Multiple functions for *Pax6* in mouse eye and nasal development

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Mouse embryos, homozygous for the small eye (*Sey*) mutation die soon after birth with severe facial abnormalities that result from the failure of the eyes and nasal cavities to develop. Mutations in the *Pax6* gene are responsible for the *Sey* phenotype. As a general disruption of eye and nasal development occurs in the homozygous *Sey* embryos, it is unclear, from the mutant phenotype alone, which tissues require functional *Pax6*. To examine the roles for *Pax6* in eye and nasal development we produced chimeric mouse embryos composed of wild-type and *Sey* mutant cells. In these embryos we found that mutant cells were excluded from both the lens and nasal epithelium. Both of these tissues were smaller, and in some cases absent, in chimeras with high proportions of mutant cells. The morphology of the optic cup was also severely affected in these chimeras; mutant cells were excluded from the retinal pigmented epithelium and did not intermix with wild-type cells in other regions. The evidence shows that *Pax6* has distinct roles in the nasal epithelium and the principal tissue components of the embryonic eye, acting directly and cell autonomously in the optic cup and lens. We suggest that *Pax6* may promote cell surface changes in the optic cup and control the fate of the ectoderm from which the lens and nasal epithelia are derived.

[Key Words: *Pax6*; *Sey*; small eye; mouse; chimera; eye; lens; nose; nasal epithelium]

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Mutations in the *Pax6* gene result in a range of abnormalities. In man these include the autosomal dominant conditions of aniridia (Ton et al. 1991; Jordan et al. 1992; Hanson et al. 1993) and Peter's anomaly (Hanson et al. 1994), which both affect the anterior chambers of the eye. In both mouse (Hill et al. 1991) and rat (Matsuo et al. 1993), *Pax6* lesions are responsible for the semidominant phenotype small eye (*Sey*). The heterozygous phenotype of *Sey* in mouse shows related manifestations seen in human conditions (Jordan et al. 1992; Hanson et al. 1994) with additional associated microphthalmia. More severe phenotypes are seen in homozygous condition in rats (Matsuo et al. 1993) and mice (Hogan et al. 1986, 1988) and in a compound heterozygous human (Glaser et al. 1994). In mice, homozygous *Sey/Sey* is lethal at birth, leads to disruption of eye development and lack of nasal cavities in the embryo (Hogan et al. 1986, 1988), and lack of olfactory bulbs and abnormal cortical plate formation in the brain (Schmahl et al. 1993).

Pax6 and other members of the *Pax* gene family have the characteristics of being important regulatory elements whose roles are central to normal development (for a recent review, see Stuart et al. 1994). We have exploited the small eye phenotype to define more clearly the developmental roles of *Pax6*, focusing primarily on the overt eye and nasal abnormalities. Eye development

proceeds from two principal tissue components: the neural ectoderm, which buds from the forebrain to form the optic vesicle, and the surface ectoderm, which forms the lens. The optic vesicle invaginates, producing the optic cup with an inner layer, which forms the neural retina, and an outer layer, which forms the retinal pigmented epithelium (RPE). The lens placode is a morphological intermediate of lens formation, characterized as an area of thickened columnar epithelium, which comes into contact with the optic vesicle. Continued eye development is dependent on the mutual interaction and invagination of these two tissues (Coulombre 1965; Smelser 1965; Saha et al. 1989; Grainger 1992). In the *Sey/Sey* homozygous mouse and rat the initial contact between the optic vesicle and head ectoderm occurs; however, there is no evidence for the formation of the lens primordium. The optic vesicle subsequently loses contact with the surface and degenerates (Hogan et al. 1986; Fujiwara et al. 1994; Grindley et al. 1995).

Like the lens, the nasal cavities form from the invagination of ectodermal placodes. Hogan et al. (1986) have considered this similarity and suggested that the eye and nasal phenotype of *Sey/Sey* mice may result from defective placode formation. In support of this suggestion, *Pax6* is initially expressed broadly in the head ectoderm and subsequently becomes localized in the placodes of these two tissues (Li et al. 1994; Grindley et al. 1995). However, in the eye, *Pax6* is also expressed in the optic vesicle and later in both the inner and outer layers of the

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optic cup, and in the distal optic stalk (Walther and Gruss 1991; Grindley et al. 1995). Defects in any of these expressing components of the eye could interfere with essential tissue-tissue interactions and lead to a phenotype resembling that of *Sey/Sey*.

To investigate the relevance of the expression pattern of *Pax6* to the *Sey/Sey* phenotype we have produced fetal mouse aggregation chimeras composed of a mixture of wild-type and small eye mutant cells. Chimeras of four genotype combinations were produced and distinguished retrospectively by PCR analysis. The distribution of mutant *Sey* cells was determined by the inclusion of three genetic markers. These studies have enabled us to investigate the developmental potential of the cells that express *Pax6* before 12.5 days gestation (E12.5) in different tissues of the developing eye and nose. We have also investigated the suggestion that the *Sey* mutation may have similar effects in the lens and nasal epithelium.

Results

Production of chimeras

Production of *Sey/Sey* ↔ *+/+* chimeras is hampered by lethality of the *Sey/Sey* mice. This means that *Sey/Sey* embryos must be produced by (*Sey/+* × *Sey/+*) matings and distinguished from other genotypes retrospectively. To facilitate identification of different genotypic classes of chimeras, we produced chimeras with compound heterozygous (*Sey^{Neu}/Sey*) embryos, rather than homozygous (*Sey/Sey*) embryos (Fig. 1). Both the *Sey* and *Sey^{Neu}* small eye alleles contain point mutations that result in loss of functional PAX6 protein (Hill et al. 1991) and are distinguishable by a PCR assay (Fig. 2). The same phenotypic abnormalities are seen in homozygous *Sey/Sey*, *Sey^{Neu}/Sey^{Neu}* (Hill et al. 1991; Schmahl et al. 1993) and *Sey^{Neu}/Sey* compound heterozygous embryos (J.C. Quinn, unpubl.). To distinguish between different classes of chimeras, we used embryos from crosses between heterozygous *Sey^{Neu}/+* females and heterozygous *Sey/+* males, both of which are pigmented (*C/C*) and homozygous for the glucose phosphate isomerase *Gpi1^b* allele. The *Sey/+* males were also homozygous (*Tg/Tg*) for the reiterated *TgN(Hbb-b1)83Clo*, β -globin transgene (Lo 1986; Lo et al. 1987; West et al. 1995). Consequently all of the embryos produced by the *Sey^{Neu}/+* × *Sey/+* cross were *Tg/-* heterozygotes. These embryos were aggregated to albino BALB/c strain embryos (*Gpi1^a/Gpi1^c*) to produce chimeric fetuses, which were analyzed at E12.5 days (Fig. 1). Four groups of chimeras were distinguished by PCR analysis of *Pax6* DNA: *Sey^{Neu}/Sey* ↔ *+/+*, *Sey^{Neu}/+* ↔ *+/+*, *Sey/+* ↔ *+/+*, and *+/+* ↔ *+/+*. The contribution of the (*Sey^{Neu}/+* × *Sey/+*) embryo was estimated in different tissues to test the developmental potential of *Sey^{Neu}/Sey* compound heterozygous cells in the *Sey^{Neu}/Sey* ↔ *+/+* fetal chimeras. This estimate was based on a combination of GPI electrophoresis, eye pigmentation, and DNA in situ hybridization to the β -globin transgene.

Figure 1. Schematic representation of the experimental design for investigation of the role of *Pax6* in eye and nasal development. Two small eye mutant strains were intercrossed to produce eight-cell stage embryos that were all genetically pigmented (*C/C*), homozygous *Gpi1^b/Gpi1^b*, hemizygous for the β -globin transgene (*Tg/-*) but differed at the small eye locus (*Sey^{Neu}/Sey*, *Sey^{Neu}/+*, *Sey/+* or *+/+*). These were aggregated to inbred BALB/c embryos (albino, *c/c*, homozygous *Gpi1^a/Gpi1^a*, nontransgenic, *-/-*, and wild-type for small eye, *+/+*) to produce aggregation chimeras. At E12.5, *Sey^{Neu}/Sey* ↔ *+/+* and the three control groups (*Sey^{Neu}/+* ↔ *+/+*, *Sey/+* ↔ *+/+* and *+/+* ↔ *+/+*) of chimeras were distinguished by DNA analysis. The contribution from the (*Sey/+* × *Sey^{Neu}/+*) embryo was quantified by DNA in situ hybridization to various tissues in the head and GPI electrophoresis of the tail, forelimb, amnion, whole yolk sac, and placenta.

Thirty-seven fetal chimeras were produced (7 *Sey^{Neu}/Sey* ↔ *+/+*, 7 *Sey^{Neu}/+* ↔ *+/+*, 7 *Sey/+* ↔ *+/+*, and 16 *+/+* ↔ *+/+*). Although the presence of GPIA and GPIB confirmed that these were all chimeric, eye pigment was not observed in any of the *Sey^{Neu}/Sey* ↔ *+/+* chimeras (discussed below). Absence of eye pigment was not attributable to retarded development, as statistical analysis revealed no significant differences, among the four groups, in median developmental stage (Kruskal Wallis test of hindlimb morphology), mean conceptus weight, fetus weight, placental weight or crown-rump length (analysis of variance). The chimeras in the other groups had variegated eyes, as expected.

Figure 2. Analysis of genomic DNA from chimeric fetuses allows identification of *Sey* genotypes. Both the *Sey* and *Sey^{Neu}* alleles have single-base-pair changes within the *Pax6* gene that give rise to novel *DdeI* and *HindIII* restriction enzyme sites, respectively. Digestion of a 148-bp PCR product with *DdeI* gives fragment sizes of 83 and 65 bp in the wild type with additional 46- and 19-bp fragments in the *Sey* mutant (lane *a* is undigested; lane *b* is digested). After digestion, the *Sey^{Neu}* mutation gives rise to 140- and 80-bp fragments from a 220-bp PCR product (lane *c* is undigested; lane *d* is digested).

Histological sections revealed several striking morphological abnormalities in *Sey^{Neu}/Sey $\leftrightarrow$ +/+* chimeras but none in the other groups of chimeras. Three (JC17, JC48, and JC50) of the seven *Sey^{Neu}/Sey $\leftrightarrow$ +/+* chimeras had abnormal bulging eyes with overgrown abnormal optic cup tissue but no lenses (Fig. 3). The lenses were absent from both eyes of chimeras JC17 (Fig. 3D) and JC50 and from the right eye of chimera JC48 (Fig. 3A). In two other chimeras (JC56 and the left eye of JC61) the lenses were present but markedly smaller than normal. This was also the case in the left eye of JC48 (Fig. 3A,C).

Thresholds for proper lens and nasal epithelium development

Initial analysis of the chimeras was based on an estimate of the overall contribution of (*Sey^{Neu}/+* $\times$ *Sey/+*) cells to the fetus [mean percent GPIIB (mean %GPIIB) in the tail and forelimb] and histology of the eye and nasal epithelium. GPI analysis showed that the contribution of the *Sey^{Neu}/Sey* cells to the *Sey^{Neu}/Sey $\leftrightarrow$ +/+* chimeras that lacked lenses was high (80% for JC17, 76% for JC50, and 56% for JC48, missing one lens). The mean %GPIIB for each of the other four *Sey^{Neu}/Sey $\leftrightarrow$ +/+* chimeras was lower (range 11%–46%; Table 1). A similar relationship was seen for the nasal epithelium.

The sizes of lenses and nasal epithelia were estimated in the *Sey^{Neu}/Sey $\leftrightarrow$ +/+* chimeras and found to be inversely correlated with the %GPIIB (*Sey^{Neu}/Sey*) contribution to the fetus (Fig. 4). This suggests that the sizes of

the lenses and nasal epithelia are quantitatively dependent on the proportion of wild-type cells in the *Sey^{Neu}/Sey $\leftrightarrow$ +/+* chimeras. In the 10 chimeras from the other three groups that were analyzed, the sizes of the lenses or nasal epithelia were not affected by the presence of *Sey^{Neu}/+* or *Sey/+* cells and showed no relationship with the percent fetal GPIIB. There was no reduction in lens size in *Sey/+* $\leftrightarrow$ *Sey $\leftrightarrow$ +/+* or *Sey^{Neu}/+* $\leftrightarrow$ *Sey $\leftrightarrow$ +/+* chimeras even though both *Sey/+* and *Sey^{Neu}/+* heterozygous adults have small eyes.

In *Sey^{Neu}/Sey $\leftrightarrow$ +/+* chimeras, lens development appeared to be more sensitive than nasal epithelium development, showing a critical threshold at ~50%–70% contribution of *Sey^{Neu}/Sey* cells (Fig. 4a). Chimeras approaching this threshold formed small lenses, and beyond this threshold lenses were not formed. The threshold is likely to exert its effect in the surface ectoderm or the lens placodes and if the proportion of *Sey^{Neu}/Sey* cells differs between left and right lens primordia this may result in unilateral effects. Several examples of chimeras with different sized lenses are shown in Table 1. The right lens of JC61 was much larger than the left lens, and JC48 had only a lens in the left eye, as shown in Figure 3A. Presumably the proportion of *Sey^{Neu}/Sey* cells was just below the critical threshold in the head ectoderm that produced the lens placode in the left eye of JC48 (with a small lens) but was above this level in the right eye (no lens).

The lens and nasal epithelia sizes are both related to the chimeric composition in the *Sey^{Neu}/Sey $\leftrightarrow$ +/+* chimeras, and either tissue may be absent unilaterally (Table 1). The shapes of the curves in Figure 4, a and b, appear to differ, and this suggests that the kinetics of the relationships between composition and tissue size may differ. In Figure 4b there is a nearly linear relationship between the extent of nasal epithelia and the %GPIIB (*Sey^{Neu}/Sey*) contribution to the fetus, whereas Figure 4a appears more sigmoidal and the size of the lens appears constant until the proportion of mutant cells reaches ~40%. It is clear that a critical percentage of wild-type cells is required to coordinate formation of both these tissues, below which they cannot form. Although there are too few animals to draw firm conclusions, it appears that different thresholds of normal cells are required for the production of the lens and nasal epithelium.

Cellular composition of lens and nasal epithelium

The tissue composition of 16 chimeras (3–5 in each group) was analyzed in more detail by estimations of the percent eye pigment and the percentage of *Tg*[−] cells, by DNA in situ hybridization to the β -globin transgene (Fig. 3). The quantitative results of the analysis of three widely spaced sections of each tissue are shown in Figure 5 and Table 1. For most of the tissues analyzed from the 11 *Sey^{Neu}/+* $\leftrightarrow$ *Sey/+* $\leftrightarrow$ *Sey $\leftrightarrow$ +/+* chimeras, the hybridization index correlated significantly with the mean percent fetal GPIIB [see Fig. 5 legend for statistical analysis]. In *Sey^{Neu}/Sey $\leftrightarrow$ +/+* chimeras, contributions from both parental genotypes in each

Figure 3. (A) Low-power photograph of a transverse histological section of the head of *Sey^{Neu}/Sey[↔]+/+* chimera JC48, showing both eyes. The right eye has no lens, and the optic cup is overgrown. The left eye has a small lens and is shown in higher magnification in C. (B) Histological section of the left eye of *+/+↔+/+* chimera JC49, showing a high contribution of *Tg/-* cells (positive signal seen as dark spots in the nuclei after in situ hybridization) in all tissues and pigmented cells in the retinal pigmented epithelium (outer layer). (C) Histological section of the left eye of *Sey^{Neu}/Sey[↔]+/+* chimera JC48 shown in A, showing complete absence of nuclei with in situ hybridization signal (absence of *Sey^{Neu}/Sey* cells) in the lens and division of the optic cup into large domains of almost entirely wild-type cells (no hybridization signal; small arrows) and almost entirely *Sey^{Neu}/Sey* cells (with hybridization signal; large arrows), respectively. Two boundaries between the domains of mutant and wild-type cells are indicated with open arrows. (D) Histological section of the left eye of *Sey^{Neu}/Sey[↔]+/+* chimera JC17 with a multilayered optic cup and no lens. The optic cup is divided into large domains of almost entirely wild-type cells (central region with no hybridization signal; small arrows) and almost entirely *Sey^{Neu}/Sey* cells (with hybridization signal; large arrows), respectively. One boundary between the domains of mutant and wild-type cells is indicated with an open arrow. (E) Histological section of the right eye of *Sey^{Neu}/Sey[↔]+/+* chimera JC61. The lens and optic cup are more normal but both lack mutant cells (no nuclei with hybridization signal). (F) Higher magnification of part of the eye shown in E. Hybridization signal can be seen in some mesoderm cells and one cell in the hyaloid plexus but not in the inner or outer layers of the optic cup. (G) Histological section of the head of *+/+↔+/+* chimera JC49 showing in situ hybridization to some cells in both the left nasal epithelium and surrounding mesoderm. (H) Histological section of the head of *Sey^{Neu}/Sey[↔]+/+* chimera JC61 showing in situ hybridization to some of the mesoderm cells but not to the nasal epithelium cells (absence of *Sey^{Neu}/Sey* cells). (ect) Ectoderm; (fb) forebrain; (hp) hyaloid plexus; (il) inner layer of optic cup; (l) lens; (m) mesoderm; (ne) nasal epithelium; (nr) neural retina; (oc) optic cup; (ol) outer layer of optic cup; (rpe) retinal pigmented epithelium.

chimera were found in regions unaffected by the *Sey* genotype, including tail, forelimb, head mesoderm, head ectoderm, proximal optic stalk, and hindbrain (Fig. 5) plus amnion, yolk sac, and placenta (not shown). In striking contrast, however, no hybridization signals to *Tg/-* (*Sey^{Neu}/Sey*) cells were found in the lenses and nasal epithelia (where present) of the five *Sey^{Neu}/Sey[↔]+/+* embryos analyzed (Fig. 3C,E,H). Hybridization signals were found, however, in all three control groups, and *+/+↔+/+* tissues are illustrated in Figure 3, B and G. Further analysis of every available section of *Sey^{Neu}/Sey[↔]+/+* eyes (13–25 sections containing lens tissue)

confirmed that mutant cells were completely absent from all of the lenses. Similarly, in the nasal epithelia, only 2–14 scattered cells were found in all available sections of the five *Sey^{Neu}/Sey[↔]+/+* chimeras (10–32 sections per chimera). This is consistent with the low false-positive frequencies seen in this (data not shown) and other (Everett and West 1996) studies. We conclude that *Sey^{Neu}/Sey* cells are effectively excluded from the nasal epithelium as well as the lens. This exclusion shows that they cannot be rescued by neighboring wild-type cells and implies that *Pax6* acts cell autonomously within prospective lens and nasal epithelium.

Table 1. Relationships between chimera composition, eye morphology, and sizes of lens and nasal epithelia in E12.5 fetal *Sey^{Neu}/Sey⁺/+* chimeras

Chimera	Side	%GPI1B in fetus ^a	Tg/- in optic cup ^b			Optic cup dysmorphology ^c	Area in section ($\mu\text{m}^2 \times 10^4$) ^d	
			inner layer	outer layer	total		lens	nasal epithelium
JC63	left	11.0	0.3	0	0.2	+	4.5	14.7
JC63	right	11.0	0.3	0	0.2	+	5.7	15.9
JC35	left	27.5	N.D.	N.D.	N.D.	+	5.5	9.8
JC35	right	27.5	N.D.	N.D.	N.D.	+	4.7	13.5
JC56	left	42.8	0.3	38.2	20.5	+++	3.9	7.4
JC56	right	42.8	0 ^e	0 ^e	0 ^e	+	5.7	11.0
JC61	left	46.9	0 ^e	0 ^e	0 ^e	+++	2.1	1.2
JC61	right	46.9	0 ^e	19.3	7.3	++	6.1	2.5
JC48	left	59.2	12.0	33.3	23.4	++++	2.6	4.9
JC48	right	59.2	N.A.	N.A.	18.1	+++++	0	2.5
JC50	left	76.8	N.A.	N.A.	N.D.	+++++	0	0
JC50	right	76.8	N.A.	N.A.	N.D.	+++++	0	0
JC17	left	80.3	N.A.	N.A.	45.6	+++++	0	2.5
JC17	right	80.3	N.A.	N.A.	29.6	+++++	0	0

Treating each eye as a separate sample, the mean percent fetal GPI1B correlated positively with the percent Tg/- cells in the total optic cup (Spearman's rank correlation coefficient, $r_s = 0.731$; $P = 0.028$), and the size of the lens correlated positively with the size of the nasal epithelium ($r_s = 0.736$; $P = 0.008$). The degree of morphological abnormality of the optic cup correlated negatively with lens size ($r_s = -0.899$; $P = 0.001$) and positively with percent Tg/- cells in the whole optic cup ($r_s = 0.781$; $P = 0.019$) and percent fetal GPI1B ($r_s = 0.933$; $P = 0.001$). Lens size also correlated inversely with percent fetal GPI1B ($r_s = -0.789$; $P = 0.004$) but not with the percent Tg/- cells in the whole optic cup ($r_s = -0.588$; $P = 0.078$).

^a%GPI1B is the mean %GPI1B in the tail and one forelimb.

^bTg/- in optic cup is the hybridization index in three widely spaced sections. Separate values are shown for the inner and outer layers of the optic cup where these layers can be clearly distinguished; otherwise the total value is shown (as plotted in Fig. 5). None of the Tg/- cells in the outer layer were pigmented and so they were not normal RPE cells. (N.D.) Not determined; (N.A.) not applicable.

^cOptic cup dysmorphology is shown on a subjective 5-point scale: (+) Normal morphology with double-cup comprising an outer layer of RPE and inner neural retina; (++) slightly abnormal; (+++) abnormal tissue present but double-cup shaped morphology largely retained; (++++ abnormal with distortion of the double-cup shape; (+++++) grossly dysmorphic and multilayered.

^dArea was estimated with a Chalkley grid as described in Materials and methods.

^eAlthough no hybridization signal was seen in the three histological sections examined for the quantitative analysis, qualitative analysis of all of the available sections revealed a few Tg/- cells.

Cellular composition of the optic cup

Development of the optic cup was also abnormal in the *Sey^{Neu}/Sey⁺/+* chimeras but not in any of the other groups of chimeras. At the stage of development examined (E12.5) the optic cup is normally composed of an outer unicellular layer of RPE and a closely opposed thicker inner layer of prospective neural retina (Fig. 3B). The state of the optic cup in the *Sey^{Neu}/Sey⁺/+* chimeras was highly variable. Some of the gross abnormalities made it difficult to assign cells to normal tissues; therefore, in Figure 5 the optic cup is considered as a whole. The extent of the morphological abnormalities was apparently influenced by the size of the lens and the proportion of the *Sey^{Neu}/Sey* cells in the chimera, two parameters that are interdependent and negatively correlated (Fig. 4; Table 1). Two distinctive classes of embryonic eyes arose in the *Sey^{Neu}/Sey⁺/+* chimeras. One class includes the optic cups that are associated with lenses, all having the characteristic double layer cup shape. Morphologically, these range from optic cups that appear normal (JC63, JC35, and JC56 right eye; Table 1) to dysmorphic cups that are distorted with ectopic tissue overgrowth (JC56 left eye, JC61 and JC48 left eye;

Table 1; Fig. 3C,E). The second class of optic cup structures have no associated lenses and are highly disorganized and overgrown (JC48 right eye, JC50 and JC17; Table 1; Fig. 3A,D).

Figure 5 shows that in *Sey^{Neu}/Sey⁺/+* chimeras, the proportion of mutant cells was lower in the optic cup than in other unaffected tissues. Although the contribution was lower in the whole optic cups (mean hybridization index=14.5) than the fetal limb and tail (mean %GPI1B=48.0), the significance of this is uncertain because there was also a difference in the other groups of chimeras (47.4% vs. 62.3%). The inner and outer layers of the optic cup were considered separately for seven *Sey^{Neu}/Sey⁺/+* eyes in Table 1. The inner layer had a lower proportion of mutant cells, which made up <1% except for the JC48 left eye (12%). Here, the mutant cells were specifically at the tip of the optic cup in a morphologically abnormal region (Fig. 3C). In the outer layer, the *Sey^{Neu}/Sey* cells contributed more significantly to three of the seven eyes (19%–38% in JC61 right, JC48 left, and JC56 left; Table 1) but were not incorporated into the thin monolayer of the RPE (Fig. 3E,F). The mutant cells were usually associated with regions of the outer layer that are either abnormally thickened or situated ectopi-

Figure 4. Relationship between the percent fetal GPI1B and the estimated sizes of both lenses (a) and both nasal epithelia (b) for different groups of chimeras. (a) The total size of both lenses showed a statistically significant negative correlation with the %GPI1B in *Sey^{Neu}/Sey ↔ +/+* chimeras ($r_s = -0.982$; $P = 0.016$) but not in the other groups of chimeras analyzed ($r_s = -0.564$; $P = 0.259$ for *Sey^{Neu}/+ ↔ +/+* plus *Sey/+ ↔ +/+* and $r_s = -0.359$; $P = 0.473$ for *+/+ ↔ +/+*). Similarly, the total size of the nasal epithelia was also negatively correlated with the %GPI1B in *Sey^{Neu}/Sey ↔ +/+* chimeras ($r_s = -0.929$; $P = 0.023$) but not in the other groups ($r_s = 0.300$; $P = 0.549$ for *Sey^{Neu}/+ ↔ +/+* plus *Sey/+ ↔ +/+* and $r_s = 0.154$; $P = 0.758$ for *+/+ ↔ +/+*). The estimated sizes of the lenses and nasal epithelia were positively correlated, both when left and right sides were pooled ($r_s = 0.946$; $P = 0.021$) or when they were considered separately (Table 1).

cally. These regions were not composed exclusively of mutant cells but contained significant proportions of wild-type cells, which occurred as segregated patches and were not intermingled with the mutant cells. The optic cups that appeared normal showed little or no *Sey^{Neu}/Sey* contribution to either layer (Table 1; Fig. 3D). Furthermore, the inability of the *Sey^{Neu}/Sey* cells (the pigmented component of the chimeras) to successfully contribute to the RPE is supported by the observation that none of the eyes of the *Sey^{Neu}/Sey ↔ +/+* chimeras showed any detectable pigmentation.

Mutant *Sey^{Neu}/Sey* cells made a significant contribution to the morphologically abnormal optic cups of the second class of eyes (with no lenses) as well as the morphologically abnormal regions of optic cups in the eyes with lenses. In both cases, the mutant cells were non-randomly distributed and not finely mixed with wild-type cells. There was a striking partitioning of the two cell types (Fig. 3D), which was not seen in the control chimeras. In the wild-type domains there were few mutant (*Tg/-*) cells and no detectable wild-type cells in the mutant domains. (Because not all mutant *Tg/-* cells show a hybridization signal, it is not known whether wild-type cells were completely absent from mutant domains or present at a low level). Segregation of cell types is unusual in mouse chimeras and implies that the interactions between *Sey^{Neu}/Sey* and *+/+* cells are abnormal so that they either fail to mix and intermingle or actively sort themselves out into homogeneous patches. This suggests that *Pax6* expression defines early cellular properties (possibly affecting the cell surface) within the optic vesicle. The evidence that *Sey^{Neu}/Sey* cells are ex-

cluded from the RPE and at a selective disadvantage in other regions of the optic cup, as well as being distributed nonrandomly, implies that *Pax6* has a direct, cell-autonomous effect not only in the lens and nasal epithelium (see above) but also in the optic cup.

Discussion

The eye is a complex organ that develops from the interaction of two embryologically distinct ectodermal layers: the head surface ectoderm and the neural ectoderm. Analysis of *Sey/Sey* homozygous and phenotypically equivalent, *Sey/Sey^{Neu}* compound heterozygous mutant mice shows that the development of the embryonic eye is disrupted but provides only a few clues as to the mechanism. Thus, a clear understanding of the role of the gene responsible, *Pax6*, is difficult to surmise. To investigate the developmental potential of the *Sey/Sey^{Neu}* compound heterozygous mutant cells, we produced mouse chimeras by aggregating mutant (*Sey/Sey^{Neu}*) and wild-type eight-cell stage embryos. The resulting *Sey/Sey^{Neu} ↔ +/+* chimeric embryos provided an environment in which wild-type and *Sey/Sey^{Neu}* mutant cells could mix and interact. Each mutant cell carried a reiterated transgene to distinguish between mutant and wild-type cells. This provided a means of testing whether *Sey/Sey^{Neu}* cells were rescued by surrounding wild-type cells in the affected tissues and, if so, whether the two types of cells mixed normally. Specifically, the failure of wild-type cells to rescue mutant cells in a tissue implies that *Pax6* gene expression is required in that tissue and acts cell autonomously. Although a system-

Figure 5. Histograms showing the contribution of (*Sey^{Neu}/+ × Sey/+*) cells to different tissues of 16 chimeric fetuses of four different genotypes. The contribution to the fetal trunk is given as the mean %GPI1B (calculated from separate estimates of %GPI1B in the tail and one forelimb). The contributions to normal RPE (where present) was estimated as the percent pigmented RPE cells. The contributions to other tissues were estimated by the corrected hybridization index (see Materials and methods) from three widely separated histological sections. (Solid bar) left; (hatched bar) right; (open bar) unpaired tissues. For eye tissues, these were the midsection of the eye and the two sections that were halfway between the midsection and the first or last section, respectively. Results for the neural retinal and RPE are presented together as the optic cup because these tissues were morphologically abnormal in some *Sey^{Neu}/Sey/+* + *+/+* chimeras. For the 11 control, *Sey^{Neu}/+ ↔ +/+*, *Sey/+ ↔ +/+* and *+/+ ↔ +/+* chimeras, the mean %GPI1B correlated well (Spearman's rank correlation coefficient, r_s) with the hybridization index for all of the tissues analysed apart from the right optic stalk: left nasal epithelium ($r_s=0.918$; $P=0.004$); right nasal epithelium ($r_s=0.964$; $P=0.004$); left lens ($r_s=0.891$; $P=0.005$); right lens ($r_s=0.842$; $P=0.012$); left neural retina ($r_s=0.945$; $P=0.003$); right neural retina ($r_s=0.927$; $P=0.005$); left RPE ($r_s=0.964$; $P=0.002$); right RPE ($r_s=0.976$; $P=0.003$); left total optic cup ($r_s=0.982$; $P=0.002$); right total optic cup ($r_s=0.952$; $P=0.004$); left optic stalk ($r_s=0.788$; $P=0.013$); right optic stalk ($r_s=0.600$; $P=0.090$); ectoderm overlying left eye ($r_s=0.900$; $P=0.004$); ectoderm overlying right eye ($r_s=0.855$; $P=0.010$); head ectoderm ($r_s=0.782$; $P=0.013$); hindbrain ($r_s=0.964$; $P=0.002$); and mesoderm ($r_s=0.961$; $P=0.002$). (*) Chimera JC48 had no lens in its left eye; (**) chimera JC17 had no lens in either eye; † tissues from the left side of the head were not analyzed from chimera JC93.

atic analysis of cell mixing was not undertaken, the distribution of the transgenic and nontransgenic cells, in various embryonic tissues unaffected by the *Sey* mutation, was apparently similar in the *Sey/Sey^{Neu} ↔ +/+* and control chimeras. In contrast, the developing eyes and nasal epithelium showed abnormal participation of the *Sey/Sey^{Neu}* cells and highlighted similarities in the developmental requirements of the lens and nasal epithelium.

Role of *Pax6* in lens and nasal epithelium formation

In the *Sey/Sey^{Neu} ↔ +/+* chimeras, *Sey/Sey^{Neu}* cells were not detected in the lens or nasal epithelium at E12.5. The inability to participate in the development of

these epithelial tissues, populated by *+/+* cells, shows that the *Sey/Sey^{Neu}* cells are incapable of rescue and that *Pax6* activity is direct and cell autonomous. Thus, *Pax6* has a cell-autonomous role at a stage of lens and nasal epithelia formation preceding E12.5. The lens and nasal epithelium are derived from the head ectoderm. Figure 5 shows that the proportion of *Sey/Sey^{Neu}* cells in other regions of the head ectoderm was similar to that in the fetal trunk (%GPI1B) and other tissues, in which *Pax6* has no apparent influence. This proportion is probably representative of the chimera as a whole. Thus, initially, the regions of head ectoderm that form the lens and nasal placodes are likely to have been composed of a similar mixture of mutant and wild-type cells. Mutant cells would subsequently be lost from the lens and nasal lineages at the stage of earliest *Pax6* function. Previous

analysis of *Sey/Sey* homozygous mice showed not only that lens and nasal cavities fail to form but that at E9.5 there is no evidence for thickening of the surface ectoderm indicative of lens and nasal placode formation (Hogan et al. 1986; Grindley et al. 1995). Placode formation is the earliest morphological indicator of lens and nasal differentiation. It was suggested (Grindley et al. 1995) that in *Sey/Sey* homozygous mice the normal transition from head ectoderm to placode formation is interrupted. The observations in the *Sey/Sey^{Neu}↔+/+* chimeras argue that *Pax6* has a direct role in this early developmental decision such that head ectodermal cells, deficient in *Pax6*, are unable to proceed to the initial stages of epithelial morphogenesis. Thus, *Pax6* is a necessary component of the system that specifies the pattern of the head. However, because the fates of lens and nasal epithelial cells are ultimately different, additional, perhaps exogenous, signals (Jacobson and Slater 1988) are necessary to specify the identity of these cells as either lens or nasal epithelium.

In the *Sey/Sey^{Neu}↔+/+* chimeras, there were inverse relationships between the %GPI1B (overall percentage of the *Sey/Sey^{Neu}* mutant cells) and the amount of both lens and nasal epithelia. In addition, the production of both of these structures is dependent on a threshold of normal cells, below which no epithelium is detectable at the embryonic stage examined (E12.5). These observations lead to two predictions. First, we predict that the size of the lens and nasal tissues is solely dependent on the number of cells that are competent to form these tissues; that is, the wild-type cells. We cannot rule out the possibilities that *Sey/Sey^{Neu}* cells are active inhibitors of lens development or that the level of a concentration-dependent exogenous signal (from outside the lens) is reduced in *Sey/Sey^{Neu}↔+/+* chimeras. However, our results, combined with failure of nasal and lens placode formation in nonchimeric, homozygous *Sey/Sey* mutants suggest that the size of these epithelia-derived structures is determined by the number of wild-type cells that constitute the placodal domains. Thus, in the chimeras, the primordia forming these specialized epithelia are smaller than normal and there is no subsequent compensatory growth mechanism.

Second, we predict that there is a critical proportion of wild-type cells in the lens and nasal primordia needed to initiate the development of these structures. This is reminiscent of an experimental phenomenon referred to as the community effect (Gurdon et al. 1993). Briefly, this suggests that in development, cells communicate with like neighbors and that a critical number of interacting cells is required to proceed to the next stage in development. Thus, in the *Sey/Sey^{Neu}↔+/+* chimeras, the presence of a high percentage of *Sey/Sey^{Neu}* cells interrupts the sparse population of wild-type cells and the critical number of interacting wild-type cells is not reached. However, the possibility also exists that lens or nasal epithelium development begins in these tissues as a mixture of mutant and wild-type cells but subsequently degenerates because of the burden of the abnormal *Sey/Sey^{Neu}* cells.

Role of *Pax6* in optic cup development

Optic vesicle-derived tissue developed in all *Sey/Sey^{Neu}↔+/+* chimeras examined, and no simple correlation was observed between size and %GPI1B. In contrast, the %GPI1B correlated with the degree of tissue abnormality in the optic cup. This we attribute, at least in part, to abnormally small or absent lenses. Lens tissue is important as a regulator of retinal growth and in the maintenance of retinal development (Saha et al. 1992). In either the absence of the lens resulting from lens ablation (Breitman et al. 1987; Kaur et al. 1989; Harrington et al. 1991) or the presence of an abnormally small lens as in the mutant aphakia (Zwaan and Webster 1985; R.E. Hill, unpubl.) convoluted overgrowth of retinal tissue occurs. However, neither of these situations show the degree of abnormality demonstrated in the *Sey/Sey^{Neu}↔+/+* chimeric eyes that lack a lens. It is not clear whether the *Sey/Sey^{Neu}* cells in the optic cup increase the degree of abnormality in this tissue.

In the *Sey/Sey^{Neu}↔+/+* chimeras the wild-type and *Sey/Sey^{Neu}* cells did not mix freely, resulting in large partitions consisting predominantly, if not exclusively, of either wild-type or mutant cells. Sharp boundaries with no detectable intermingling occurred between the cell types. In the lensless eyes the ocular tissue was highly convoluted and normal inner and outer layers were not identifiable. In these eyes the partitioning of the two cell types suggests a mechanism, operating at an earlier developmental stage, which sorts the cells into large patches of like cells. This failure of cell types to mix illustrates that differences exist between the wild-type and *Sey/Sey^{Neu}* cells consistent with differences in cellular properties that define identity. It is likely that these differences lie at the cell surface.

It has been suggested that homeo domain-containing proteins control the expression of genes encoding extracellular matrix proteins and cell adhesion molecules (Edelman and Jones 1992; Jones et al. 1993; Goomer et al. 1994; Holst et al. 1994). Furthermore, *Pax6* and other members of the *Pax* gene family are known to interact with the promoters of cell surface molecules and, in particular, those that belong to the immunoglobulin superfamily. For example, the evidence is strong that *Pax5* regulates the B-lymphocyte receptor CD19 (Kozmik et al. 1992), and *Pax6* interacts with the neural cell adhesion molecule L1 promoter region in *in vitro* DNA-binding assays (Chalepakidis et al. 1994). Thus, *Pax6* deficiency may influence the expression of adhesion molecules or other cell surface molecules to effect the cell sorting.

In the eyes of *Sey/Sey^{Neu}↔+/+* chimeras, the optic cup appears more normal in the presence of a lens than in those where the lens is absent. In addition, there appears to be a relationship between the size of the lens (and therefore the overall contribution of *Sey/Sey^{Neu}* cells; %GPI1B) and the degree of tissue abnormalities. In the eyes with lenses the mutant cells were excluded more rigorously from the inner layer of the optic cup than from the outer layer. In all but one case (Fig. 3C), no appreciable patches of *Sey/Sey^{Neu}* cells were detected in

the inner layer. Most *Sey/Sey^{Neu}* cells in these optic cups were in the outer layers, either in abnormally thickened or ectopic tissue. In no cases examined were these cells found in the single cell layer of RPE. In the embryos with low %GPIIB and larger lenses, the degree of tissue abnormalities was reduced, coincident with more extreme reduction of mutant cells in the optic cup. We suggest that the presence of a lens provides an environment in which the developmental conditions for the optic cup are more stringent. Therefore, the optic cup develops more normally and the mutant cells make only a very low overall contribution. The evidence that *Sey/Sey^{Neu}* cells were excluded from the RPE and apparently at a selective disadvantage in other regions of the optic cup, as well as being distributed nonrandomly, implies that *Pax6* has a direct, cell-autonomous effect on the optic cup as well as the lens and nasal epithelium.

These chimeras provide evidence that *Pax6* has a role in all the primary tissues of the eye, including the inner and outer layers of the optic cup. *Pax6* exerts an effect prior to E12.5 at a time when overt differentiation is still undetectable in the inner layer. Because the fates of cells in the inner and outer layers differ and the optic cup is of different embryological origin than the lens and nasal epithelium, we suggest that *Pax6* has multiple roles in eye and nasal development.

Evolution of the role of *Pax6*

Pax6 is highly conserved in evolution, and homologs have been found in *Drosophila* and *Caenorhabditis elegans*. Quite surprisingly, *Drosophila Pax6* is also involved in eye development being responsible for the *eyeless* phenotype (Quiring et al. 1994; Halder et al. 1995). The similarity of the phenotype led to the suggestion that *Pax6* may have a homologous role in the development of these highly divergent eye structures separated by extreme evolutionary distances. In *C. elegans Pax6* has a role in patterning the head region (Chisholm and Horvitz 1995; Zhang and Emmons 1995). Thus, in vertebrates the roles of *Pax6* in head ectoderm and in the eye may have descended from very ancient conserved functions.

A problem with generalization of conserved function based on phenotype alone is the possible fortuity of the observations (Hill and Davidson 1994). *Pax6* is expressed in multiple domains of the head and brain in both mouse and *Drosophila*. Thus, a role for *Pax6* may incidentally overlap in the eye. In addition, eye structure and development differ dramatically between the two species. Thus, similar phenotypic characteristics observed in *Pax6* mutants in the two species may well be attributable to unrelated developmental defects. However, analysis of the *Sey/Sey^{Neu} ↔ +/+* chimeric mice provides evidence that *Pax6* has an early role in neural retina development, prior to the appearance of differentiating retinal cells. In comparison, the *Drosophila eyeless* mutant shows that *Pax6* also functions in retinal development and development is defective in the mutant at a stage prior to differentiation (Quiring et al. 1994). This

provides further evidence that the *Pax6* homologs have equivalent functions in development, strengthening the argument for conserved developmental roles.

Materials and methods

Mice

Inbred BALB/c/Eumm mice were purchased from the Department of Medical Microbiology, University of Edinburgh. The founder stock of *Sey^{Neu}/+* mice was kindly supplied by Dr. Jack Favor, Institut für Genetik, Neuherberg, Germany. *Sey^{Neu}* arose on a (C3H×102)_{F1} genetic background and was crossed to a CBA/Ca genetic background. The *Sey* allele (Roberts 1967) was maintained on a partially congenic CBA/Ca-*Sey*/+ stock. Other stocks were produced and maintained in the Centre for Reproductive Biology (Edinburgh). Transgenic strain 83 (Lo 1986) carries the reiterated β-globin, TgN(Hbb-b1)83C10 transgene (abbreviated to Tg) and was used to derive the random-bred, homozygous *Tg/Tg* stock TGB on a largely (C57BL/Ola×CBA/Ca) genetic background (Keighren and West 1994; West et al. 1995). A *Sey*/+ stock (SEYTG), which was homozygous for the transgene (*Tg/Tg*), was produced by intercrossing CBA/Ca-*Sey*/+ and TGB and backcrossing *Sey*/+ individuals to TGB; *Sey*/+, *Tg/Tg* mice were used in the experiment after two to four backcross generations. The albino, *Gpi1^c/Gpi1^c*, CF₁ hybrid stock was produced from crosses between C57BL-*Gpi1^c*, c/Ws females and BALB/c-*Gpi1^c*/Ws males (West and Flockhart 1994).

Production and dissection of chimeras

Eight-cell embryos were obtained from two crosses. CBA/Ca-*Sey^{Neu}/+* females were mated to SEYTG (*Sey*/+, *Tg/Tg*) males to produce a mixture of *Sey^{Neu}/Sey*, *Sey^{Neu}/+*, *+/Sey*, and *+/+* embryos, all of which were *Gpi1^b,C/Gpi1^b, C- /Tg*. Inbred BALB/c mice were intercrossed to produce wild-type, albino embryos (*Gpi1^a,c/Gpi1^a,c*) without the transgene. Chimeras were made by aggregating embryos from the two crosses and the four groups of chimeras (*Sey^{Neu}/Sey ↔ +/+*, *Sey^{Neu}/+ ↔ +/+*, *Sey/+ ↔ +/+*, and *+/+ ↔ +/+*) were distinguished retrospectively by PCR analysis as described below.

BALB/c and CBA/Ca-*Sey^{Neu}/+* females were induced to ovulate by intraperitoneal (i.p.) injection of 5 IU of pregnant mares' serum gonadotrophin (PMSG) at ~12 noon, followed 48 hr later by i.p. injection of 5 IU of human chorionic gonadotrophin (hCG). Females were caged overnight with males of the appropriate stock, and mating was confirmed the following morning by the presence of a vaginal copulation plug; the day of the vaginal plug was designated 0.5 day postcoitum (d.p.c.). On the same day as vaginal plugs were identified, CF₁ females in estrus were paired overnight with vasectomized CF₁ males and mating was verified the following morning by the presence of a vaginal plug. These provided homozygous, *Gpi1^c/Gpi1^c* pseudopregnant females for embryo recipients.

Embryos were flushed from the CBA/Ca-*Sey^{Neu}/+* and BALB/c females at 2.5 d.p.c. with HEPES-buffered M2 handling medium (Quinn et al. 1982). Aggregations were carried out according to methods used by West and Flockhart (1994). After overnight culture, the aggregated embryos were surgically transferred to the uteri of pseudopregnant CF₁ females. Pregnancies were timed according to the pseudopregnant female, with the plug day being 0.5 d.p.c.; the recipient female was sacrificed at 12.5 d.p.c. (E12.5). This age was chosen because normal RPE pigmentation is well established and provides a marker for the chimera analysis (see below).

Fetuses were dissected into cold PBS. The weight of the total conceptus, fetus, and placenta were recorded along with the crown-rump length and morphological index based on hind-limb development (McLaren and Buehr 1990; Palmer and Burgoyne 1991). Different samples of the conceptus were analyzed by GPI electrophoresis, PCR, and in situ hybridization to the reiterated transgene (Fig. 1). Tissue samples dissected for GPI electrophoresis into H₂O/glycerol 50:50 from the tail, forelimb, amnion, whole yolk sac, and placenta. Methods used for GPI electrophoresis as described in West and Flockhart (1994). The trunk was digested for 24 hr at 55°C in 0.5 ml of proteinase K buffer [5 mM Tris, 25 mM KCl, 25 mM MgCl₂ at pH 7.8, 0.45% NP-40, 0.45% Tween, 0.1 mg/ml of gelatin] plus 300 µg of proteinase K. The head of each fetus was fixed in ethanol/acetic acid [3:1 (vol/vol)] and then processed to wax for analysis by DNA-DNA in situ hybridization.

Analysis of genomic DNA

The four groups of chimeras (*Sey^{Neu}/Sey* ↔ +/+, *Sey^{Neu}/+* ↔ +/+, *Sey/+* ↔ +/+, and *+/+* ↔ +/+) were distinguished by PCR analysis of *Pax6* genes in samples of fetal trunk DNA. After digestion in proteinase K buffer (see above), the resulting suspension was denatured at 95°C for 5 min. One microliter was then added to a PCR mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.1% Triton X-100, 0.1 mM of each dNTP, and 1.5 units of *Taq* DNA polymerase (Boehringer Mannheim) per 50-µl volume. Primers *Hax-5* (ATGGAACCTGATGTGAAGGAGG) and *G15* (GCAACACTCTAGTCACATTCC) or *MC130* (CTTTCTCCAGGCGCTCAATCTG) and *G459* (GCAACAGGAAGGAGGGGAGAG) were used to amplify 220- and 148-bp fragments, respectively, from within the *Pax6* gene for identification of the *Sey^{Neu}* and *Sey* mutations. For the analysis of the *Sey^{Neu}* mutation, 20 µl of PCR product was digested with 9 units of *Hind*II, and for the *Sey* mutation 30 µl of PCR product was digested with 30 units of *Dde*I (as per manufacturer's instructions; Boehringer Mannheim). Samples were loaded onto a 15% polyacrylamide gel (19:1, acrylamide/bis-acrylamide) and subjected to electrophoresis in TBE buffer at 280 V for 2 hr. Restriction fragments were fixed in the gel by washing twice in 10% ethanol, 0.5% acetic acid, before incubation for 10 min in 6 mM AgNO₃. Gels were washed twice in distilled water and then stained for 20 min in 375 mM NaOH, 3 mM NaBH₄ and 0.16% formaldehyde to visualize fragments (C.M. Abbott, unpub.). After transfer to distilled water, the gels were photographed.

To verify the genotype of chimeras where mutant sequences were present in low proportion, a second "nested" PCR reaction was carried out to reduce possible low-level nonspecific sequence amplification. The PCR mixture and enzyme concentrations were as before. First round primers for *Sey* fragments were *B509/3* (TGCCAGCAACAGGAAGGAGG) and *F769* (GGGCAAAGACATCTGGATAATG), and for *Sey^{Neu}* were *Hax-5* (see above) and *F772* (CATTATCCAGATGTGTTTCCC). These were subjected to 15 cycles at 95°C for 0.5 min, 56°C for 0.75 min, and 72°C for 1.0 min. One microliter of the resultant PCR mixture was transferred to a new tube containing either second-round *Sey* primers *MC130* and *G459* or *Sey^{Neu}* primers *Hax-5* and *G15*. This mixture was then subjected to a further 30 cycles at 95°C, for 0.5 min, 55°C for 0.75 min, and 72°C for 1.0 min. Nested PCR products were of identical size to original PCR assay, with *Sey* being 148 bp and *Sey^{Neu}* being 220 bp. Fragments were digested and subjected to polyacrylamide gel electrophoresis and silver staining as described above. Original genotype results for chimeras where the proportion of mutant sequence was very low (e.g., chimera JC63 where ~5% of

genomic DNA contained the *Sey* or *Sey^{Neu}* sequence) were duplicated using the nested PCR procedure and thus specificity and sensitivity of the original genotyping procedure confirmed.

DNA-DNA In Situ Hybridization

Sections were cut at 7 µm and mounted on slides coated with 3-aminopropyltriethoxysilane (Sigma) and analyzed by in situ hybridization to the transgene as described by Keighren and West (1993). Hybridized digoxigenin-labeled DNA probe was detected by diaminobenzidine (DAB) staining for peroxidase-labeled antibody (nonradioactive DNA labeling and detection kit; Boehringer Mannheim). Slides were counter stained with hematoxylin and eosin and examined by bright-field and phase-contrast light microscopy.

Analysis of E12.5 chimeras

The %GPIIB contribution was estimated by scanning densitometry of GPI electrophoresis plates (West and Flockhart 1994). The average %GPIIB in the fetal tail and forelimb was used as an estimate of the overall contribution of (*Sey^{Neu}/+* × *Sey/+*) cells to the fetus.

After DNA in situ hybridization, the percentage of transgene-positive cells in each of the selected test tissues was estimated (James et al. 1995) in each of the fetuses examined by phase-contrast light microscopy. The contribution of transgenic (*Tg/-*) cells was estimated for each tissue in three widely spaced sections. For tissues in the eye, the midsection and the two sections that were halfway from the mid- to the first or last section were selected for examination. An eyepiece grid and a standardized sampling protocol was used for each separate tissue, and ~150–1000 cells were classified, depending on the tissue. For some tissues, for example, where cells were densely packed, individual cells were not classified but the total number of cells and the total number of hybridization signals were counted in the same field of view. For morphologically normal RPE, (*Sey^{Neu}/+* × *Sey/+*) cells were identified as pigmented and/or positive for the hybridization signal.

The primary estimates of percent hybridization-positive cells were corrected to allow for the failure to detect a hybridization signal in a proportion of the *Tg/-* (*Sey^{Neu}/+* × *Sey/+*) cells. The tissue-specific correction factors were derived from the following estimates of percent hybridization for tissues from two hemizygous transgenic (*Tg/-*) E12.5 fetuses: nasal epithelium, 105.5%; lens, 83.3%; optic cup, 84.5%; overlying ectoderm (overlying the eye), 64.5%; head ectoderm, 56.8%; hindbrain, 89.1%; head mesoderm, 88.5%. Most of these estimates are below 100%, because in histological sections, a proportion of nuclei fail to include a hybridization signal. In densely packed tissues, the number of hybridization signals may be greater than the number of identifiable nuclei in the field of view, because of overlapping nuclei (e.g., nasal epithelium estimate of 105.5%). The final corrected hybridization index may also exceed 100% if the contribution of *Tg/-* cells in the chimera is close to 100% and the correction factor overcompensates.

Estimation of lens and nasal epithelium size

The area of the lens or nasal epithelium in one histological section (where the area was maximum) was used as a measure of tissue size. Areas were estimated (Curtis 1960) using a modified Chalkley grid (Graticules Ltd., Tonbridge Kent, UK).

Statistical analysis

Statistical tests were performed on an Apple Macintosh computer using the statistical package StatView 4.1 (Abacus Concepts Inc., Berkeley, CA).

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