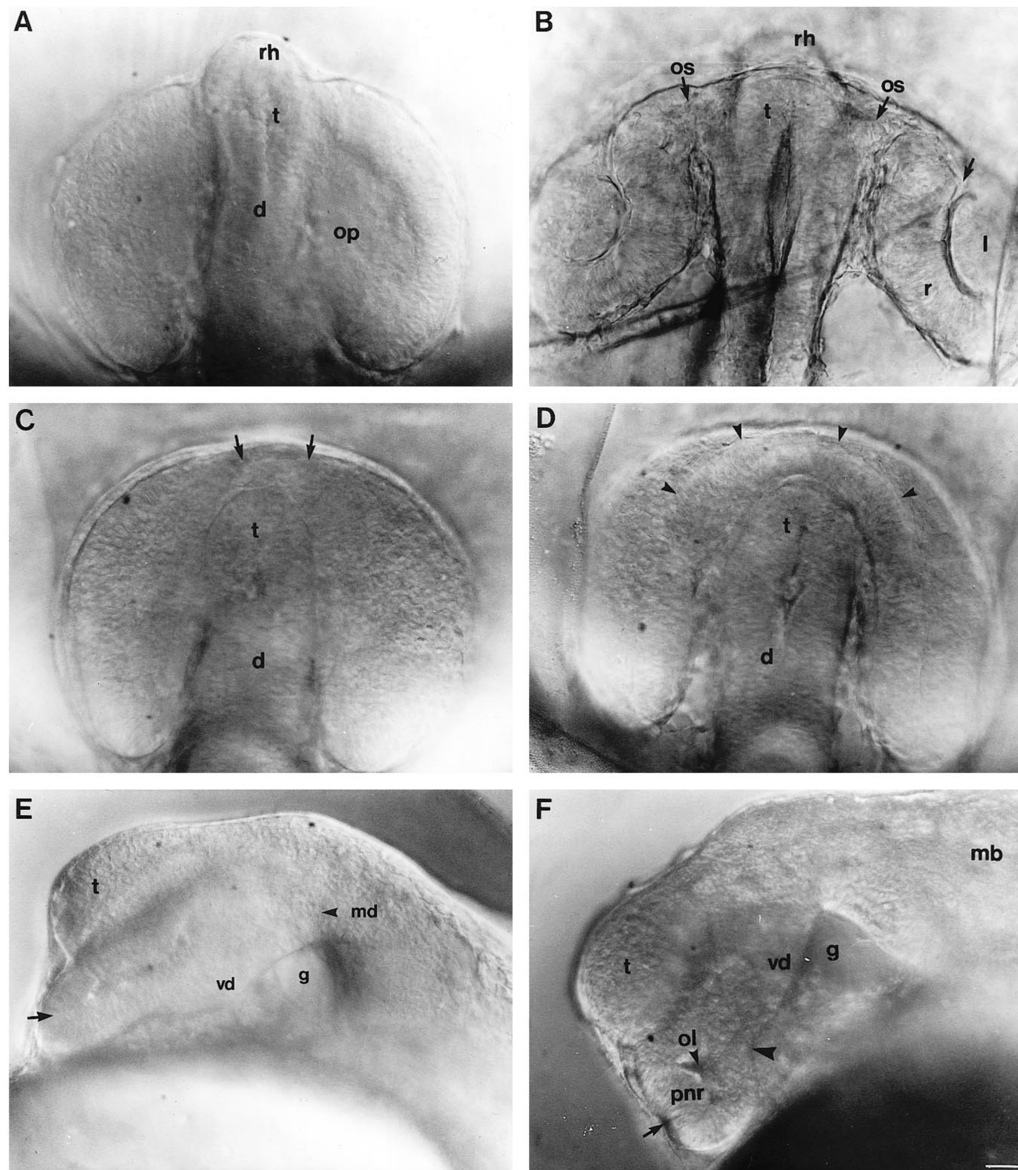

Fig. 1. Development of the eye in living wild-type embryos and embryos homozygous for the *cyclops* mutation. (A,B) Wild-type embryos, (C-F) *cyclops* embryos. (A) 13s, dorsal view. (B) 24 hour, dorsal view. The arrow indicates the rostral limit of optic cup invagination. The position of the telencephalon is indicated although it is above the plane of focus. (C) 13s, dorsal view. The arrows indicate the bridge of fused retina around the anterior pole of the neural keel. The anterior hypothalamus (see A and B) is missing from mutant embryos. (D) 20-22s, dorsal view. The arrowheads indicate minor invagination in the bridge of fused neural retina. (E) 16s, lateral view focused on the midline. The arrow indicates the area of prospective retinal fusion. A large gap is present beneath the diencephalon. (F) 20-22s, lateral view focused on the midline. The arrow indicates a small amount of invagination in the bridge of prospective retinal tissue. The arrowhead indicates the caudal limit of retinal fusion. Abbreviations: d, diencephalon; g, gap in mid-diencephalic neuroepithelium; l, lens; mb, midbrain; md, mid-diencephalon; ol, optic lumina; op, optic primordia; os, optic stalks; pnr, presumptive neural retina; r, retina; rh, rostral hypothalamus; t, telencephalon; vd, ventral diencephalon. Scale bar, 25 μm.

analysed for changes in gene expression and/or changes in eye morphogenesis. In support of previous observations which showed that overexpression of *shh* leads to eye defects (Krauss et al., 1993; Barth and Wilson, 1995), we found that approximately 90% of injected embryos had readily detectable changes in gene expression and morphogenesis of the eyes (see below). In contrast, embryos injected with RNA encoding β-galactosidase failed to show any comparable alterations in eye development or in gene expression patterns (data not shown and see Barth and Wilson, 1995).

Although optic vesicle evagination invariably did occur in *shh* injected embryos, the size of the optic vesicle was generally reduced as compared to wild type embryos (compare Fig. 4B with 4A). Confirming previous observations (Barth and Wilson, 1995), injection of *shh* RNA led to a failure of separation of the eye primordium from the diencephalon such that the eye and the diencephalon were fused over a large region. As we describe in detail below, we interpret this phenotype as being caused by hypertrophy of the optic stalks at the expense of pigment epithelium and neural retina. Defects in retinal development were readily apparent in living *shh* injected embryos such that retinal tissue, most obviously pigment epithelium, was reduced and occasionally absent (Fig. 4C-E).

Overexpression of *shh* leads to a reduction in the numbers of cells expressing Pax6 and ectopic expression of Pax2

Pax6 and Pax2 distributions were analysed in *shh*-injected embryos fixed between 6-8s and 30s (24 hours). In 89 of 101 embryos examined in detail, overexpression of *shh* led to major reduction in the number of Pax6-containing cells within the optic primordia at all stages of development examined (Fig. 5A and compare Fig. 5C with 5B). The remaining Pax6 protein was distributed in more distal/posterior regions of the optic primordia (Fig. 5C). Pax6 was also reduced in other regions of the embryo including the lens epithelium, dorsal diencephalon (Fig. 5C) and rhombomeres of the hindbrain (not shown).

In marked contrast to the observed reduction in Pax6, Pax2 was widely ectopically expressed in the optic primordia in 80 of 91 *shh*-injected embryos (Fig. 5D and compare Fig. 5F with

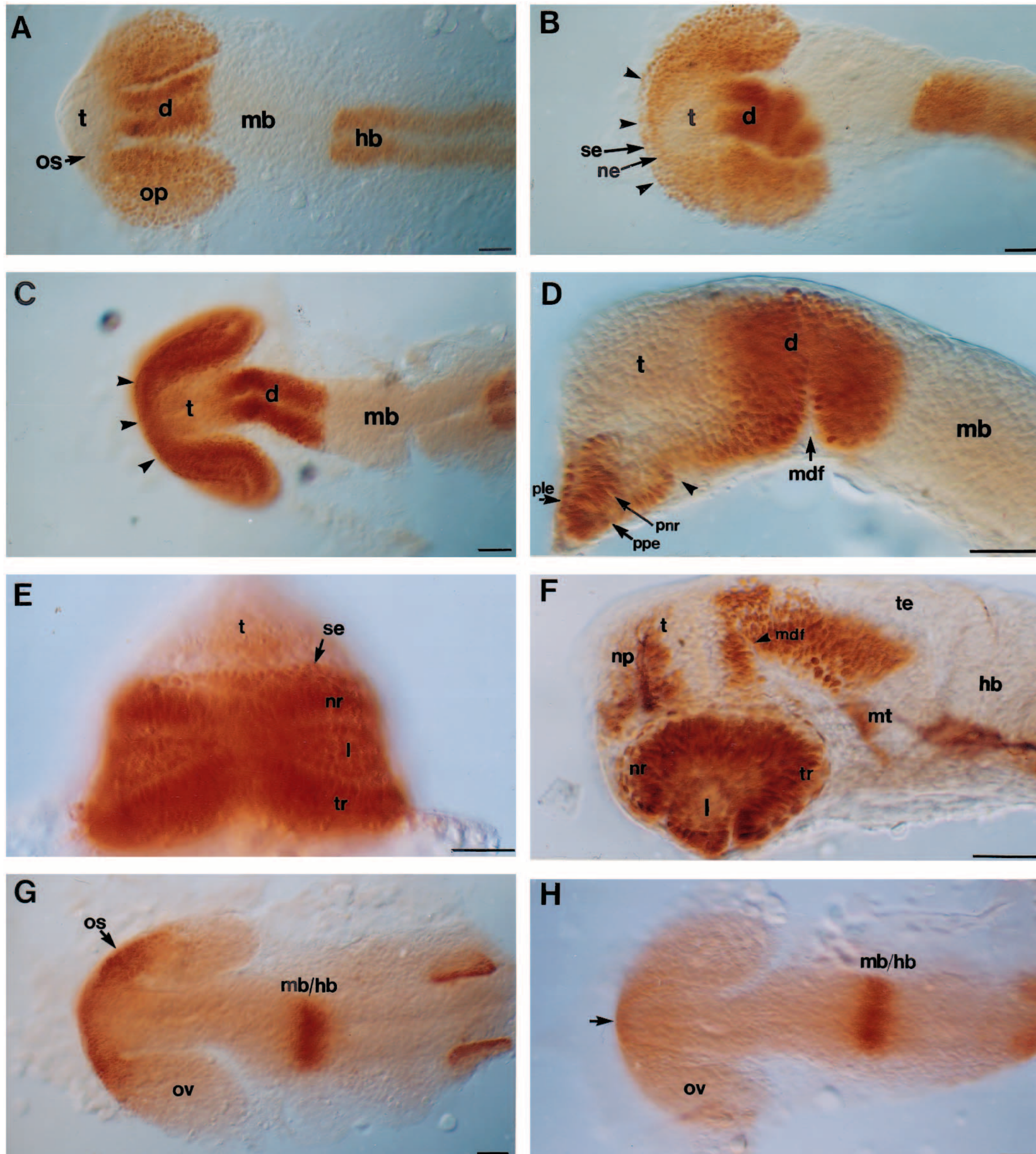

Fig. 2. Pax protein distribution in wild-type and *cyclops* mutant embryos. Whole-mounted embryos labelled with anti-Pax6 antibody (A-F), or labelled with anti-Pax2 antibody (G,H). (A,B) Dorsal views of 15-16s wild-type and *cyclops* embryos respectively. The arrowheads in B indicate the bridge of rostral retinal fusion. (C,D) Dorsal and lateral (focused on the midline) views respectively of 20s *cyclops* embryos. The arrowheads indicate the area of retinal fusion in C. In D the lateral portions of the cyclopic eye have been removed. The arrowhead indicates the caudal limit of eye fusion. (E) Frontal view of 28s *cyclops* mutant embryo. (F) Lateral view of 28 hour *cyclops* mutant embryo also labelled with anti-tubulin antibody (black labelling of axons). This embryo was also examined in a different study (Macdonald et al., 1994). (G,H) 18-20s dorsal views of wild-type (G) and *cyclops* (H) embryos labelled with anti-Pax2 antibody. The arrow in H indicates a few cells near the midline weakly labelled with anti-Pax2 antibody. Abbreviations: d, diencephalon; hb, hindbrain; mb, midbrain; mdf, mid-diencephalic furrow; l, lens; mt, midbrain tegmentum; ne, neural ectoderm; nr, nasal retina; os, optic stalks; np, nasal placode; op, optic primordia; ov, optic veiscle; ple, presumptive lens ectoderm; pnr, presumptive neural retina; ppe, presumptive pigment epithelium; se, surface ectoderm; t, telencephalon; te, tectum; tr, temporal retina; vd, ventral diencephalon. Scale bars, 50 µm.

5E). Ectopic Pax2 was present thoughout much of the optic primordia although it was usually absent from the posterior/distalmost regions that retained *pax6* expression (compare Fig. 5F with 5C). Widespread ectopic Pax2 expression was not observed elsewhere in the embryo although we did not analyse other regions of Pax2 distribution in detail (Fig. 6A).

To confirm that the changes in Pax protein distribution reflected changes in RNA distribution, we examined a further

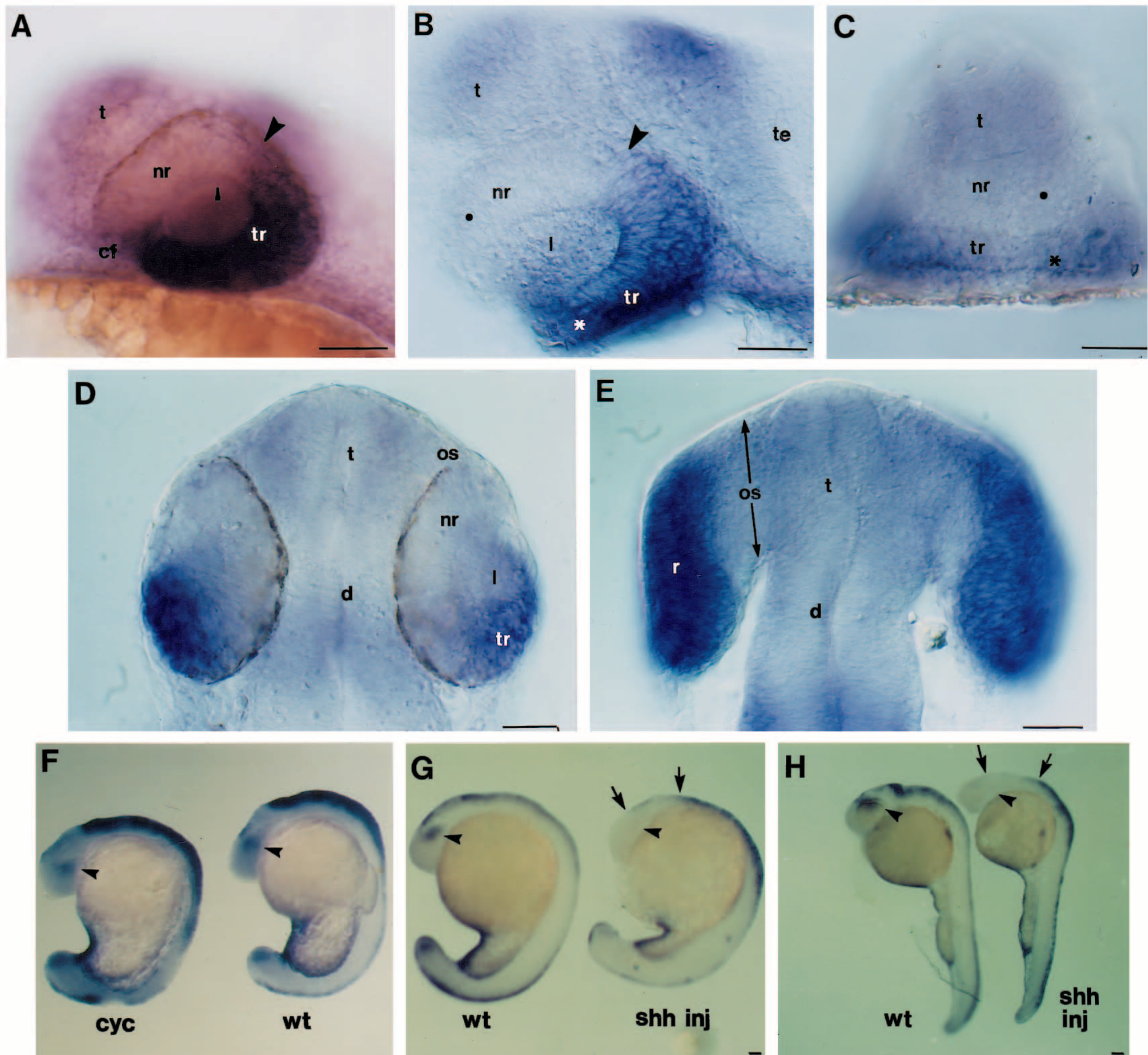

Fig. 3. *rtk2* and *msxC* expression in wild-type, *cyclops* and *shh*-injected embryos. Whole-mounted embryos with rostral to the left (A,B,F-H) or up (D,E). (A,B) Lateral views of *rtk2* expression in 24 hour wild-type (A) and *cyclops* mutant (B) embryos. The arrowheads indicate the boundary between nasal and temporal retina at the posterior groove. The asterisk and filled circle indicate equivalent positions of the eye in B and C. (C) Frontal view of *rtk2* expression in a 20s *cyclops* mutant embryo. *rtk2* expression in temporal retina is continuous around the rostral pole of the brain. (D,E) Dorsal views of *rtk2* expression in 24 hour wild-type and *shh*-injected embryos respectively. *rtk2* expression is widespread thoughout the retinal tissue in the *shh*-injected embryo. (F) Lateral views of *msxC* expression in the eye (arrowheads) of wild-type (right) and *cyclops* mutant (left) 18s embryos. (G,H) Lateral views of *msxC* expression in the eyes of *shh* injected (right) and wild-type (left) 18-20s (G) and 24 hour (H) embryos. In addition to an absence of *msxC* expression in the eyes (arrowheads), *msxC* is reduced throughout the head (arrows). Abbreviations: cf, choroid fissure; d, diencephalon; l, lens; nr, nasal retina; os, optic stalk; t, telencephalon; te, tectum; tr, temporal retina. Scale bars, 50 µm.

100 embryos using in situ hybridisation to determine the distribution of *pax6* and *pax2* transcripts. Complementing the changes in protein distribution in the optic primordia, the number of *pax6*-expressing cells was reduced whereas the number of *pax2*-expressing cells was increased (Fig. 5G-J).

Ectopic expression of Pax2 correlates with an increased amount of optic stalk-like tissue and reduced pigment epithelium and neural retina

The region of attachment of the optic primordia to the diencephalon was greatly increased in *shh*-injected embryos as compared to wild-type embryos (compare Fig. 5F with 5E). During later stages of development this region of the optic primordia of injected embryos continued to express Pax2 and remained as a grossly oversized optic stalk-like structure (Fig. 6E). The optic stalks of wild-type embryos are only a few cell diameters thick by 24 hours (Fig. 6B,D) whereas the stalks linking the reduced retinae of injected embryos to the forebrain are much broader (compare Fig. 6C with 6B, and 6E with 6D). Optic cup and lens formation were restricted to areas of the

optic primordia distal and posterior to the hypertrophied optic stalk-like structures (Fig. 6C).

To determine if the reduced domain of Pax6-expressing cells differentiated as retina in *shh*-injected embryos, we examined eyes at stages when pigment epithelium and neural retina have normally differentiated. We found that differentiation of both layers of the retina did occur (Fig. 6F-H). However pigment epithelium was more severely reduced than neural retina (Fig. 6F-H). This is perhaps not surprising as prospective pigment epithelial cells are likely to arise from more medial parts of the optic primordium (which showed the most severe changes in Pax protein distribution) than prospective neural retinal cells (Fig. 7A). The proximal extent of pigment epithelial differentiation coincided with the distal limit of Pax2 expression (Fig. 6F). The reduced neural retina showed normal lamination, however, the retinal ganglion cell layer appeared reduced as compared to the photoreceptor and inner nuclear layers (Fig. 6G,H).

Based upon their location in the distal/posterior region of the optic primordia, we assumed that the retinal cells that remained in *shh*-injected embryos may correspond to cells in the dorsal and temporal region of the normal eye. To test this possibility we assessed the expression of *rtk2* and *msxC* in the eye primordia of *shh*-injected embryos. *rtk2* was widely expressed in the eye primordia of all embryos examined suggesting that retinal cells with temporal identity were present (*n*=48, Fig. 3E). In contrast, *msxC*, which is normally expressed in a narrow region of the dorsal retina, was absent from the optic primordia in 23 out of 24 *shh*-injected embryos (Fig. 3G,H). However, this result may not imply that the cells that normally express *msxC* do not form retina in injected embryos as there was considerable downregulation of *msxC* thoughout the embryo, particulary in the head (Fig. 3G,H).

The distributions of Pax proteins in wild-type, *cyclops* and *shh*-injected embryos are summarised in Fig. 7.

DISCUSSION

Spatial regulation of Pax2 and Pax6 distributions by Shh

In this study we show that the partitioning of the optic primordium into optic stalk and retina is dependent upon midline signalling. Two genes potentially involved in subdividing the optic primordia are *pax6,* which is expressed in the presumptive pigment epithelia and neural retinae, and *pax2,* which is expressed primarily in the presumptive optic stalks. We show that expression of these two closely related genes is affected in a complimentary manner by the midline signalling molecule Shh. Overexpression of *shh* leads to ectopic expression of *pax2* and complimentary loss of *pax6* expression. In contrast, *cyclops* mutant embryos which lack early CNS expression of *shh* (Krauss et al. 1993) exhibit reciprocal changes in gene expression such that the domain of *pax2* expression is reduced whereas *pax6* expression is expanded.

If Shh is directly responsible for regulating Pax2 distribution then it may act over considerable distance as the distalmost Pax2-containing cells within the optic primordia are many cell diameters from the site of *shh* expression at the base of the optic stalks (Fig. 7A and see Barth and Wilson 1995). However, it is possible that presumptive optic stalk cells may be closer to the site of *shh* expression at the time at which they initiate *pax2* expression and that subsequent migration takes them to more distal regions of the optic primordia. There is evidence that members of the Hedgehog family in vertebrates and invertebrates can have both short range and long range signalling abilities. For instance, the amino-terminal peptide of *Drosphila* Hh acts as a short range signal that regulates the transcription of *wingless* (*wg*) and *decapentaplegic* (*dpp*) (Basler and Struhl, 1994; Ingham and Fietz, 1995; Fietz et al., 1995; Porter et al., 1995) and in vertebrates, Shh has been implicated in the

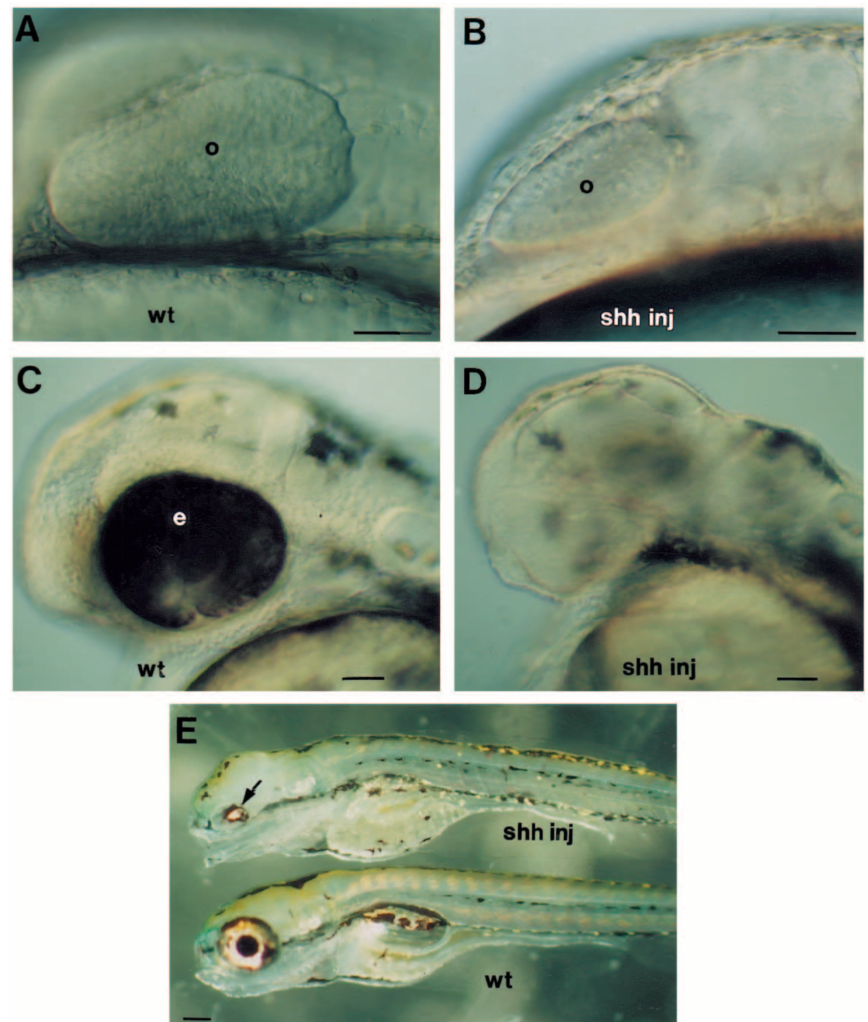

Fig. 4. Development of the eye in living wild-type embryos and embryos injected with *shh* RNA. Lateral views with rostral to the left. (A,B) 10-12s, wild-type and injected embryos respectively. (C,D) 48 hour, wild-type and injected embryos respectively. The injected embryo has virtually no pigment epithelium and very little neural retina. (E) Comparison of injected (upper) and wild-type (lower) embryos at 3-4 days of development. The arrow indicates the much reduced retina in the injected embryo. Abbreviations: e, eye; o, optic primodium. Scale bars, (A-D) 50 μm; (E) 200 μm.

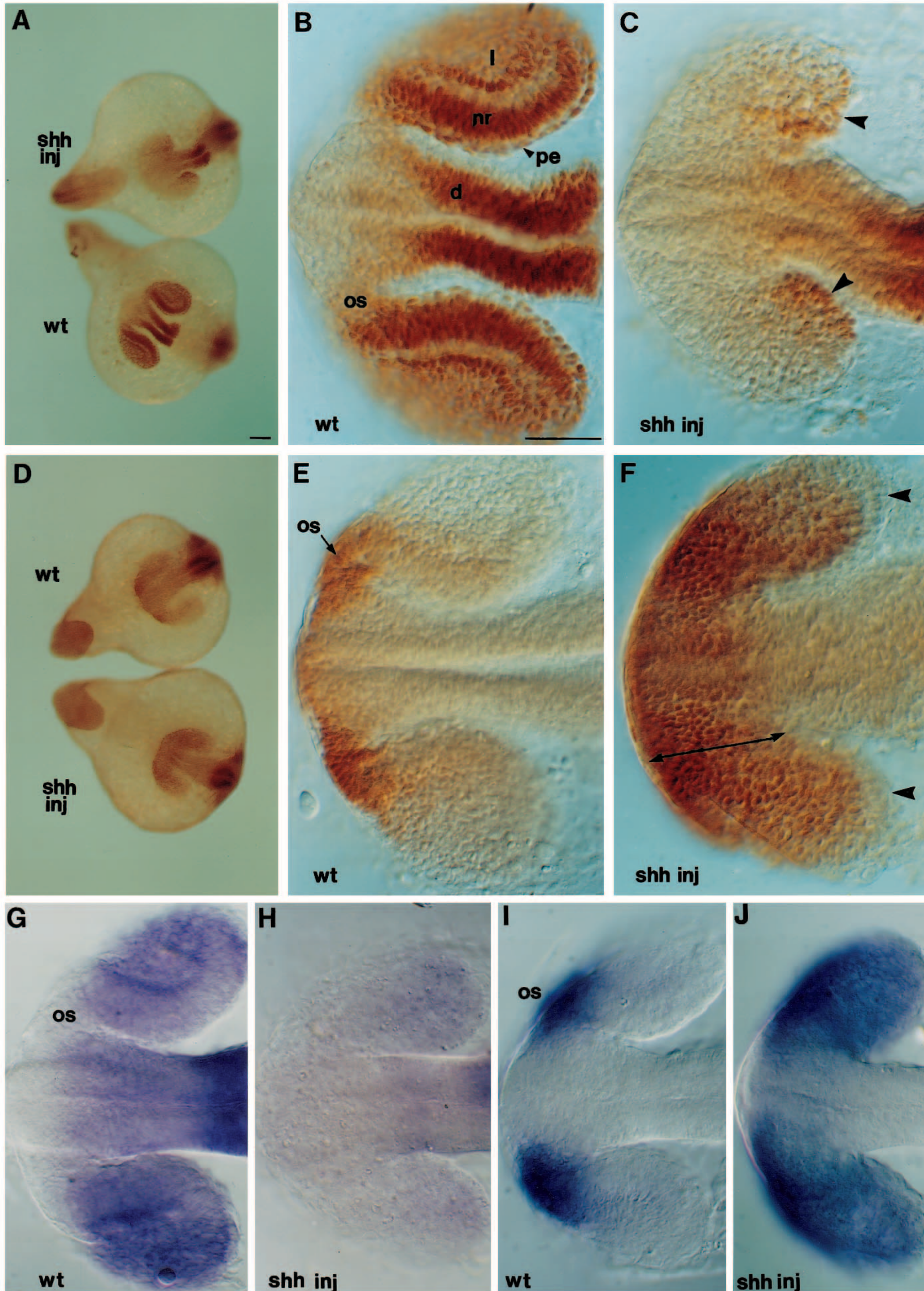

Fig. 5. *shh* has opposite effects upon Pax2 and Pax6 expression in the optic primordia. Whole-mounted 16-18s embryos labelled with antibodies to Pax6 (A-C) or Pax2 (D-F), or hybridised with antisense RNA probes to *pax6* (G,H) or *pax2* (I,J). Rostral CNS is to the left. (A) Dorsal views of Pax6 distribution in *shh*-injected (upper) and wild-type (lower) embryos. (B,C) Higher magnification views showing Pax6 distribution in the forebrain and eyes of wild-type (B) and injected (C) embryos. Some anti-Pax6 antibody labelling persists in the caudal portions of the optic primordia of the injected embryo (arrowheads in C). (D) Dorsal view of Pax2 distribution in intact wild-type (upper) and injected (lower) embryos. (E,F) Higher magnification views showing Pax2 distribution in the forebrain and optic primordia of wild-type (E) and injected (F) embryos. The most caudal cells of the optic primordia of the injected embryo are not labelled with the anti-Pax2 antibody (arrowheads in F). The area of attachment of the optic primordia to the diencephalon is greatly increased in the *shh*-injected embryo (double headed arrow in F). (G,H) Dorsal view of *pax6* expression in the eye primordia of wild-type (G) and *shh*-injected (H) embryos. (I,J) *pax2* expression in the optic primordia of wild-type (I) and *shh*-injected (J) embryos. Abbreviations: d, diencephalon; l, lens; nr, neural retina; os, optic stalk; pe, pigment epithelium. Scale bars, 50 µm.

contact-dependent induction of floorplate cells by the notochord (Roelink et al., 1994, 1995; Marti et al., 1995). Several lines of evidence have recently suggested that the amino-terminal cleavage product of Shh may be diffusible and may therefore also have longer range signalling ability. For instance, Shh can induce sclerotomal markers in adjacent presomitic mesoderm over distances up to about 150 µm and can still induce these markers when separated from the presomitic tissue by a nucleopore filter (Fan and Tessier-Lavigne, 1994; Fan et al., 1995). Furthermore, Shh can induce motor neuron differentiation in a dose-dependent manner that is independent of cell contact (Marti et al., 1995; Roelink et al., 1995; Tanabe et al., 1995). In addition, it has recently been shown that cells expressing Shh can also induce neuronal differentiation within forebrain tissue in the absence of cell contact, suggesting that Shh may be able to diffuse through the forebrain neuroepithelium (Ericson et al., 1995) and hence may be able to regulate *pax* gene expression at a distance from the site of *shh* expression.

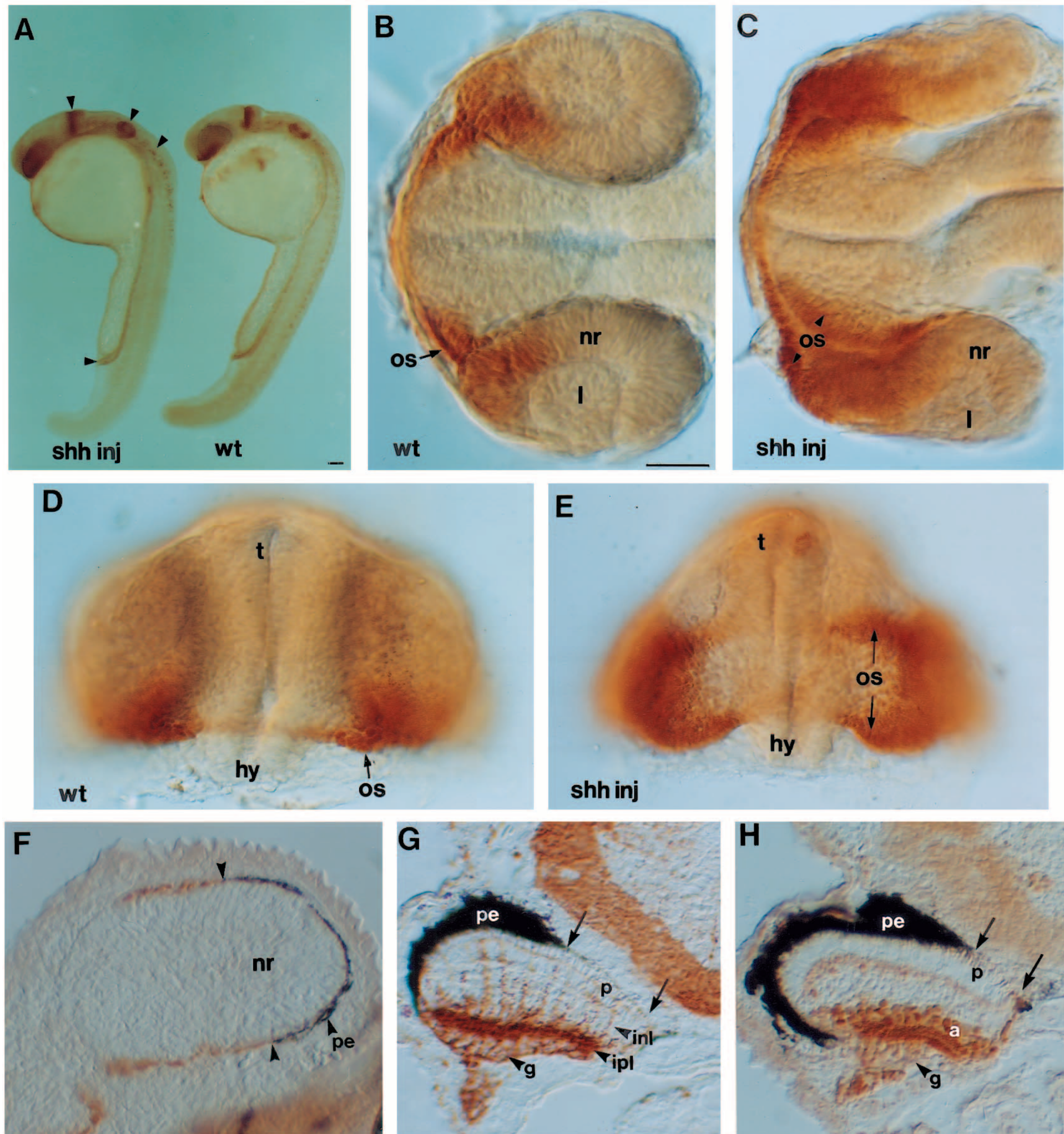

Fig. 6. Altered Pax protein distribution correlates with abnormal segregation of the optic primordia into optic stalks and retinae in embryos injected with *shh* RNA. Rostral is to the left in A-C and F. All embryos in this figure showed only moderate reduction in the extent of the retinal tissue. Embryos are labelled with anti-Pax2 (A-F), HNK1 (G) or anti-Pax6 (H) antibodies. (A) Lateral views of 24 hour injected (left) and wild-type (right) embryos. The arrowheads indicate sites of Pax2 expression at the midbrain/hindbrain boundary, in the otocysts, in hindbrain neurons and in the nephric duct. (B,C) Ventral views of wild-type (B) and injected (C) embryos. (D,E) Frontal views of wild-type (D) and injected (E) embryos. The arrows in E indicate the expansion of Pax2 expression in the optic stalk region of the injected embryo. (F) Horizontal section through the retina of an *shh*-injected embryo at 2 days of development. The distal extent of Pax2 distribution (arrowheads) is coincident with the proximal limit of pigment epithelium formation. Most of the cells in the deeper layers of the retina still contained Pax6 at this stage as judged by anti-Pax6 labelling of adjacent serial sections. (G,H) Frontal sections through the retina of an *shh*-injected 5-day old fish labelled with HNK1 antibody (G; brown labelling of axons) or anti-Pax6 antibody (H). At this stage Pax6 protein is restricted mainly to amacrine cells (Macdonald and Wilson, unpublished observations). The antibody labelling reveals relatively normal lamination of the neural retina. The arrows indicate medial regions of the neural retina over which there is no pigment epithelium. Abbreviations: a, amacrine cell layer; g, ganglion cell layer; hy, hypothalamus; inl, inner nuclear layer; ipl, inner plexiform layer; l, lens; nr, neural retina; os, optic stalk; p, photoreceptor layer; pe, pigment epithelium; t, telencephalon. Scale bars, 50 μm.

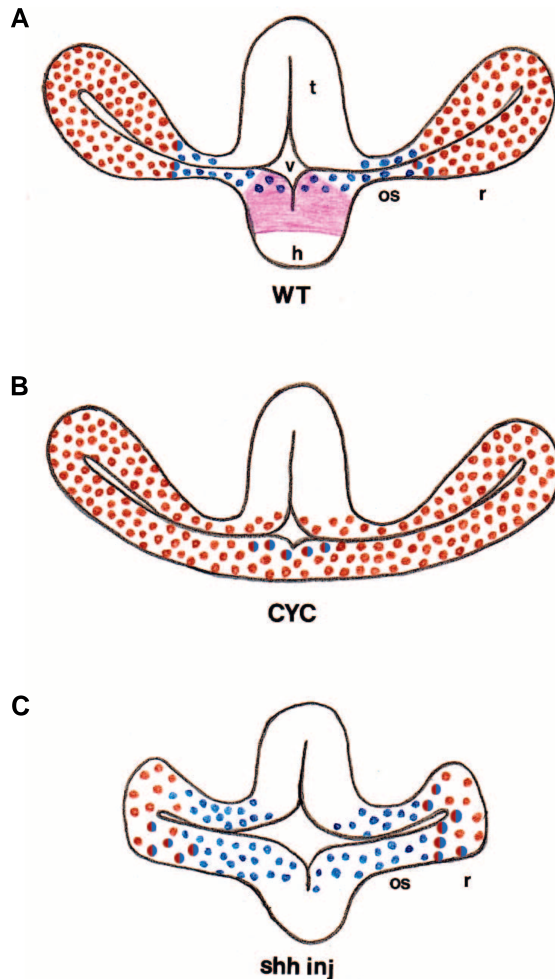

Fig. 7. Schematic representations of Pax6 (red) and Pax2 (blue) protein distributions in wild-type, *cyclops* and *shh*-injected embryos. The drawings represent frontal views of the CNS similar to those shown in Fig. 6D,E. For clarity, the eye primordia are shown simply extending laterally from the forebrain. (A) In the wild-type embryo, Pax6 is present in the pigment epithelial and neural layers of the retina and Pax2 is present in the optic stalks and a small region of the ventral anterior retina. The Pax6-expressing region of the optic primordium later invaginates with more distal cells forming neural retina and more proximal cells forming the pigment epithelium which covers the scleral surface of the neural retina. The distribution of *shh* transcripts (purple) is based upon Krauss et al. (1993) and Barth and Wilson (1995). (B) In *cyclops* mutant embryos, Pax6 protein is present in cells across the rostral midline of the forebrain, Pax2 is almost absent and most if not all cells differentiate as retina. (C) In *shh*-injected embryos, Pax6 protein is absent from most cells in the optic primordia whereas Pax2 expression is expanded. Optic stalk-like tissue is hypertrophied and the retina is greatly reduced. Abbreviations: h, hypothalamus; r, retina; os, optic stalk; t, telencephalon; v, ventricle.

An additional mechanism by which Hh proteins may elicit long range responses is through the local induction of other signalling molecules that subsequently either diffuse over greater distances or establish a cascade of cell to cell signalling events (Johnson and Tabin, 1995). However, although homologues of the signalling molecules wg and dpp, targets of Hh regulation in *Drosophila* (Ingham and Hidalgo, 1993; Basler and Struhle, 1994; Ingham and Fietz, 1995) are expressed in the developing vertebrate embryo, we are currently unaware of any that are expressed in regions of the forebrain appropriate to play a role in the spatial regulation of *pax* gene expression in the eyes.

It is interesting to note that some of the changes in eye development that occur following *shh* injection resemble the changes that occur in embryos that have been exposed to altered levels of retinoic acid during the early period of eye development. For instance, inhibition of retinoic acid synthesis results in embryos which lack ventral/anterior retina (Marsh-Armstrong et al., 1994) while treatment of embryos with retinoic acid results in thickened optic stalks (Marsh-Armstrong et al., 1994; Dowling, Hyatt and Schmitt, personal communication). However, exogenous retinoic acid also causes retinal duplication, a feature we have not observed in our experiments. Despite this difference, the fact that retinoic acid can induce *Shh* expression within the limb bud (Riddle et al., 1993) raises the possibility that at least some of the effects of retinoic acid upon the developing eye may be elicited through alterations in the levels of Shh.

Pax proteins and eye development

The domain of Pax6 protein distribution in the forebrain of *cyclops* mutant embryos is expanded to include cells of the rostral neural keel which normally form structures such as the optic stalks and rostral hypothalamus. One consequence of ectopic Pax6 expression appears to be that these rostral cells are respecified to become retina. An alternative possibility is that presumptive optic stalk cells are not present in the neural keel of *cyclops* mutant embryos and that Pax6 containing cells migrate from the existing retinal fields to the rostral midline thereby fusing the two eye primordia. We favour the first of these possibilities as the complementary changes in *pax* gene expression observed in *cyclops* and *shh* injected embryos favour a model by which midline signals regulate the expression of *pax* genes rather than the survival of retinal or optic stalk precursor cells. Supporting this possibility are experiments in the chick which have suggested that signals emanating from the notochord can inhibit *pax6* expression in the ventral spinal cord (Goulding et al., 1993) – in the absence of these signals, *pax6* expression spreads to more ventral cells which normally do not express the gene.

Our results suggest that the presumptive retinae of *cyclops* mutant embryos are expanded whereas the retinae of *shh*-injected embryos are reduced. These changes in the extent of retinal tissue are reflected by alterations in the distribution of Pax6 in the optic primordia, suggesting that Pax6 is a key regulator of retinal development. This conclusion is supported by analysis of the phenotype of *Small eye* mice which carry a mutation in the *Pax6* gene and exhibit severe retinal defects in both heterozygous and homozygous embryos (Hill et al., 1991; Hogan et al., 1988; Schmahl et al., 1993). It is interesting that although retinae were always reduced in *shh* injected embryos, outgrowth of the optic primordia did occur even though Pax6 was reduced or absent from most presumptive retinal cells. This suggests that Pax6 may not be required for the initial formation of the optic vesicle, and indeed the optic vesicle does initially evaginate in *Pax6* mutant mice (Hogan et al., 1988, Schmahl et al., 1993).

The relatively normal spatial distribution of *rtk2* and *msxC* transcripts and appropriate lamination of neurons in the retinae of *cyclops* mutant embryos indicates that while the signalling pathway(s) affected by the *cyclops* mutation may be required to

partition the optic primordia, they appear to be less critical for the differentiation of the retina. The consequences of *shh* overexpression upon the regional patterning of the remaining retinal tissue in *shh* injected embryos is less clear. Based upon the morphogenesis of the eye of *shh*-injected embryos, we believe that dorsal and temporal regions of the neural retina are least affected by Shh (this study and see Barth and Wilson, 1995). Supporting this interpretation, we found that the retinal tissue of *shh*-injected embryos always expressed the temporal retinal marker, *rtk2*. However, *msxC*, a gene normally expressed in the dorsal retina adjacent to the posterior groove, was absent from the retinae of *shh*-injected embryos. This could mean that cells that normally express *msxC* do not form retinae in *shh*-injected embryos. However, because we observed widespread overall reduction in *msxC* expression in dorsal regions of the embryo, we favour an alternative interpretation: that ectopic Shh may directly or indirectly downregulate the expression of *msxC*. Supporting the notion that ectopic Shh can elicit widespread inhibition of genes normally expressed in dorsal tissue, it has recently been shown that Shh can inhibit the expression of *Pax3*, *Sim1* and *Pax7* within dorsal regions of the developing somites (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994).

The expression of Pax2 in the developing optic stalks raises the possibility that this protein may play a role in optic stalk differentiation analogous to the role of Pax6 in retinal development. Indeed, the expanded domain of Pax2 distribution following *shh* injection correlates with enlarged optic stalk-like structures. However, it is difficult to assess just how closely the hypertrophied optic stalk-like structures of *shh*-injected embryos resemble the normal optic stalks of wild-type embryos, as the normal development of optic stalk cells has not been well studied. Although it is known that the optic stalks of wild-type embryos decrease in diameter over time (Schmidt and Dowling, 1994), their ultimate fate has not been conclusively determined. We believe that it is likely that the medial regions of the optic primordia of *shh*-injected embryos do not form simply enlarged but otherwise normal stalk tissue. For instance, we know of at least one other gene which is abnormally expressed in this tissue: the homeobox gene *nk2.2* is normally restricted to the most proximal cells of the optic stalks but following *shh* injection it is widely expressed in the hypertrophied optic stalk-like tissue (Barth and Wilson, 1995).

Given the complementary distributions of Pax6 and Pax2 in wild-type, *cyclops* and *shh*-injected embryos it is tempting to speculate that there may be some regulatory interactions between these two closely related proteins. Indeed, in vitro binding assays of Pax6 and Pax2 have shown that the proteins can bind to similar DNA sequences (Epstein et al., 1994) raising the possibility that they may compete for the same target sequences in vivo. Perhaps the simplest model for an interaction between Pax2 and Pax6 would be one in which Pax2 represses the expression of Pax6 thus allowing cells towards the midline of the optic primordia to differentiate as optic stalk tissue and not as pigment epithelium or neural retina.

The effects of ectopic expression of Shh have been assessed at various different sites in the developing vertebrate embryo (for example, Roelink et al., 1994; Johnson et al., 1994). In each group of cells that are affected, the alterations in gene expression resulting from ectopic expression of *shh* appear to be quite specific. For instance, Pax2 is ectopically expressed within cells of the optic primordia but not in cells of the adjacent diencephalic tissue, and *nk2.2* is ectopically expressed in both diencephalic cells and the optic primordia (Barth and Wilson, 1995), whereas the *HNF-3β* homolog, *axial,* is never expressed in the optic primordia but is ectopically expressed in other regions (Krauss et al., 1993; Barth and Wilson, 1995). These observations suggest that Shh can evoke a response in cells that lie outside its normal domain of function and that the consequences of Shh signalling are critically dependent upon the position of a cell within the developing embryo; thus the same signal can have very different consequences dependent upon the context of the responding cell.

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Note added in proof

A recent independent study by Ekker et al., presents similar data and conclusions to the work in this paper (Ekker, S. C., Ungar, A. R., Greenstein, P., von Kessler, D. P., Porter, J. A., Moon, R. T. and Beachy, P. A. (1995). Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. *Current Biology* (in press).