

Different roles for *Pax6* in the optic vesicle and facial epithelium mediate early morphogenesis of the murine eye

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SUMMARY

Chimaeric mice were made by aggregating *Pax6*^{-/-} and wild-type mouse embryos, in order to study the interaction between the optic vesicle and the prospective lens epithelium during early stages of eye development. Histological analysis of the distribution of homozygous mutant cells in the chimaeras showed that the cell-autonomous removal of *Pax6*^{-/-} cells from the lens, shown previously at E12.5, is nearly complete by E9.5. Most mutant cells are eliminated from an area of facial epithelium wider than, but including, the developing lens placode. This result suggests a role for *Pax6* in maintaining a region of the facial epithelium that has the tissue competence to undergo lens differentiation.

Segregation of wild-type and *Pax6*^{-/-} cells occurs in the optic vesicle at E9.5 and is most likely a result of different adhesive properties of wild-type and mutant cells. Also, proximo-distal specification of the optic vesicle (as assayed

by the elimination of *Pax6*^{-/-} cells distally), is disrupted in the presence of a high proportion of mutant cells. This suggests that *Pax6* operates during the establishment of patterning along the proximo-distal axis of the vesicle.

Examination of chimaeras with a high proportion of mutant cells showed that *Pax6* is required in the optic vesicle for maintenance of contact with the overlying lens epithelium. This may explain why *Pax6*^{-/-} optic vesicles are inefficient at inducing a lens placode. Contact is preferentially maintained when the lens epithelium is also wild-type.

Together, these results demonstrate requirements for functional *Pax6* in both the optic vesicle and surface epithelia in order to mediate the interactions between the two tissues during the earliest stages of eye development.

Key words: *Pax6*, Mouse chimaera, Chimera, Eye morphogenesis

INTRODUCTION

Correctly regulated expression of wild-type *Pax6* is necessary for normal development of the mammalian CNS and sensory organs (Hill et al., 1991; Ton et al., 1991; Matsuo et al., 1993; Mastick et al., 1997; Warren and Price, 1997; Carić et al., 1997; Grindley et al., 1997). The developing eye is particularly sensitive to *Pax6* gene dosage (Schedl et al., 1996): rat and mouse small eye phenotypes, and human Aniridia, result from heterozygous null mutations in *Pax6* (Hill et al., 1991; Ton et al., 1991; Fugiwara et al., 1994), and homozygous mutants fail to develop eyes (Roberts, 1967; Hogan et al., 1986).

Pax6 is expressed in several eye tissues throughout development (Walther and Gruss, 1991; Grindley et al., 1995; Hitchcock et al., 1996) and is probably required at several stages. *Pax6* is expressed in both the optic vesicle and the facial epithelium prior to and during the interaction between the two tissues that leads to lens placode formation and invagination (Grindley et al., 1995; Furuta and Hogan, 1998). This interaction represents the final determinative event of the

complex multistep process of lens induction (Grainger et al., 1988; Grainger, 1992). After lens placode invagination, *Pax6* continues to be expressed in the lens and optic cup.

In *Pax6*^{-/-} mice, the optic vesicle is misshapen, but meets the prospective lens epithelium as normal. Subsequently, the interaction between the optic vesicle and the epithelium does not occur – no lens placode is formed, the optic vesicle loses contact with the head surface and fails to develop into an optic cup (Grindley et al., 1995). Thus *Pax6* is required during the interaction, but studies of homozygous mutants do not distinguish whether it is required in the optic vesicle, or lens epithelium, or both.

One way to investigate which tissues have a requirement for *Pax6*, or other genes, during development is to make chimaeric mice by incorporating mutant cells into an otherwise normal embryo, and analysing the developmental potential of the mutant cells (reviewed in Rossant and Spence, 1998; West, 1999). In a previous study by Quinn et al. (1996), analysis of mouse embryos which were chimaeras of *Pax6*^{-/-} and wild-type cells at E12.5 demonstrated an autonomous requirement

for Pax6 in cells of the lens and the retinal pigmented epithelium (RPE). The chimaeras fell into two groups: 'low-percentage' chimaeras (<50% Pax6^{-/-} cells) that formed a relatively normal eye, within which Pax6^{-/-} cells were eliminated from the lens and much reduced in the neural retina and RPE, and 'high-percentage' chimaeras, in which no lens formed and the disorganised optic cup contained segregated regions of Pax6^{-/-} and Pax6^{+/+} cells.

For this study a series of chimaeras was analysed at E9.5 in order to examine the roles of Pax6 at very early stages of eye development (during the interaction between the optic vesicle and the prospective lens epithelium), to define further the stages at which Pax6 is required during lens formation and to investigate the significance of cellular adhesiveness for eye morphogenesis.

The results define previously undescribed roles for Pax6 in both the prospective lens epithelium and optic vesicle at very early stages of eye development, prior to full morphological development of the lens placode. The cell-autonomous roles for Pax6 in lens and retinal differentiation that were shown at E12.5 can be traced to these early stages of eye development. The study has also revealed unexpected roles for Pax6 in the optic vesicle: it acts during proximo-distal specification, and is the primary determinant of maintenance of contact with the lens epithelium.

MATERIALS AND METHODS

Mice

Mouse stocks, their maintenance and the production of chimaeric embryos by aggregation, have been described previously (Quinn et al., 1996). Two null mutations of Pax6 were used, Pax6^{Sey} and Pax6^{Sey-Neu} (Hill et al., 1991). Briefly, embryos were obtained from a cross between pigmented (C/C) heterozygote Pax6^{Sey/+} males, homozygous for a reiterated silent β-globin transgene marker *TgN(Hbb-b1)83Clo* (here referred to as *Tg*) and the glucose phosphate isomerase *Gpi1^b* allele (Pax6^{Sey/+}; C/C; *Tg/Tg*; *Gpi1^{b/b}*), and superovulated Pax6^{Sey-Neu/+} females that did not carry the transgene (Pax6^{Sey-Neu/+}; C/C; *Gpi1^{b/b}*). 8-cell embryos were flushed from the uterus at E2.5, and aggregated with 8-cell embryos from an albino, *Gpi1^{a/a}* Pax6^{+/+} wild-type cross (BALB/c × A/J)F2 (Pax6^{+/+}; *c/c*; *Gpi1^{a/a}*), also not carrying the transgene. After overnight culture, chimaeric embryos were transferred into the uteri of pseudopregnant 'CF1' (West and Flockhart, 1994) female mice on day 2.5 of pseudopregnancy, this being taken as E2.5.

Analysis of chimaeras

Embryos were dissected into cold phosphate-buffered saline (PBS) at E9.5. Any phenotype was described carefully and embryos were staged according to the criteria of forelimb development described by Wanek et al. (1989). The head was removed and fixed in 3:1 ethanol:acetic acid. The tail was removed and washed again in PBS before transfer into 20 μl Proteinase K buffer for PCR genotyping (see below). The rest of the body was washed in PBS then transferred to 20 μl water and freeze/thawed three times. 4 μl of the cell suspension was taken for colorimetric quantitative GPII analysis (West and Flockhart, 1994), giving a global value of the percentage of cells derived from each aggregated 8-cell embryo in the chimaera. Extreme care was taken to avoid cross contamination between samples of different embryos.

Heads were fixed for 6 hours, then washed in 70% ethanol and processed to wax. Histological analysis of the distribution of cells from the Pax6^{Sey-Neu/+} × Pax6^{Sey/+} cross was facilitated by performing DNA in situ hybridisation on 7 μm sections using digoxigenin-labelled β-

globin probe to detect the reiterated transgene carried homozygously by the Pax6^{Sey/+} male mice (described in Keighren and West, 1993). Hybridised digoxigenin-labelled DNA probe was detected by diaminobenzidine staining for peroxidase-conjugated antibody. Slides were counterstained with haematoxylin and examined by bright-field microscopy. Cells (whatever their Pax6 genotype) derived from the Pax6^{Sey-Neu/+} × Pax6^{Sey/+} aggregated embryo were thus visualised by the presence of a brown spot in the nucleus.

After in situ hybridisation, the percentage of transgene-positive cells in various regions of the eye and head was counted. Primary estimates of the percentage of hybridisation-positive cells were corrected on a tissue-by-tissue basis to allow for failure to detect the signal in some *Tg^{+/-}* cells, due to sectioning through the nucleus. The percentage of apparent hybridisation-positive nuclei in each tissue of several E9.5 (Pax6^{Sey-Neu/+}; *Tg^{-/-}*) × (Pax6^{Sey/+}; *Tg^{+/+}*) embryos (non-chimaeric, therefore 100% *Tg^{+/-}*) was calculated, and these percentages used as correction factors when counting the hybridisation-positive cells in the same tissues in chimaeras. Comparative data presented here in Fig. 3 and Table 1 represent percentages corrected in this way.

Tg^{+/-} cells in proximal and distal regions of the optic vesicle were counted separately for each chimaeric eye using a graticule grid on the light microscope. The number of *Tg^{+/-}* cells per graticule square (25×25 μm at 400× magnification) was counted for as many squares, on as many tissue sections, as possible. Fig. 2B shows the areas defined as 'proximal' and 'distal' for this analysis. The average number of cells per graticule square, both proximally (P) and distally (D), were calculated for each eye.

Genotyping

PCR genotyping of chimaeras was performed retrospectively. The tail of each E9.5 embryo was digested with Proteinase K in a total volume of 20 μl, for 1 hour at 55°C, as described by Grindley et al. (1995), then denatured for 5 minutes at 95°C. 2 μl was used per PCR reaction.

The Pax6^{Sey} allele was detected using a mutagenically separated PCR technique (MS-PCR) (Rust et al., 1993). Two forward primers were designed (SP1, SP2) with specific sequence and mismatches, which caused them either to preferentially recognise the Pax6^{Sey} point mutation (G to T) allele (SP1) or the wild-type allele (SP2). Their sequences were as given, where letters in lower case represent mismatches and * denotes the site of the Pax6^{Sey} point mutation. SP1, 5'-GAGAACACCAACTCCATCAGTTCTAAgT*-3'; SP2, 5'-AGCAACAGGAAGGAGGGGGAacGAACACCAACTCCATCAGTTCTtACG*-3'. The reverse primer was MC130 (Quinn et al., 1996): 5'-CTTTCTCCAGAGCCTCAATCTG-3'.

All three primers were used in the reaction. PCR conditions were 1 μM SP1, 0.08 μM SP2, 0.1 μM MC130, 20 μM NTPs, 1 mM MgCl₂. 1 unit of Taq polymerase was used per reaction. Cycling conditions were: first cycle: 95°C, 2 minutes; 55°C, 1 minute; 72°C, 1 minute. 43 cycles: 95°C, 40 seconds; 55°C, 1 minute; 72°C, 1 minute. Final cycle: 95°C, 40 seconds; 55°C, 1 minute; 72°C, 5 minutes.

The Pax6^{Sey} allele produces a 129 bp SP1/MC130 band, and the wild-type allele a 148 bp SP2/MC130 band, resolved on a 4% NuSieve agarose gel. Pax6^{Sey} alleles were reliably detected in chimaeras with as few as 3% mutant cells using the conditions described.

Detection of the Pax6^{Sey-Neu} allele was performed using primers Hax5 and G15 as described in Quinn et al. (1996). Digestion with *HindII* did not restrict the wild-type or Pax6^{Sey} 220 bp PCR fragment, but produced 140 bp and 80 bp bands from Pax6^{Sey-Neu}. These were resolved on a 4% NuSieve agarose gel.

RESULTS

42 E9.5 chimaeras were produced. Genotypic analysis showed that ten chimaeras were of the (Pax6^{Sey/Sey-Neu} ↔ Pax6^{+/+})

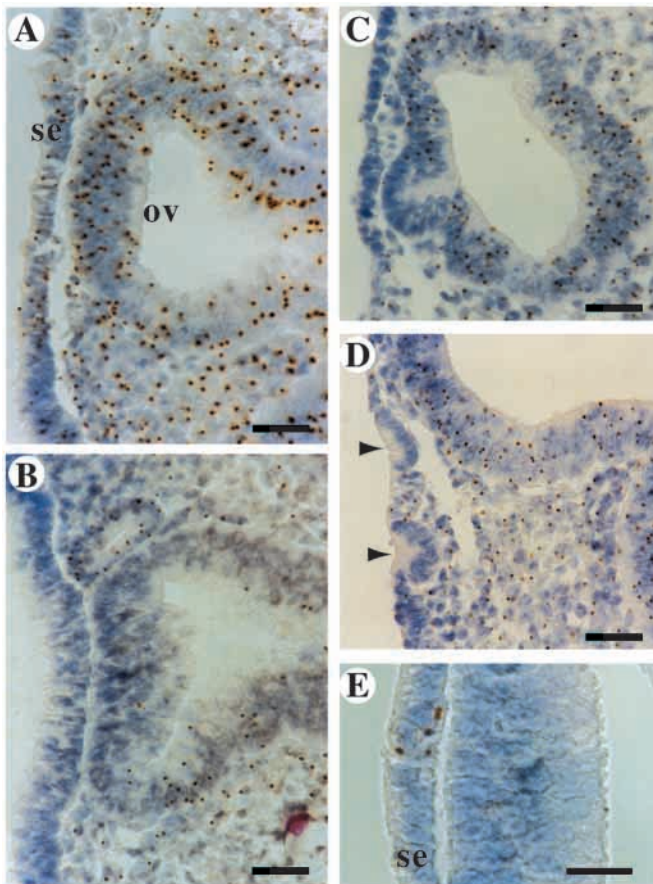


Fig. 1. (A) Control heterozygote ($Pax6^{+/+} \leftrightarrow Pax6^{+/Sey}$) chimaera, showing random distribution of Tg^+ cells throughout eye. (B) Similar stage eye of low-percentage ($Pax6^{+/+} \leftrightarrow Pax6^{Sey/Sey-Neu}$) chimaera (28% GPII-B). Homozygous mutant cells are not present in distal optic vesicle, nor surface epithelium. There is an adjacent ectopic mutant vesicle. (C) High-percentage ($Pax6^{+/+} \leftrightarrow Pax6^{Sey/Sey-Neu}$) chimaera, 74% GPII-B. A small patch of wild-type cells in the optic vesicle uniquely maintains contact with wild-type placodal epithelium. (D) Arrowheads indicate wild-type ectopic structures (apparently placodal) adjacent to a high-percentage mutant eye. (E) One section of a clump of $Pax6^{Sey/Sey-Neu}$ cells retained in the epithelium overlying the optic vesicle of a low-percentage mutant chimaera. The mutant cells are grouped together, and are slightly dysmorphic, in contrast to the regular alignment of the surrounding wild-type cells. se, surface epithelium; ov, optic vesicle. Bars, 50 μm (A-D), 15 μm (E).

combination (i.e. a mixture of $Pax6^{Sey/Sey-Neu}$ and wild-type cells), eight were ($Pax6^{Sey-Neu/+} \leftrightarrow Pax6^{+/+}$), eight were ($Pax6^{Sey/+} \leftrightarrow Pax6^{+/+}$) and 16 were ($Pax6^{+/+} \leftrightarrow Pax6^{+/+}$). As described in Materials and Methods, the ratio of GPII-A and GPII-B allozymes in the trunk of each embryo was determined by quantitative electrophoresis: %GPII-B was taken as an estimate of the percentage of cells derived from the ($Pax6^{Sey-Neu/+} \times Pax6^{Sey/+}$) 8-cell embryo in the E9.5 chimaera. The distribution of these cells was identified histologically by DNA in situ hybridisation on wax sections using a digoxigenin-labelled probe, which recognised the β -globin transgene Tg carried homozygously by the male $Pax6^{Sey/+}$ stud mice.

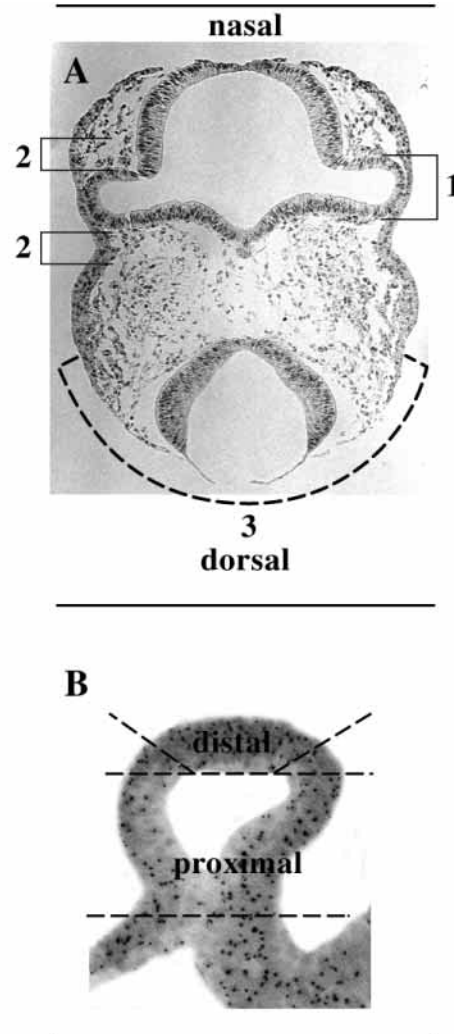


Fig. 2. (A) Diagrammatic description of regions of the head surface epithelium over which the distribution of $Tg^{+/-}$ cells was counted in transverse section. Region 1: prospective lens epithelium; percentage of $Tg^{+/-}$ cells = 'L'. Region 2: 'outside-lens' epithelium; percentage of $Tg^{+/-}$ cells = 'O'. Region 3: head epithelium; percentage of $Tg^{+/-}$ cells = 'H'. (B) Definition of 'proximal' and 'distal' optic vesicle. Note this example is the same optic vesicle as Fig. 1A. Areas underlying the surface epithelium were defined as 'distal'. A small amount of tissue in each vesicle was not identified as either distal or proximal.

In all cases where cells from the small eye cross were $Pax6^{+/+}$, $Pax6^{Sey-Neu/+}$ or $Pax6^{Sey/+}$ (control chimaeras), there was random mixing with cells from the wild-type embryo. No morphological abnormalities occurred and at this stage of development the $Pax6^{+/-}$ cells are functionally equivalent to $Pax6^{+/+}$ (see below) (Fig. 1A).

Mutant chimaeras ($Pax6^{Sey/Sey-Neu} \leftrightarrow Pax6^{+/+}$) could be broadly classified into two groups: (1) 'low-percentage chimaeras' which, on the basis of GPI measurements and $Tg^{+/-}$ cell-counting, contained fewer than approximately 45% mutant cells in the eyes (4 chimaeras) and (2) 'high-percentage chimaeras', which were $\geq 49\%$ mutant (6 chimaeras).

In the former category, there was a striking loss of $Pax6^{Sey/Sey-Neu}$ cells from the developing lens placode and a

Table 1. Analysis of distribution of Pax6^{+/+}, Pax6^{+/-} and Pax6^{Sey/Sey-Neu} cells in chimaeric epithelia

Pax6 genotype	%GPI1B in trunk	H (% Tg ⁺ cells in head epithelium)	L (% Tg ⁺ cells in lens epithelium)	O (% Tg ⁺ cells in outside-lens epithelium)	L/H	O/H
+/-	12	13.96 (155)	14.46 (95)	14.27 (222)	1.056	1.022
+/+	17	9.59 (663)	8.32 (350)	8.41 (392)	0.852	0.861
+/+	17	9.59 (663)	9.31 (303)	11.73 (443)	0.953	1.202
+/-	29	17.10 (166)	16.00 (284)	16.72 (235)	1.145	0.978
+/+	40	39.85 (270)	43.41 (203)	-	1.089	-
+/-	45	53.78 (727)	54.23 (390)	40.83 (658)	1.008	0.759
+/-	45	53.78 (727)	45.02 (402)	37.84 (422)	0.837	0.703
+/+	54	65.74 (483)	63.83 (239)	63.93 (113)	1.047	0.972
+/+	54	65.74 (483)	65.03 (244)	61.57 (212)	0.989	0.937
+/+	64	55.94 (653)	54.04 (635)	49.82 (435)	0.966	0.891
+/+	64	55.94 (653)	64.34 (431)	52.47 (314)	1.150	0.938
+/+	66	46.99 (587)	54.96 (381)	53.51 (360)	1.170	1.139
+/+	66	46.99 (587)	47.79 (325)	50.37 (244)	1.017	1.072
+/-	79	65.10 (582)	71.09 (324)	71.32 (446)	1.092	1.096
+/-	79	65.10 (582)	63.21 (156)	57.93 (350)	0.971	0.890
-/-	9	12.39 (416)	2.00 (294)	3.02 (168)	0.161	0.243
-/-	9	12.39 (416)	1.29 (274)	1.31 (194)	0.104	0.150
-/-	16	13.69 (985)	1.10 (307)	2.08 (426)	0.080	0.152
-/-	16	13.69 (985)	8.51 (373)	2.73 (241)	0.621	0.199
-/-	22	12.38 (1089)	0.21 (551)	0.98 (388)	0.017	0.079
-/-	22	12.38 (1089)	1.99 (532)	1.48 (683)	0.160	0.120
-/-	28	44.48 (524)	4.20 (280)	3.89 (548)	0.094	0.087
-/-	28	44.48 (524)	9.84 (244)	7.15 (372)	0.260	0.161
-/-	49	56.48 (373)	45.90 (246)	39.33 (435)	0.813	0.696
-/-	49	56.48 (373)	43.96 (286)	31.68 (556)	0.778	0.561
-/-	51	49.26 (917)	33.63 (297)	29.83 (329)	0.683	0.606
-/-	51	49.26 (917)	52.99 (251)	53.47 (640)	1.074	1.085
-/-	60	57.38 (486)	40.82 (213)	46.08 (341)	0.711	0.803
-/-	60	57.38 (486)	39.40 (498)	37.05 (537)	0.687	0.645
-/-	74	61.66 (676)	56.99 (499)	60.00 (734)	0.924	0.973
-/-	74	61.66 (676)	55.63 (226)	58.69 (447)	0.902	0.952
-/-	78	70.87 (695)	70.02 (433)	69.21 (520)	0.988	0.977
-/-	78	70.87 (695)	67.66 (462)	61.80 (607)	0.955	0.872

See text and Fig. 2 for further explanation.

Each row represents one chimaeric eye. For each value of H, L and O, the number of cells counted in the analysis is given in parentheses.

restricted area of epithelium surrounding it. This suggests a role for Pax6 in the region of head ectoderm which is competent to form lens tissue (Fig. 1B). Pax6^{Sey/Sey-Neu} cells were also virtually absent from the distal optic vesicle, which made good contact with the prospective lens (Fig. 1B). There was some cell segregation in the optic vesicle, including the formation of ectopic vesicles of mutant cells.

In six high-percentage chimaeras, there was cell segregation in both the region of surface epithelium and the distal optic cup (Fig. 1C), but no efficient elimination of Pax6^{Sey/Sey-Neu} cells. Optic vesicles were misshapen and wild-type cells preferentially retained contact with the overlying epithelium (Fig. 1C). In the most striking examples, several small ectopic placode-like structures formed, entirely Pax6^{+/+}; these did not always maintain contact with the optic vesicle (Fig. 1D). These phenomena are described below.

Role for Pax6 in the facial epithelium before placode formation

The area of facial epithelium which expresses Pax6 is wider than that which is actually contacted by the optic vesicle and which subsequently undergoes lens placodal differentiation. In the four low-percentage mutant chimaeras, Pax6^{Sey/Sey-Neu} cells were clearly underrepresented both in the epithelium of the putative lens placode and the surrounding regions that will not

contribute to the lens (Fig. 1B). This was the case even in those less developmentally advanced chimaeras which had yet to form a morphologically distinguishable placodal structure. To quantify this effect, the percentage of Tg^{+/+} cells was counted separately in three regions of the epithelium, as indicated in Fig. 2A. These were (1) lens placodal epithelium overlying the optic vesicle (the prospective lens), (2) epithelium outside the lens area to a distance of 125 µm and (3) non-specialised surface epithelium of the head, dorsal to region 2. This was performed for (Pax6^{+/+} ↔ Pax6^{+/+}), (Pax6^{Sey/+} ↔ Pax6^{+/+}), (Pax6^{Sey-Neu/+} ↔ Pax6^{+/+}) and (Pax6^{Sey/Sey-Neu} ↔ Pax6^{+/+}) chimaeras. Each eye was analysed separately.

Table 1 presents the data from this analysis. For (Pax6^{+/+} ↔ Pax6^{+/+}), (Pax6^{Sey/+} ↔ Pax6^{+/+}) and (Pax6^{Sey-Neu/+} ↔ Pax6^{+/+}) control chimaeras, irrespective of the percentage of cells in the embryo derived from the small eye cross (estimated as %GPI1-B), the proportions of Tg^{+/+} cells in regions 1, 2 and 3 were approximately equal. Some deviation is expected due to sampling errors and stochastic variation in the distribution of cells. The ratio of %Tg^{+/+} cells in the lens epithelium (L) over the percentage in the head epithelium (H) and the ratio of Tg^{+/+} cells in the 125 µm outside the lens (O) over H were calculated separately for each eye. Both ratios were close to 1.0 (Fig. 3).

Repeating this exercise for (Pax6^{Sey/Sey-Neu} ↔ Pax6^{+/+}) mutant chimaeras confirmed gross underrepresentation

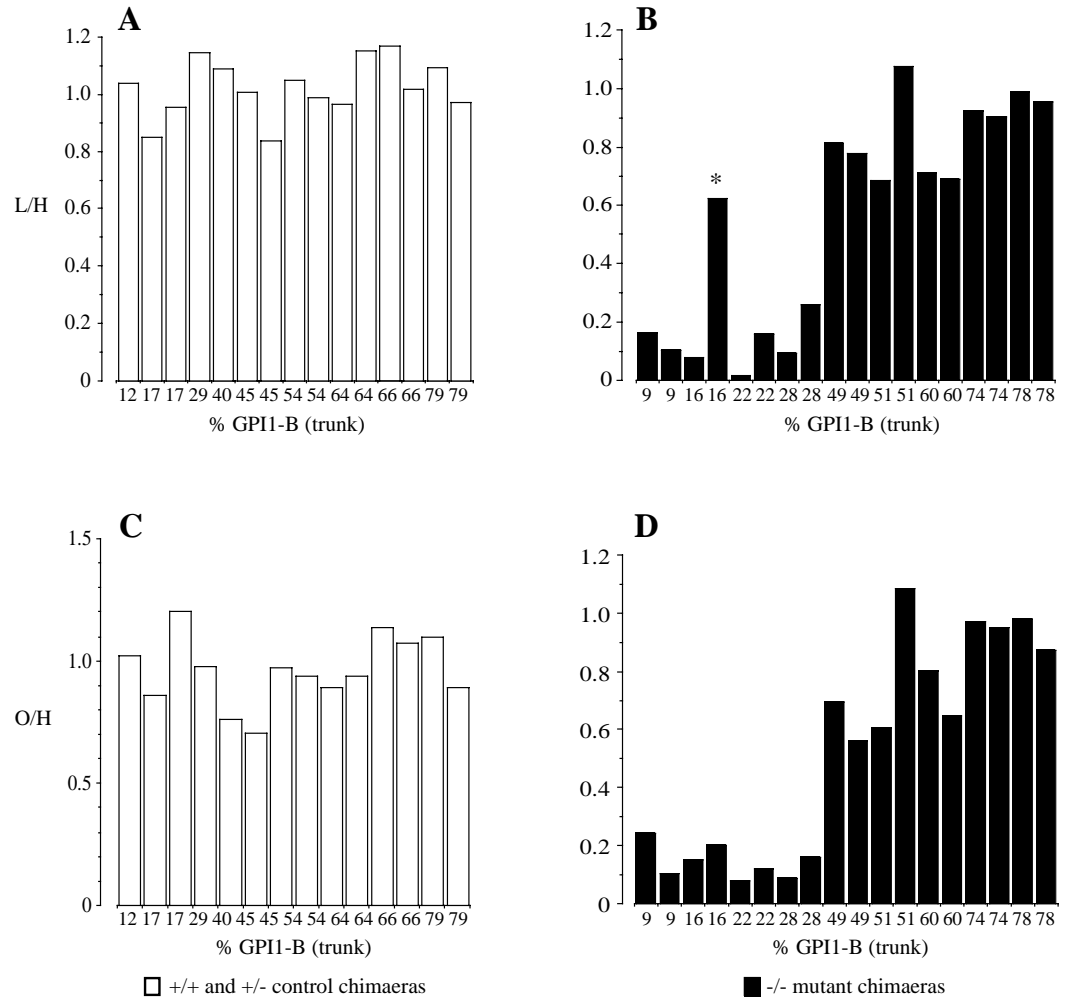
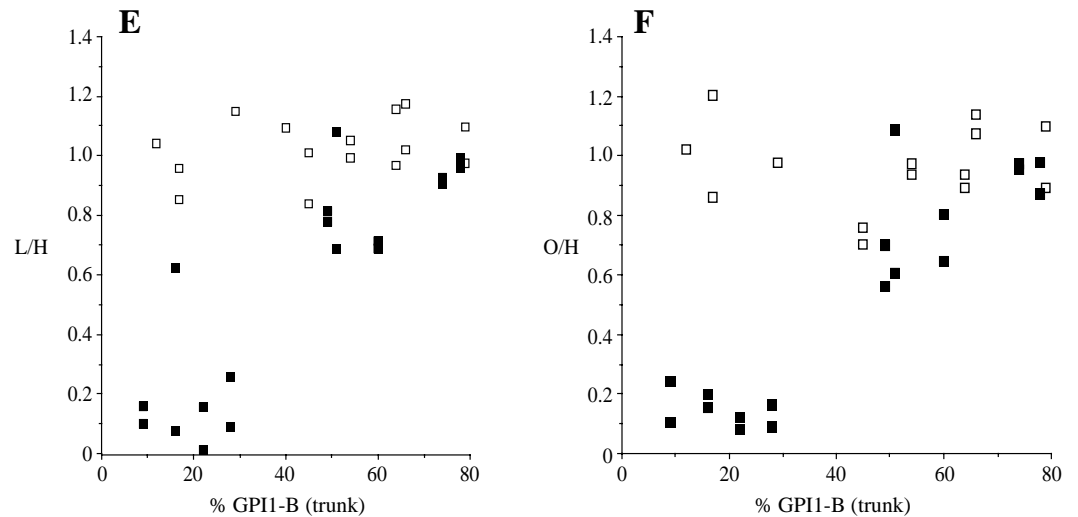


Fig. 3. (A,C) Ratios L/H (A) and O/H (C) (as described in text) plotted for each eye of ($Pax6^{+/+} \leftrightarrow Pax6^{+/+}$) and ($Pax6^{+/+} \leftrightarrow Pax6^{+/-}$) chimaeras. The percentage of GPII-B of each chimaera is on the horizontal axis. (B,D) L/H and O/H, plotted similarly for ($Pax6^{+/+} \leftrightarrow Pax6^{Sey/Sey-Neu}$) chimaeras. The bar marked with the asterisk is referred to in the text. (E,F) Scatter graphs of L/H and O/H, respectively, against percentage of GPII-B for each eye. White squares, control chimaeras; black squares, mutant chimaeras.



(normally five- to tenfold) of $Pax6^{Sey/Sey-Neu}$ cells in low-percentage chimaeras in the presumptive lens and the region outside (L/H and O/H $\ll 1$) (Figs 3, 1B; Table 1). Those few mutant cells that were retained in the lens epithelium were not

randomly spaced, but clumped. They often comprised an area of irregular cell-packing, such as in the single eye of this group that shows significant numbers of $Pax6^{Sey/Sey-Neu}$ cells (Fig. 1E; * in Fig. 3B).

For high-percentage chimaeras (above 49% GPII-B), the underrepresentation of *Pax6^{Sey/Sey-Neu}* cells in the presumptive lens region broke down. Segregation was visible in the surface epithelium, and several small *Pax6^{+/+}* placodal structures formed in these eyes, but they were interspersed with large numbers of mutant cells (Fig. 1C,D).

The data from low-percentage chimaeras shows that *Pax6^{Sey/Sey-Neu}* cells tend to be excluded prior to and during formation of the lens placode by *Pax6^{+/+}* cells. Quinn et al. (1996) established a cell-autonomous requirement for *Pax6* in the formation of the lens in low-percentage chimaeras. This study demonstrates that this cell-autonomous requirement is manifest at E9.5 during the earliest stages of placode formation. The unexpected loss of *Pax6^{Sey/Sey-Neu}* cells from an area which is wider than the lens placode, before the placode develops, suggests that there is a very early requirement in the broad area of *Pax6*-expressing pre-placodal head ectoderm. This is consistent with a role for *Pax6* in maintaining those cells which have the properties of classical lens competence or bias (Grainger, 1992); these are stages in the lens formation pathway which are prior to appearance of the lens placode (see Discussion).

Requirement for *Pax6* during proximo-distal patterning of the optic vesicle

Pax6 is normally expressed in the first manifestation of the evaginating optic vesicle in the forebrain, at E8.0. Subsequently it is expressed at high levels in the optic vesicle prior to and during contact with the facial epithelium. Expression is higher distally than proximally, and subsequently becomes restricted to the neural retina and distal optic cup structures such as the iris and ciliary body (Grindley et al., 1995).

Low-percentage mutant chimaeras had very few *Pax6^{Sey/Sey-Neu}* cells in distal regions of the optic vesicle. A consistent and extraordinary feature of these chimaeras was the presence of small ectopic vesicles budding from the side of the 'wild-type' vesicle, which were composed entirely of *Pax6^{Sey/Sey-Neu}* cells (Fig. 1B). These were never seen in chimaeras with *Pax6^{+/+}* or *Pax6* heterozygous cells.

The optic vesicles of high-percentage chimaeras showed segregation and clumping into pure patches of wild-type and *Pax6^{Sey/Sey-Neu}* cells. Clumps of mutant cells were, however, not excluded from distal regions of the vesicle (Fig. 1C).

To quantify possible cell-autonomous roles for *Pax6* during patterning of the optic vesicle, a comparison was made of the numbers of *Tg^{+/-}* cells in the distal and proximal regions of the optic vesicles of chimaeric eyes – regions which will form the inner and outer layers, respectively, of the optic cup. This analysis was performed using a graticule grid on tissue sections as described in Materials and Methods. Average proximal (P) and distal (D) scores were calculated for each eye; P, D and the ratio P/D were plotted against %GPII-B, as measured previously (Fig. 4).

Irrespective of genotype, the density of *Tg^{+/-}* cells proximally (P) rose linearly with increasing trunk %GPII-B, there being little difference between homozygote *Pax6* mutant chimaeras and controls (Fig. 4A). Distally, D rose linearly with %GPII-B for control chimaeras. For mutant chimaeras (black squares in Fig. 4B) the graph confirms that very few *Pax6^{Sey/Sey-Neu}* cells were retained in the distal optic vesicle of

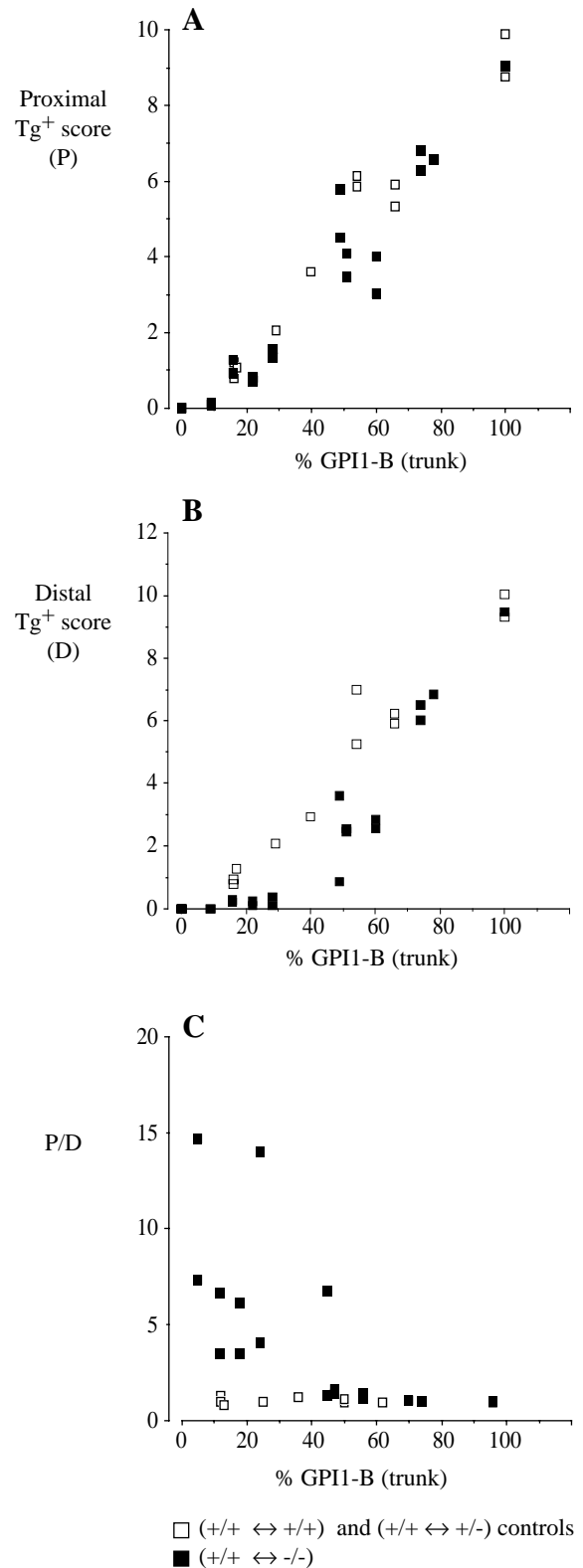


Fig. 4. Average number of *Tg⁺* signals per 25×25 μm square calculated for distal (D) and proximal (P) regions of optic vesicle. Black squares represent (*Pax6^{+/+}* ↔ *Pax6^{Sey/Sey-Neu}*) chimaeras, white squares are control chimaeras. Each point is one eye. (A) P plotted against percentage of GPII-B of the embryo. (B) D against percentage of GPII-B. (C) P/D for each eye against percentage of GPII-B.

low-percentage chimaeras, although at higher percentages, no underrepresentation was discernible.

Fig. 4C, the plot of P/D against %GPI1-B, shows that for control chimaeras, there were approximately equal numbers of $Tg^{+/-}$ cells proximally and distally. Low percentage mutant chimaeras had a high P/D ratio, due to $Pax6^{Sey/Sey-Neu}$ cells being largely absent distally. There was no proximal overrepresentation (Fig. 4A) as would be expected if distal mutant cells were being pushed proximally. The P/D ratio is variable for these because it depends critically on the exact value of the very small D denominator.

At approximately 49% $Pax6^{Sey/Sey-Neu}$ cell composition, the distal underrepresentation effect disappears: P/D for high-percentage mutant chimaeric eyes returns to 1 (Fig. 4C). There is no evidence of a gradual increase in the proportion of $Tg^{+/-}$ cells in distal regions, which would be expected if the exclusion of $Pax6^{Sey/Sey-Neu}$ cells was dependent only on the numbers of $Pax6^{+/+}$ cells present to displace them. In contrast, it appears that once the contribution of wild-type cells to these chimaeric optic vesicles falls below 50%, they are no longer capable of excluding $Pax6^{Sey/Sey-Neu}$ cells distally at all. This suggests that exclusion of mutant cells depends also on some element of proximo-distal patterning in the vesicle and that this element of patterning is not maintained in the presence of a high percentage of $Pax6^{Sey/Sey-Neu}$ cells. It is concluded, therefore, that *Pax6* itself operates during the specification or maintenance of this proximo-distal patterning – either intrinsically, or via interactions with other tissues such as the lens placode.

Different roles for *Pax6* in the optic vesicle and lens placode

Eye development requires an interaction at E9.5 between the prospective lens epithelium and the contacting optic vesicle, leading to lens placode induction. In *Pax6* homozygous mutants, this interaction does not occur; however, since both optic vesicle and lens epithelium normally express *Pax6*, it is not obvious whether *Pax6* has roles in the lens epithelium only, or the optic vesicle, or in both. The property of cell segregation in high-percentage mutant chimaeric eyes was therefore exploited to look at situations where only one tissue, lens epithelium or optic vesicle, was mutant, compared to areas where both or neither had functional *Pax6*.

All chimaeras were staged according to forelimb development (Materials and Methods). Of the six high-percentage ($Pax6^{Sey/Sey-Neu} \leftrightarrow Pax6^{+/+}$) chimaeras, three were limb stage 3 (W3) and three were less advanced, W1 or W2. A series of non-chimaeric $Pax6^{Sey/Sey-Neu}$ embryos showed that in homozygous mutant eyes, the optic vesicle loses its transitory contact with the surface ectoderm by stage W3, whereas there may still be some area of contact at W1 and W2. The interaction between the lens epithelium and the optic vesicle was therefore primarily studied in eyes of chimaeras at stage W3, to ensure that any contact was a specific effect of the genotype of the cells in the tissues concerned.

All sections through the region of contact or near-contact between the optic vesicle and the surface epithelium in all eyes were divided by graticule into 25 μm blocks. Each block was analysed and scored according to four parameters. (1) Genotype of surface epithelium (e); lengths of wild-type epithelium were scored e^+ , and $Pax6^{-/-}$ stretches e^- . (2)

Genotype of optic vesicle (o); this was scored o^+ or o^- according to genotype, as with the overlying epithelium. (3) Contact between optic vesicle and surface epithelium (c); areas where the two were in contact were scored c^+ , but c^- when there were mesenchymal cells between the two. (4) Placodal development (p); areas of surface epithelium which showed thickening, close adhesion and columnar cell appearance characteristic of incipient lens placode development were scored p^+ , and p^- where no placodal differentiation was visible. For example, a 25 μm block (or half-block where appropriate) where the presumptive lens epithelium was wild-type and placodal, but the optic vesicle cells were mutant and not in contact with the placode, was scored $e^+o^-c^-p^+$.

The summed data for frequency of occurrence of each combination of e,o,p,c scores across all sections of the eyes is presented in Table 2.

(i) Correlation between epithelial genotype and placode formation

For the three W3-staged chimaeras, a χ^2 test was used to confirm whether the formation of placodal structures (p) was dependent on the genotype of the epithelial cells (e), by adding up the lengths of e^+p^+ , e^+p^- , e^-p^+ and e^-p^- epithelia. The null hypothesis of random association was tested as a normal 2 \times 2 contingency χ^2 (Table 3i).

The χ^2 value of 123.5 (1 df; $P < 0.0001$) indicates a strong overrepresentation of e^+p^+ and e^-p^- at the expense of the other two combinations. This confirms the cell-autonomous requirement for *Pax6* during lens placode formation. The role of the optic vesicle in placode formation is discussed in (iii) below.

(ii) The genotype of the optic vesicle is the primary determinant of adhesion with the prospective lens epithelium

The association between genotype of the optic vesicle ($o^{+/-}$) and the presence or absence of contact with the overlying epithelium ($c^{+/-}$) was investigated similarly, comparing the observed frequency of combinations with that expected by random assortment.

Table 3iia ($\chi^2=130.3$, $P < 0.0001$) shows a highly significant correlation between the presence of functional *Pax6* in the optic vesicle and the maintenance of contact with the lens epithelium. Areas of o^+c^- and o^-c^+ were almost entirely restricted to the edges of the optic vesicle, or were due to irregularities in the optic vesicle preventing proper contact between the tissues.

There is also a similar clear correlation in the less advanced (limb stages W1/2) chimaeras (Table 3iib; $\chi^2=35.51$, $P < 0.001$), although the situation is complicated by the presence of mutant stretches of optic vesicle which have made, but not yet lost, contact with the lens epithelium.

Whether or not the *Pax6*-dependent contact between the optic vesicle and the lens epithelium was independent of the genotype of the lens epithelium ($e^{+/-}$) was also investigated for both sets of three chimaeras, i.e. comparing observed $e^{+/-}$ and $c^{+/-}$ combinations. This result (Table 3iic; $\chi^2=11.74$, $P < 0.001$) suggests a correlation between functional *Pax6* in the epithelial cells and their contact with the underlying optic vesicle. However, the χ^2 value is much less than that for the association between contact ($c^{+/-}$) and the genotype of the optic vesicle ($o^{+/-}$). When the same analysis was performed for the three

Table 2. Analysis of the interaction between optic vesicle and epithelia in high-percentage chimaeras

	Epithelial genotype (e) +/-	Optic vesicle genotype (o) +/-	Contact (c) +/-	Placode formation (p) +/-	Total
(i) Older chimaeras, limb stage 3					
	+	+	+	+	20.0
	+	+	+	-	6.5
	+	-	-	-	18.5
	+	-	-	+	26.0
	-	+	+	-	13.5
	-	-	+	-	10.0
	-	-	-	+	2.0
	-	+	-	-	4.0
	+	-	+	-	2.0
	+	-	+	+	2.0
	-	-	-	-	134.0
	+	-	-	?	5.0
	+	+	-	?	11.0
	-	?	+	-	1.5
	?	+	+	-	11.0
(ii) Younger chimaeras, limb stage 1-2					
	+	+	+	n/a	63.5
	+	-	+	n/a	16.0
	+	-	-	n/a	24.0
	+	+	-	n/a	7.0
	-	+	+	n/a	20.0
	-	+	-	n/a	4.0
	-	-	+	n/a	26.0
	-	-	-	n/a	22.5

For details, see text.

Each row represents a combination of the e^{+/-}, c^{+/-} etc. possibilities, and the total is the sum of the occurrences of each combination. Thus the top row in (i) represents areas where both neural and surface epithelia were wild type and in contact and where a placode was forming. 20 graticule divisions (25 µm blocks) fulfilled these criteria across all sections through these six eyes.

It was sometimes impossible to identify placodes unequivocally in younger embryos, so 'p' scores were omitted from (ii).

younger chimaeras (Table 3iii; $\chi^2=1.628$, $P=0.23$) no significant deviation from random association was shown.

Together, these results suggest that there is preferential adhesion between the optic vesicle and the surface epithelium if both have functional *Pax6*. However, the genotype of the *optic vesicle* cells is the primary and earlier determinant of adhesion. There are significant stretches of wild-type lens epithelium that do not maintain contact with the optic vesicle, and regions of mutant epithelium that make contact (e⁺c⁻ and e⁻c⁺ in Tables 2, 3iii). This shows that wild-type *Pax6* in the epithelium is neither necessary nor sufficient to maintain this contact, although it does facilitate it.

(iii) The role of the optic vesicle in inducing a lens placode

Although the presence of an optic vesicle is not always necessary to induce lens in epithelial tissue in experimental situations (Grainger, 1992), during normal development it has been shown that the optic vesicle is a lens inducer. The investigation into whether *Pax6* is required in the optic vesicle in order to perform this function is complicated, in this study, by the association between the genotype of optic vesicle cells and the prolonged maintenance of contact with the prospective lens epithelium. It can be imagined that the production of signal from the optic vesicle to the lens may be independent of *Pax6* but that, irrespectively, transduction of this signal may be dependent on the maintenance of contact between the tissues. A *Pax6^{Sey/Sey-Neu}* optic vesicle could be capable of producing inductive signals, but if contact were required to transfer the signal to the epithelium, no induction would occur.

A straightforward χ^2 analysis of the relationship between optic vesicle genotype and placode formation (o^{+/-}, p^{+/-}) suggests a significant deviation from random assortment (Table 3iv; $\chi^2=11.73$, $P<0.001$).

Thus wild-type optic vesicle preferentially underlies placodes. However, this relationship is expected, given the associations described (above), for contact to be maintained optimally when wild-type optic vesicle meets wild-type surface epithelium, and the requirement for *Pax6* in the epithelium during placode formation. No e⁻o⁺p⁺ combinations are noted in Table 2i which, in combination with data from other groups (Fujiwara et al., 1994; Enwright and Grainger, 1999), confirms that a wild-type optic vesicle cannot induce a placode in homozygous mutant prospective lens epithelium.

Misplaced placodes

One further extraordinary feature of the high-percentage mutant chimaeras was the generation of small ectopic placodal structures in the epithelium around the eyes (Fig. 1D). 11 of these putative placodes were noted, around five eyes – some eyes had two or three whereas others had none. They were entirely composed of wild-type cells and tended to invaginate precociously. They were never noted in low percentage mutant chimaeras or in controls.

DISCUSSION

This study has demonstrated different functions for *Pax6* in the optic vesicle and the surface epithelium of the face, including

Table 3. Test for associations between the parameters involved in interactions between optic vesicle and lens epithelium in high-percentage mutant chimaeras

Interactions tested					Statistical significance
(i) Epithelial genotype/placode combination					
	e ⁻ p ⁻	e ⁻ p ⁺	e ⁺ p ⁻	e ⁺ p ⁺	
Observed	163.0	2.0	27.0	48.0	$\chi^2=123.5, P<0.0001$
Expected*	130.6	34.4	59.4	15.6	
(ii) Optic vesicle genotype/contact combination					
(a) W3 chimaeras					
	o ⁻ c ⁻	o ⁻ c ⁺	o ⁺ c ⁻	o ⁺ c ⁺	
Observed	185.5	14.0	15.0	51.0	$\chi^2=132.4, P<0.0001$
Expected*	150.7	48.8	49.8	16.6	
(b) W1/2 chimaeras					
	o ⁻ c ⁻	o ⁻ c ⁺	o ⁺ c ⁻	o ⁺ c ⁺	
Observed	83.5	42.0	11.0	46.5	$\chi^2=35.51, P<0.0001$
Expected*	64.8	60.7	29.7	27.8	
(iii) Epithelial genotype/contact combination					
(a) W3 chimaeras					
	e ⁻ c ⁻	e ⁻ c ⁺	e ⁺ c ⁻	e ⁺ c ⁺	
Observed	140.5	25.0	60.5	30.5	$\chi^2=11.74, P<0.001$
Expected*	129.7	35.8	71.3	19.7	
(b) W1/2 chimaeras					
	e ⁻ c ⁻	e ⁻ c ⁺	e ⁺ c ⁻	e ⁺ c ⁺	
Observed	26.5	46.0	31.0	79.5	$\chi^2=1.628, P=0.23$
Expected*	22.8	49.7	34.7	75.8	
(iv) Optic vesicle genotype/placode combination					
	o ⁻ p ⁻	o ⁻ p ⁺	o ⁺ p ⁻	o ⁺ p ⁺	
Observed	164.5	30.0	35.0	20.0	$\chi^2=11.73, P<0.001$
Expected*	155.5	39.0	44.0	11.0	

For details, see text.

*Expected values for random association between parameters, calculated for standard 2x2 contingency chi-squared test.

the prospective lens. Several groups have postulated multiple roles for *Pax6* during CNS development (Quinn et al., 1996; Cvekl and Piatigorsky, 1996; Mastick et al., 1997; Warren et al., 1997). Future research will determine the extent to which these 'multiple roles' are mediated through the same limited sets of downstream effector genes.

The results are consistent with and extend the previous study by Quinn et al. (1996). In both studies, wild-type cells appear to direct near normal eye development in chimaeras with a low-percentage of mutant cells, with loss or exclusion of these cells from lens and retinal tissues. High percentage chimaeras lose any distinction between distal (inner) and proximal (outer) optic cup, with segregation of *Pax6^{Sey/Sey-Neu}* and *Pax6^{+/+}* cells. There is clearly some 'critical mass' of wild-type cells which must be present in the eye for normal lens and retinal development (shown by the tight threshold between 'low' and 'high-percentage' chimaeras), and this community effect is manifested at E9.5.

The role of *Pax6* in lens development

It is well established that there is a cell-autonomous

requirement for *Pax6* in the developing lens (Quinn et al., 1996; Altmann et al., 1997). What had not previously been established, however, is the stage of lens development at which this requirement manifests.

The fate of mutant cells in the facial epithelium

This study has shown that the cell-autonomous exclusion of *Pax6^{Sey/Sey-Neu}* cells from the lens of low-percentage chimaeras occurs because mutant cells are lost from a broad region of the head epithelium, at and before lens placode formation. In chimaeras with <45% mutant cells, there was a severe underrepresentation of *Pax6^{Sey/Sey-Neu}* cells at E9.5 in both the prospective lens placode (in contact with the optic vesicle) and a significant area of epithelium outside the lens region. Not all mutant cells were eliminated from the lens placode, and the developmental potential of these remaining cells is undefined (although no mutant cells have been observed in lenses of older chimaeras). It is likely that the partial elimination seen at E9.5 is nearing the end of a complete clearing. Where these missing cells go is also undefined; apoptotic death is perhaps most likely. There is also no evidence that mutant cells are displaced

into other areas of the head epithelium. The possibility of differential rates of cell cycling between wild-type and mutant cells is being investigated; a role for Pax6 in maintaining normal rates of cell cycling in facial epithelia would possibly explain the underrepresentation of Pax6^{Sey/Sey-Neu} cells in low-percentage chimaeras at E9.5. However, no overt deficiency of proliferation in Pax6^{Sey/Sey-Neu} cells in the facial epithelium has been observed in initial experiments using bromodeoxyuridine to label dividing cells (data not shown), nor is there any evidence of proliferation defects in the normal numbers of Pax6^{Sey/Sey-Neu} cells present in the epithelium of high-percentage chimaeras.

In the previous study at E12.5, low-percentage mutant chimaeras had lenses that were entirely wild type, but smaller than normal. This may be explained either if the chimaeras have a smaller than normal lens placode, or if the mutant cells which remain in the lens placode after E9.5 cannot subsequently differentiate, and die.

The failure to remove Pax6^{Sey/Sey-Neu} cells from the facial epithelium of high-percentage chimaeras requires further analysis. Fig. 3E,F does not resolve whether there are intermediate levels of elimination at the transition from low to high-percentage chimaeras. Taken together, the data suggest that elimination is a result of interaction between wild-type and mutant cells and that larger clumps of Pax6^{Sey/Sey-Neu} cells are not eliminated at this stage.

Tissue competence and lens induction

The processes leading to lens induction are best understood in *Xenopus laevis*. In this species, a region of lens competent anterior epithelium arises early in development, which then becomes increasingly biased to form a lens by a series of inductive interactions prior to contact by the optic vesicle (Grainger et al., 1988; Grainger, 1992). The area of the face which is competent to form a lens is larger than that which will normally do so in final response to contact by the optic vesicle (reviewed in Grainger, 1992). Why this should be so is not known, though the dimensions of the lens-competent region presumably reflect the extent of the inductive signals that the epithelium receives. Although extreme caution must be observed when extrapolating to other vertebrates, data from the chicken and, more recently, the mouse provide good evidence that similar phenomena of lens competence and bias, prior to specification of the lens, occur in these groups too (Piatigorsky, 1981; Barabanov and Fedtsova, 1982; Enwright and Grainger, 1999). The domains of lens competent or biased epithelium in mouse and chicken have not been accurately mapped, although the expression domain of Pax6 has been implicated as marking out a region of lens-forming potential, from which the lens will be specified (Li et al., 1994). It is not yet clear whether, in the mouse, the region of lens competence is established before or after the onset of expression of Pax6. The broad domain of Pax6 expression in facial epithelia prior to placode formation is a feature of all vertebrates so far studied, and it appears that only a subset of those cells which express Pax6 will subsequently contribute to the lens (Pueschel et al., 1992; Li et al., 1994; Grindley et al., 1995; Mizuno et al., 1999). We therefore assume that the expression of Pax6 in the surface epithelium is a response to prior inductive signals, and that contact with the optic vesicle determines where the lens finally forms. Other tissues which, in experimental situations, are able

to transdifferentiate into lens (Okada, 1991) also express Pax6, and it has been suggested that expression may correlate with lens-forming competence. Our data support this model; clearly the expression of Pax6 has qualitative functional effects on the behaviour of cells in the facial epithelium, including the prospective lens. We conclude, therefore, that Pax6 is important for maintenance of the tissue competence which allows this epithelium to respond to lens-inductive signals from the optic vesicle.

The roles of Pax6 in the optic vesicle

Cellular adhesiveness and patterning

A consistent feature of all mutant chimaeras was segregation of cells into separate populations of Pax6^{+/+} and Pax6^{Sey/Sey-Neu} cells, with budding of ectopic mutant vesicles from some eyes. Cell segregation was previously noticed in chimaeras at E12.5; this study has shown that Pax6 expression mediates cell segregation from at least E9.5. The best explanation is that expression of Pax6 controls target genes which affect the suite of molecules at the surface of the optic vesicle cells, leading to differential homotypic adhesiveness.

Although consistent with dysmorphology of the E12.5 chimaeras produced by Quinn et al. (1996), an unexpected result in the light of previous work (Grindley et al., 1995) was the role of Pax6 in directing some aspects of the proximo-distal patterning of the optic vesicle. At E9.5, Pax6 is expressed throughout the optic vesicle, strongest distally. Elimination of Pax6^{Sey/Sey-Neu} cells occurred distally in low percentage chimaeras, but in high percentage chimaeras there was no evidence of a proximo-distal difference in cell segregation (which occurred throughout the optic vesicle) or for cell elimination (for which there was no evidence at all). The data show that above approximately 49% Pax6^{Sey/Sey-Neu} cells in the optic vesicle, there is a precipitous loss of some elements of proximo-distal patterning (Fig. 4C) and hence Pax6 must be responsible for this patterning. This effect may be intrinsic to the optic vesicle, or may require a minimum threshold signal from the overlying lens placode, not available in the high percentage chimaeras.

The fate of mutant cells that are excluded from the distal optic vesicle in low-percentage mutant chimaeras is uncertain. It is possible that these cells can be physically excluded, and are the source of cells which form the ectopic mutant vesicles (Fig. 1B). However, the total number of cells in these vesicles cannot account for all the missing cells – some other factor, such as apoptosis or decreasing proliferation, must be responsible also. The fate of Pax6^{Sey/Sey-Neu} cells that remain distally at E9.5 has not been determined.

Maintenance of contact with the head surface ectoderm

The adhesion of cells within an undifferentiated epithelium like the optic vesicle is very different from the adhesion between epithelia, as seen when the optic vesicle makes contact with the surface epithelium of the head. Nevertheless, it is a striking result of this study that Pax6 is required for maintenance of contact between the lens placode and optic vesicle. It reinforces the evidence that expression of Pax6 affects the expression of cell-surface and/or extracellular adhesion molecules.

While the adhesion of the prospective placode to the optic vesicle is facilitated by the expression of wild-type Pax6 in the placodal epithelium, this in itself is insufficient to prevent loss

of contact. This study showed that it is the genotype of the optic vesicle cells which, specifically and cell-autonomously, is the primary determinant of whether contact is maintained.

Expression of *Pax6* in the optic vesicle ensures that it remains in intimate association with the induced lens placode, and this role is likely to be extremely important during the subsequent invagination of the lens and the mechanics of normal physical interaction between the tissues.

There is no overwhelming evidence from this study that the optic vesicle needs wild-type *Pax6* in order to produce lens-inducing signals. This is consistent with data from Furuta and Hogan (1998), which showed that BMP4 is a lens-inducing signal – its expression is unaffected in *Pax6^{Sey-Neu/Sey-Neu}* mice. If maintenance of contact is important for transduction of lens-inducing signals from the optic vesicle to the prospective lens epithelium, then the failure of contact could explain the apparent inefficiency of lens induction by *Pax6*-null optic vesicles (this study; Enwright and Grainger, 1999).

If the apparent ectopic placodes seen in high-percentage mutant chimaeras really are displaced developing lenses, then they may be interpreted as a consequence of the failure of the contact between optic vesicle and surface epithelia due to mechanical stress during growth. Alternatively, they may indicate a loss of lateral inhibition of placodal development in the absence of a large lens placode. Until the molecular nature of these apparent placodes is defined, it is not useful to speculate further on this.

Conclusion: expression of *Pax6* mediates the interaction between the optic vesicle and pre-placodal facial epithelium

Although there remain several questions yet to be answered about the behaviour of mutant cells in this set of chimaeras, our data have clearly shown that the full gamut of interactions between the optic vesicle and the head-surface epithelium only occur if *both* are expressing functional *Pax6*. The role of *Pax6* in the surface epithelium starts before full lens placode differentiation, which is consistent with the hypothesis that it is important for the maintenance of this pre-placodal tissue.

In the optic vesicle, the evidence suggests that expression of *Pax6* affects the adhesive properties of the cells. This probably effects the morphogenesis of the optic vesicle, and may be the molecular basis behind the misshapen vesicles seen in *Pax6* homozygous mutants (Grindley et al., 1995). We have shown that expression of *Pax6* in the optic vesicle is one of the mechanisms which generates its proximo-distal polarity, and also maintains the 'spot-weld' between it and the developing lens that is necessary during the next stages of eye development.

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