Development 121, 1433-1442 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

The role of *Pax-6* in eye and nasal development

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SUMMARY

***Small eye* (*Sey*) mice homozygous for mutations in the *Pax-6* gene have no lenses and no nasal cavities. We have examined the ontogeny of eye and nasal defects in *Sey/Sey* embryos and have related the defects seen to the pattern of *Pax-6* mRNA expression in the mouse during normal eye and nasal development.**

There are two principal components of the early eye, the neural ectoderm of the optic vesicle, which forms the retina, and the overlying surface ectoderm, which forms the lens and cornea. By studying these interacting tissues in normal and *Sey/Sey* embryos, we have identified processes for which Pax-6 is important and can thus suggest possible roles for the *Pax-6* gene.

Pax-6 is essential for the formation of lens placodes from surface ectoderm. In normal development, early *Pax-6* mRNA expression in a broad domain of surface ectoderm is downregulated, but expression is specifically maintained in the developing lens placode. Moreover, other *Pax-6*-expressing tissues are frequently those that have can trans-differentiate into lens. Thus, phenotype and expression together suggest a role for Pax-6 in lens determination.

At least some functions of Pax-6 can be separated from the influence of other tissues. Early *Sey/Sey* optic vesicles are abnormally broad and fail to constrict proximally. These defects occur prior to the time of lens placode formation and probably reflect a requirement for Pax-6 in neural ectoderm. In surface ectoderm domains, where *Pax-6* expression is known to be independent of the presence of an optic vesicle, Pax-6 function is required for the maintenance of its own transcription.

The mutual dependency of lens and optic vesicle development can also be studied using the *Small eye* mutation. Using region-specific markers we find that, in the morphologically abnormal *Sey/Sey* optic vesicles, aspects of normal proximo-distal specification nevertheless persist, despite the complete absence of lens.

Like the lens, the nasal cavities develop from ectodermal placodes that normally express *Pax-6* mRNA, fail to form in *Sey/Sey* mice and show Pax-6-dependent *Pax-6* mRNA regulation. Analysis of patterns of programmed cell death and absence of nasal region expression from an *Msx-1* transgene in *Sey/Sey* embryos suggest a requirement for Pax-6 in the transition from presumptive nasal ectoderm to placode, and that *Msx-1*, or genes regulating it, are possible targets for Pax-6.

Key words: *Pax-6*, *Small eye*, craniofacial development, optic vesicle, nasal placode, lens placode, mouse

INTRODUCTION

Pax-6, independently isolated by homology to *gooseberry distal* (Walther and Gruss, 1991), and from positional cloning at the aniridia locus (Ton et al., 1991), encodes two DNA-binding motifs, a paired domain (Bopp et al., 1986; Treisman et al., 1991) and a *paired*-like homeodomain (Frigerio et al., 1986).

Members of the Pax gene family (Walther et al., 1991), have regulatory roles during development (Chalepakis et al., 1991; Epstein et al., 1991; Baldwin et al., 1992; Tassabehji et al., 1992). A variety of anterior segment congenital eye abnormalities result from heterozygosity for mutations in the mouse, rat or human *Pax-6/PAX6* gene. These include mouse and rat *Small eye* (Hill et al., 1991; Matsuo et al., 1993), human Peters' anomaly (Hanson et al., 1994) and aniridia, congenital lack of iris (Jordan et al., 1992; Glaser et al., 1992; Hanson et al., 1993). These phenotypes can result from deletions encompassing the entire *Pax-6/PAX6* gene, suggesting that the mutations result in loss of protein function and that the phenotype is a result of haplo-insufficiency (Hill et al., 1991).

Small eye is a semidominant mutation. Homozygous mutant mice have no eyes and no nasal cavities. Since, like the lens, nasal cavities form from the invagination of an ectodermal placode, these phenotypes have been suggested by Hogan et al. (1986) to result from a failure of early placode differentiation. *Sey/Sey* embryos also have no olfactory bulbs (Hogan et al., 1986) and have defects in neuronal differentiation and migration that lead to abnormal cortical plate formation (Schmahl et al., 1993). *Pax-6* is thus necessary for the normal development of eyes, nose and brain. The range of abnormalities seen in *Sey* mice may indicate that *Pax-6* has a number of roles during normal development. We have concentrated our attention on the eye and nasal phenotypes.

MATERIALS AND METHODS

Embryonic material

We used non-pigmented Swiss mice as parents for all in situ hybridi-

sation experiments. Embryonic development is assumed to have begun at midnight of the night of mating. The allele of *Small eye* used is *Sey* (Lyon and Searle, 1989). Homozygous *Small eye* mice die at birth (Hogan et al., 1986), thus the *Sey/Sey* embryos used were obtained from crosses of heterozygous *Small eye* parents, which were selected on the basis of eye size. Littermates from these matings (*Sey*/+ and +/+ embryos) provided control embryos of matched developmental stage.

In those cases where we illustrate comparisons between *Sey/Sey* embryos and their littermates, we have also compared the littermates with wild-type embryos derived from wild-type parents. This was done using our own results and by reference to published data on patterns in normal mouse development for the markers that we have used (Steel et al., 1992; Walther and Gruss, 1991; Dong and Chung, 1991; Sulik et al., 1988).

Probes for in situ hybridisation

A gene-specific 1 kb template for making mouse *Pax-6* RNA probe was made by polymerase chain reaction, PCR, from a 1.6 kb cDNA clone, pm1 (Ton et al., 1991), using oligonucleotide C128 (ATGGTTTTCTAATCGAAGGG) from the 3′ end of the *Pax-6* homeobox, and a T7 promoter oligonucleotide (AATACGACTCACTATAG) matching vector sequence. A similar procedure was used to make an *entactin* probe: oligonucleotides D932 (CAGTGTCACCACAGCACTGGC) and T3 promoter oligonucleotide (ATTAACCCTCACTAAAG) were used to amplify a 1.6 kb region at the 3′ end of clone p633, a gift of Professor Albert E. Chung. PCR product was extracted by phenol-chloroform followed by ethanol precipitation before being transcribed. The template for the *Tyrp2* probe, a gift of Ian Jackson, was the 1.2 kb clone p5A7 described in Steel et al. (1992), digested with *Hin*dIII.

In situ hybridisation

Embryos were removed into ice-cold phosphate-buffered saline (PBS) and then fixed in 4% paraformaldehyde (PFA) in PBS at 4°C and processed routinely for histology. In situ hybridisation using ^{35}S-labelled riboprobe was performed as described in Monaghan et al. (1991). Control probes *Msx-1* and *Msx-2*, which detect transcripts of genes with known expression patterns, were used to identify non-specific signal. Where expression patterns in *Sey/Sey* and normal were compared, sections were hybridised in the same experiment, generally on the same slide. Autoradiographs using K5 Emulsion (Ilford), were exposed for 2.5 to 6 weeks and sections were stained with methyl green. Computer-aided overlaying of dark-field and bright-field images was performed as previously described (Monaghan et al., 1991).

PCR genotyping of young embryos

The *Sey* mutation (Hill et al., 1991) creates a diagnostic *Dde*I site that allows embryonic material to be genotyped by PCR amplification and *Dde*I digestion. For genotyping, paraformaldehyde-fixed embryos were bisected using tungsten needles in PBS at the level of the midgut (8 day) or heart (8.5 day and later embryos). Anterior portions were aligned a few millimetres apart and embedded in a 3% low melting point agarose gel made with PBS. The agarose block was cut in such a way that each embryo could later be unambiguously identified and then fixed in 4% PFA, processed routinely for histology and embedded in wax. In this way, similar sections of embryos of different genotypes appear on the same slide and are hybridised equivalently. Posterior embryo parts were individually washed twice in PBS then digested in 20 μl with 6 μg/ml proteinase K for 1 hour at 55°C in 1× PCR buffer, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin and 0.1% Triton X-100, with added non-ionic detergents, 0.4% Tween 20 and 0.4% NP40. Proteinase K was inactivated at 95°C for 10 minutes. 1-2 μl of digested sample was taken for each PCR reaction. PCR reactions were performed in total volumes of 25 μl using 1 pmole/reaction of each primer B509 (TGCCAGCAACAGGAAGGAGG) and E723 (CTTTCTCCAGAGCCTCAATCTG) with 0.2 mM of each dNTP in the 1× PCR buffer described above. Taq polymerase (1 unit/ reaction) was added after an initial denaturing step of 4 minutes at 95°C. 35 cycles (95°C for 30 seconds, 61°C for 30 seconds and 72°C for 48 seconds) were performed followed by a single extension step of 72°C for 5 minutes. Following phenol-chloroform extraction and ethanol precipitation, samples of PCR products were digested with *Dde*I. The 154 bp PCR product was cut to give 83 bp and 71 bp fragments for wild-type alleles and 83 bp, 51 bp and 20 bp fragments for *Sey* alleles. These digestion products were analysed on 4% NuSieve GTG agarose gels (FMC) in 1× TBE buffer.

Detection of cell death

We detected cell death using Nile Blue Sulphate staining (Sulik et al., 1988). Freshly dissected embryos were stained in 1:20,000 Nile Blue Sulphate in either PBS or PBT (PBS with 0.01% Tween 20) for 15 minutes and photographed immediately using 64T film on Wild M400 photomicroscope. The specificity of this procedure was confirmed by comparing the pattern of dark-staining cells with that generated using acridine orange DNA stain (Graham at al., 1993).

Msx-1 transgenic mice

Production of the transgenic mice carrying the reporter construct will be described elsewhere. Briefly the reporter construct contains 5 kb of sequence upstream of the mouse *Msx-1* gene and generates a fusion transcript with *lacZ* in frame with the *Msx-1* gene from an *Nco*I site 127 bp 3′ of the start of the *Msx-1* coding region. The construct uses SV40 poly(A) addition and transcription termination sequences. Transgenic mice were produced on a (CBA × C57BL/6)F_1 hybrid background. From matings of Swiss *Sey* mice to transgenics, *Sey* males carrying the transgene were identified by Southern hybridisation and PCR analysis. These were mated to *Sey* females. Embryos from these matings were obtained at E9.0 to E11.5 days and fixed in 2% formaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3), 2 mM $MgCl_2$, 5 mM EGTA, then stained for β-galactosidase activity (Beddington and Lawson, 1990). Fixation times were 10-30 minutes depending upon embryo size. Staining was for 5 hours in the dark at 37°C.

RESULTS

Optic vesicle development

Homozygous mutant *Sey/Sey* embryos can first readily be distinguished from littermates at about 9.5 days by the abnormal shape of the optic vesicle and forebrain (Fig. 1A,B). We have confirmed this morphological characterisation by PCR analysis of the genotype (see Materials and methods). The optic vesicle is constricted proximally in the wild-type embryo but not in the *Sey/Sey* embryo. This defect persists for some time: E10.5-E11.5 *Sey/Sey* embryos typically have a uniformly broad optic vesicle, distorted towards the distal end (Fig. 1D) giving the appearance of an abortive process of optic cup formation (Fig. 4A-C,E,G). In normal eye development, the optic vesicle interacts with the lens pit, growing around it to form a bilayered optic cup. The thin outer layer and thicker inner layer of the optic cup go on to form pigmented retinal epithelium and neural retina, respectively. Optic vesicles in E11.5-E15.5 S*ey/Sey* embryos display a bilayered neuroepithelial structure. The *Sey/Sey* optic stalk retains a lumen, unlike the normal optic nerve, which is a dense bundle of axons by E15.5.

Transmission EM analysis of *Sey/Sey* optic vesicles (data not shown) reveals that they make local contact with overlying

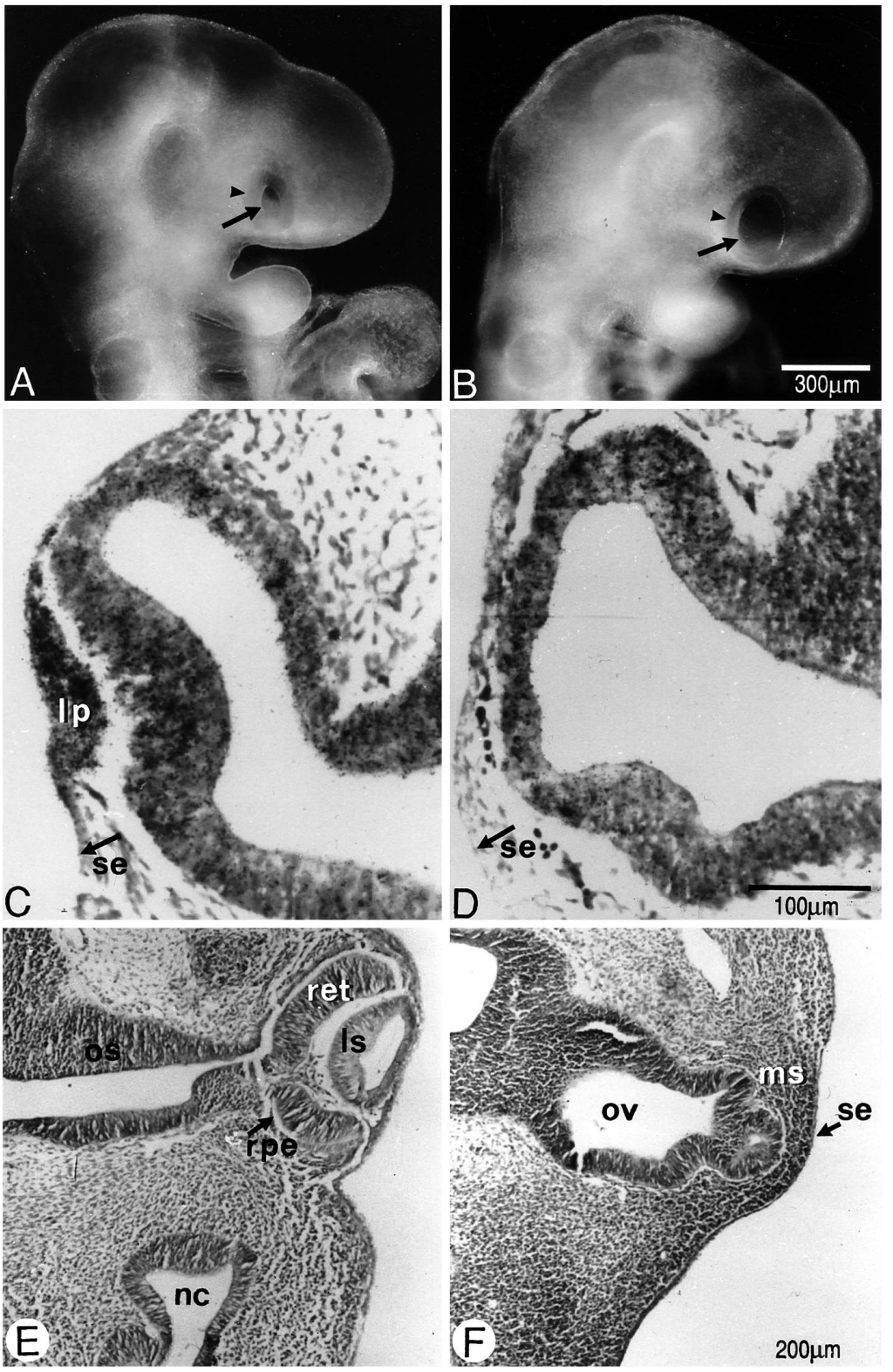

Fig. 1. Early phenotype of *Sey/Sey* embryos. Characteristic appearance of the eye at E9.5 of (A) wild-type embryo and (B) *Sey/Sey* embryo. *Sey/Sey* optic vesicle is broader than normal and has failed to constrict proximally. Arrows, proximal restriction of optic vesicles. Arrowheads, extent of the eye region. Histology of the eye at (C,D) E9.75 and (E,F) E11.5. At E9.75, the lens placode (lp), a prominent thickening of the surface ectoderm (se) is present in the littermate (C), but absent from the *Sey/Sey* embryo (D). Later *Sey/Sey* embryo, at E11.5 in F lacks the developing lens (ls) and nasal cavity (nc), present in littermate (E). Normal littermate optic vesicle has produced an optic stalk (os) and optic cup with distinct retina (ret), and pigmented retinal epithelium (rpe). *Sey/Sey* optic vesicle (ov), is broader than normal, is distorted at the distal end and is separated from surface ectoderm (se) by intervening mesenchymal-like cells (ms).

surface ectoderm at E9.5, but that this contact is progressively lost (shown at E15.5 in Fig. 4A) as mesenchymal cells intervene between the two tissues.

Absence of lens placodes in *Sey/Sey* embryos

Histological analysis of *Sey/Sey* embryos at E9.5 to E11.5 showed an absence of the thickened surface ectodermal component of the eye, the lens placode, lens pit and subsequent lens vesicle, which are present in their normal and *Sey*/+ littermates. At E9.75 when lens placodes are normally well developed (Fig. 1C), *Sey/Sey* embryos have no thickening of the surface ectoderm in the eye region (Fig. 1D). As a result,

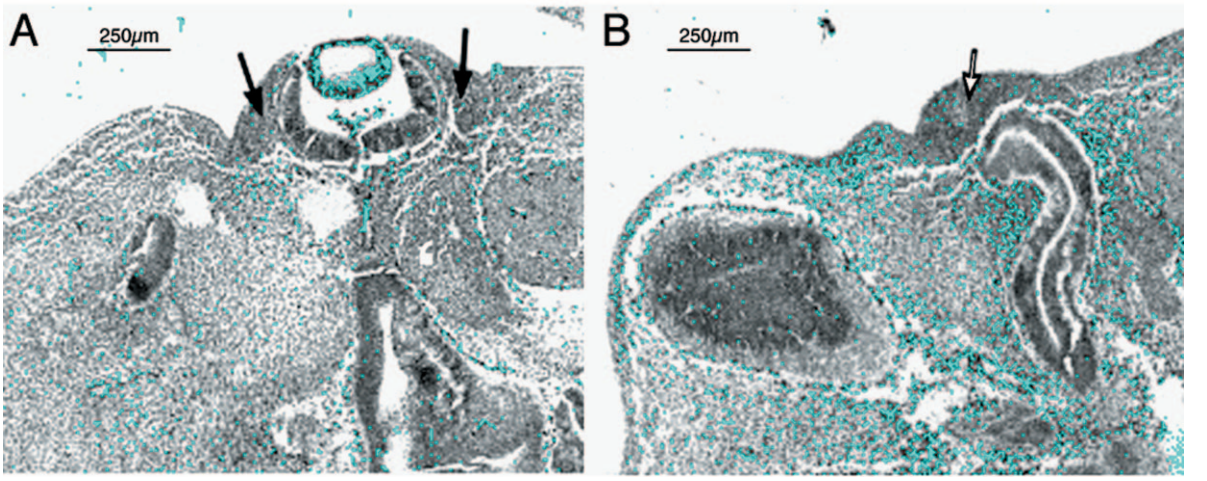

Fig. 2. Radioactive in situ hybridisation detecting expression of *entactin* mRNA, blue, in transverse sections at E12 of (B) *Sey/Sey* embryo and (A) normal littermate. Developing lens expresses high levels of *entactin* mRNA from E10 onwards, whereas condensing mesenchymal cells (arrows) around the optic cup in A have low levels of expression. A region of dense tissue (open arrow) present at the surface adjacent to the distal optic vesicle in B is typical for *Sey/Sey* embryos and has the appearance and expression characteristics of condensed mesenchyme rather than of developing lens. Anterior of sections is to the left.

there is no developing lens detectable in *Sey/Sey* embryos at E11.5 (Fig. 1F).

A region of dense tissue near the surface adjacent to the distal optic vesicle is typically present in homozygous *Small eye* mutants from E10 onwards. This is not abortive or delayed lens development, but is probably a condensation of mesenchymal cells. Developing lens (Fig. 2A) normally strongly expresses mRNA for the extracellular matrix protein gene *entactin* from the lens pit stage onwards (Dong and Chung, 1991). In contrast, the regions of dense tissue in *Sey/Sey* embryos express *entactin* mRNA at low levels (Fig. 2B) more comparable to the level of expression in mesenchymal cells that condense adjacent to the anterior optic cup in normal embryos. By high magnification microscopy, we confirmed that the regions of dense tissue seen at the surface in *Sey/Sey* mutants were condensations of mesenchymal-like cells under a non-placodal epithelial layer (data not shown).

For stages E12.5 onwards, when lens fiber differentiation could be detected in

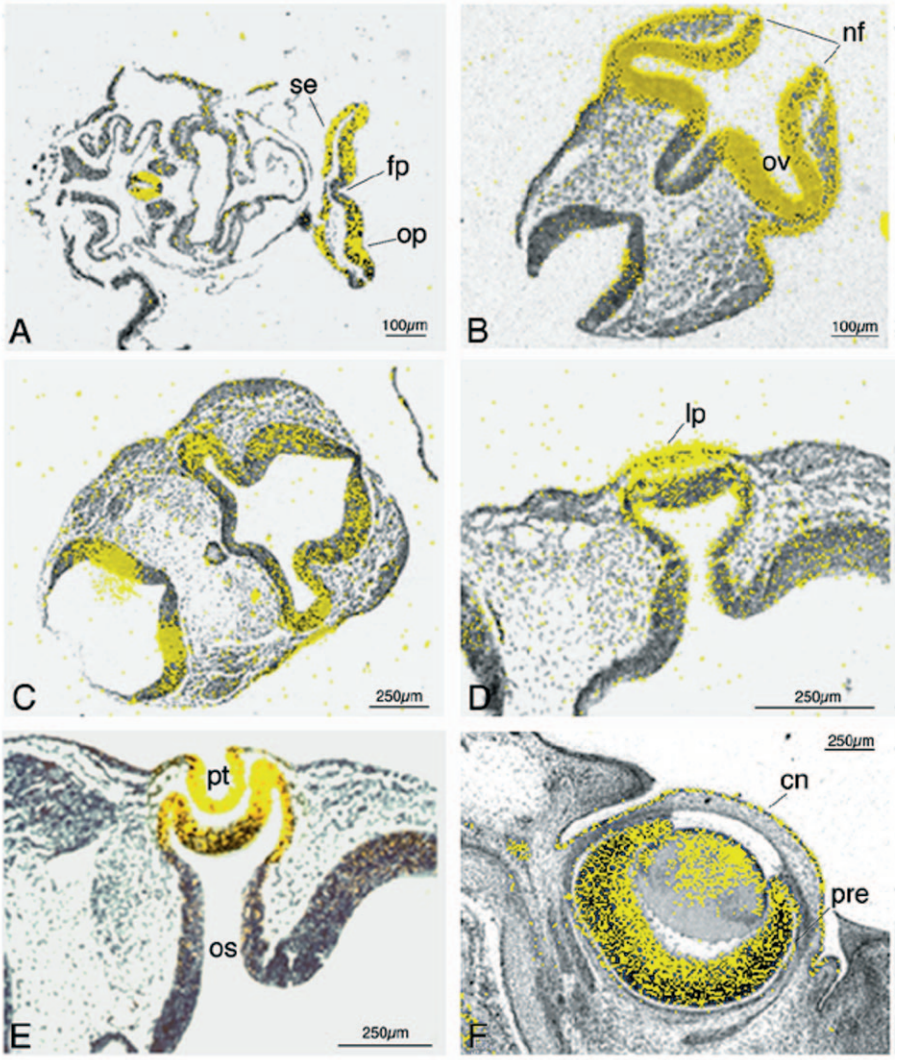

Fig. 3. Normal expression of *Pax-6* mRNA, yellow, during early eye development. Radioactive in situ hybridisation on transverse sections at embryonic ages (A) E8.0; (B) E8.5; (C) E9.25; (D) E9.5; (E) E10.5 and (F) E15.5. Surface ectoderm (se), optic pit (op), floorplate (fp), neural folds (nf),optic vesicle (ov), lens placode (lp), lens pit (pt), optic stalk (os), pigmented retinal epithelium (pre), cornea (cn).

controls, we found no evidence of lenses, lentoid bodies or of cells with the eosin-staining properties of lens fibers in any homozygous mutant examined.

Pax-6 expression in the neural ectoderm

We have examined *Pax-6* transcription in wild-type mice from 8 days to 15.5 days. At the earliest stage examined, E8.0, Pax-6 transcripts were detected over an extensive region of head neural ectoderm, including the optic pit, the first morphologically detectable indication of the eye region (Fig. 3A). There was no expression in the floorplate. At E8.5, shortly before headfold closure, much of the complexity of the later forebrain *Pax-6* expression pattern has been established. In the presumptive diencephalon, expression is mainly dorsal, reaching the neural folds, and is already segmented as has been reported for later stages (Walther and Gruss, 1991; Figdor and Stern, 1993; Puelles and Rubenstein, 1993). Transverse sections through the head at this stage typically show expression to be strong in the optic vesicle, weaker more rostrally in the presumptive telencephalon, with the neural ectoderm expression being undetectable or only weakly detectable at the neural folds (Fig. 3B).

Pax-6 expression within the optic vesicle is polarised distally. Thus, from E9.5 onwards expression in the optic vesicle is strongest around the rim of the developing optic cup (Fig. 3D) and is consistently weaker both in the back of the optic cup and in the proximal optic vesicle structures such as the developing optic stalk (Fig. 3E). In the early optic cup, *Pax-6* is expressed in both the prospective pigmented retinal epithelium (PRE) layer and prospective neural retina (Fig. 3E). This pattern is dynamic. Thus, by E15.5 expression within the PRE is only seen in anterior regions, i.e. near the rim of the optic cup. (Fig. 3F).

Restriction of *Pax-6* mRNA expression in surface ectoderm of wild-type mice

At E8.0, *Pax-6* mRNA is expressed in a broad region of head surface ectoderm covering the prosencephalon but not the hindbrain region or the presumptive first branchial arch (Fig. 3A). This expression in the surface ectoderm is still broad at E8.5 and extends rostrally to the neural folds. This is in contrast to the neural ectoderm expression at this stage, where strong expression is localised to the optic vesicle and presumptive diencephalon (Fig. 3B). Over the next 24 hours, expression in the surface ectoderm becomes restricted to the developing lens placode (Fig. 3D) nasal placode and immediately adjacent tissues. *Pax-6* mRNA expression in the lens placode forms part of a larger domain that extends in the dorsal-caudal direction (Fig. 3C). As lens pit formation proceeds, this expression domain is further restricted until it lies exclusively within the developing eye region (Fig. 3E). Similarly the expression in the ectoderm between the lens and nasal placodes persists longer than most non-placodal expression but, by E9.75, the lens and nasal placodes are clearly separated by a region of non-expressing ectoderm. Consistent with a previous report (Walther and Gruss, 1991), we find *Pax-6* mRNA expression continues in the parts of the eye derived from surface ectoderm until the last stage examined, E15.5 (Fig. 3F). This includes the lens pit, the lens vesicle and the lens as well as the developing cornea.

Pax-6 and *Tyrp2* expression domains in optic vesicles of *Sey/Sey* embryos

To characterise the structures formed by the optic vesicle in

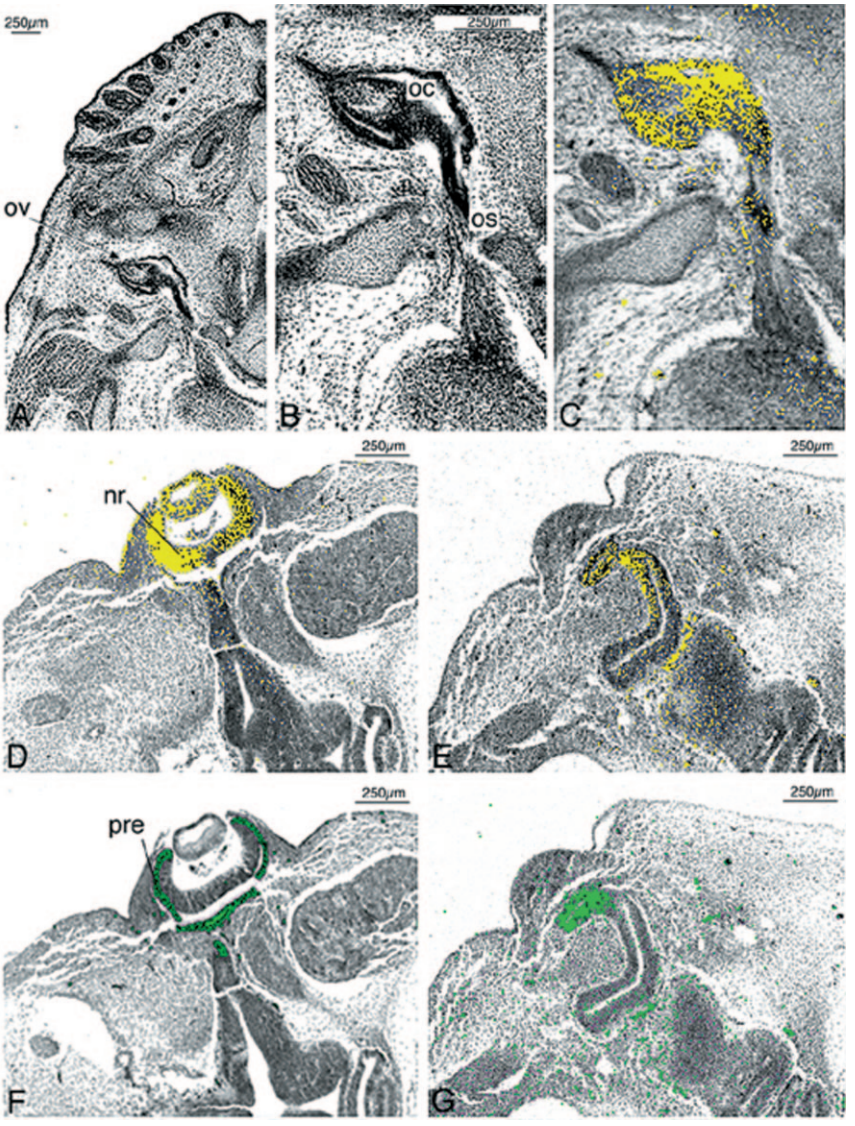

Fig. 4. (A-C) Optic vesicle phenotype of *Sey/Sey* embryos at E15.5. (A) Transverse sections through optic vesicle (ov), showing separation from skin by intervening tissue. (B) Detail of bilayered optic cup structure (oc) and optic stalk (os). (C) *Pax-6* mRNA expression, predominantly in cup-like structure. (D-G) Expression domains of *Pax-6* mRNA (D,E) and *Tyrp2* mRNA (F,G) in transverse sections of control (D,F) and *Sey/Sey* (E,G) eyes at E12.5. Developing pigmented retinal epithelium (pre); developing neural retina (nr). Anterior is to the left.

the absence both of functional Pax-6 protein and of lens, we combined our histological analysis with in situ hybridisation using probes for gene transcripts that normally display region-specific expression patterns within the optic vesicle. The single base change in the *Sey* allele, whilst predicted to result in a truncated Pax-6 protein (Hill et al., 1991), will not affect the in situ hybridisation assay for *Pax-6* mRNA expression, thus Pax-6 can also be used as a marker in this way. We have also used the tyrosinase-related protein gene *Tyrp2* as an early optic vesicle marker that becomes restricted to RPE, the pigmented retinal epithelium layer of the optic cup, by about E10.5 (Steel et al., 1992). We examined *Sey/Sey* and control embryos hybridised with antisense probes for *Pax-6* (stages E8.0 to E15.5) or *Tyrp2* (stages E11.5-E12.5)

Pax-6 expression in *Sey/Sey* optic vesicles is initially indistinguishable from that in +/+ and *Sey*/+ littermates (Fig. 5B). As optic vesicle abnormalities become apparent in *Sey/Sey* mutants, *Pax-6* expression retains features seen in the expression patterns of littermates. Thus at E9.5 to E10.5, *Pax-6* expression is strongest in most distal structures (Fig. 5C). Later still, *Sey/Sey* optic vesicles form distinct optic stalk-like and bilayered optic-cup-like structures, shown at E15.5 in Fig. 4A,B. As for normal eyes, it is the two neuralectodermal layers of the optic-cup-like structure that express *Pax-6* mRNA (Fig. 4C) and there is little expression in the optic stalk.

Using the *Tyrp2* probe, there is typically a small domain of intense signal in the most distal regions of the *Sey/Sey* optic vesicle (Fig. 4G). Where the optic vesicle forms a cup, *Tyrp2* transcripts are most abundant at the rim of this cup, but are present in both layers. Unlike the clearly differentiated pigmented retinal epithelium and neural retina in the optic cups of normal embryos, the two neuroepithelial layers in those structures in *Sey/Sey* embryos that resemble cups are of similar thickness and appearance (Fig. 4E,G). In these structures, *Tyrp2* is almost exclusively expressed within the larger, but still distally restricted, *Pax-6* expression domains (Fig. 4E). This is in contrast to normal embryos, where there is a considerable region of pigmented retinal epithelium that expresses *Tyrp2 (*Fig. 4F) but not *Pax-6 (*Fig. 4D).

Surface ectoderm Pax-*6* mRNA expression in homozygous mutant mice

PCR genotyping (see Materials and methods) allows identification of *Sey/Sey* embryos prior to the appearance of morphologicaly recognisable defects. As mentioned above, the single base change in the *Sey* allele does not affect the in situ hybridisation assay for *Pax-6* mRNA expression, thus we have been able to analyse *Pax-6* expression in early *Sey/Sey* embryos.

Genotyped embryos were sectioned and hybridised with antisense *Pax-6* probe. *Sey/Sey* embryos showed no differences from the normal pattern of expression at E8.0 or E8.5 (Fig. 5A,B). By E9.5-E9.75, however, when in normal embryos expression was confined to around the lens and nasal placodes, we detected no expression in the surface ectoderm anywhere in the heads of homozygous mutants, including the eye region (Fig. 5C).

Pax-6 expression and nasal placode development

We found *Pax-6* mRNA (Fig. 6A) in nasal placode, though at a lower level than in lens placode. We find that this expression continues in placodal epithelium during the formation of nasal pits (Fig. 6B) and that, subsequently, expression is detectable in the developing olfactory epithelium, as has been previously reported by Walter and Gruss (1991). No nasal placodes could be detected on serial sections through the nasal region of *Sey/Sey* embryos at E9.75, whereas littermates at this stage have well-formed nasal placodes undergoing the first stages of invagination to form a nasal pit. The nasal placode *Pax-6* mRNA detectable in littermates (Fig. 6C) is absent from *Sey/Sey* embryos at this stage (Fig. 6D) although *Sey/Sey* embryos retain expression in the neuroepithelium of the developing forebrain.

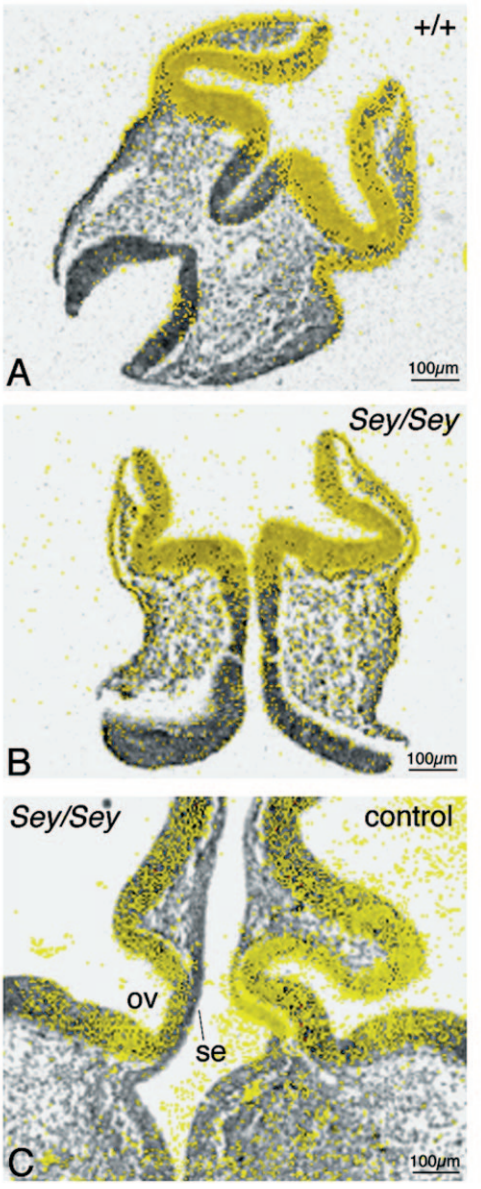

Fig. 5. *Pax-6* mRNA expression, yellow, for (A) wild-type embryo and (B) *Sey/Sey* embryo, at E8.5. *Sey/Sey* embryos at this stage have the same *Pax-6* mRNA expression pattern as their littermates. (C) By E9.75 surface ectoderm (se) expression adjacent to optic vesicle (ov) in littermate (right) is absent from *Sey/Sey* embryo (left).

Msx-1 transgene expression in the nasal placode region

In +/+ and *Sey*/+ mice carrying the the *Msx-1* transgene, lateral placodal epithelial cells express the *lacZ* marker at a high level, whereas cells in non-placodal ectoderm cells, underlying mesenchyme and medial nasal placode do not. This specific expression is detectable at E9.5 and develops into an arc of strong expression in the placodal ectoderm of the prospective lateral nasal process by E10.5 (Fig. 7A,B). X-gal staining of *Sey/Sey* embryos (Fig. 7C) and littermates (Fig. 7B) from E9.0 to E11.5 showed that this early marker for nasal placode differentiation is never expressed in the absence of functional Pax-6 protein.

Cell death in nasal placode region

In normal embryos, a narrow arc of cell death appears around the edge of the developing nasal placode prior to invagination (Fig. 7D). Patterns of cell death, detected by Nile Blue Sulphate (NBS) staining, can be used as markers to study the fate of cells in the nasal region in *Sey/Sey* embryos. In contrast to the restricted arcs of NBS-staining cells in normal embryos, we found diffuse patches of NBS-stained cells in the nasal region of E9.25-E9.75 *Sey/Sey* embryos (Fig. 7E). The number of stained cells varied considerably from embryo to embryo, and between left and right sides of the same embryo, but consistently these patches were diffuse. Analysing the cell death patterns at E10.5, we find that the majority of NBS-staining regions are identical in *Sey/Sey* embryos and their littermates, but there are three differences. (1) Cell death normally occurs at the point of fusion of the lateral and medial nasal processes. The position of the processes formed in *Sey/Sey* embryos and the cell death pattern on them identifies these as medial nasal processes, the cell death domains of the lateral nasal processes being absent. This supports the conclusions of previous scanning electron microscopy studies by Heinzmann et al. (1991) that the lateral nasal processes are absent in *Sey/Sey* embryos at this stage. (2) In 10.5 day *Sey/Sey* embryos, we observe bilateral ectopic bands of subectodermal NBS staining over the anterior forebrain. This may identify a population of midbrain neural crest cells that, in homozygous rat *Smalleye* (*rSey*) embryos, fail to reach the nasal region (Matsuo et al., 1993), and may die. (3) The normally narrow
band of NBS staining along the midline of the brain is broadened in *Sey/Sey* embryos into a diamond shape patch over the telencephalon-diencephalon boundary.

DISCUSSION

Early optic vesicle abnormalities in *Sey/Sey* embryos

The optic vesicle and surface ectoderm both express *Pax-6* during normal development and both show abnormalities in *Sey/Sey* mice. In normal eye development, there are multiple interactions between these two tissues. The optic vesicle is thought to be important for correct positioning and growth of the lens (reviewed by Grainger, 1992). Equally, the developing lens appears to be important for growth of the retina. (Coulombre and Coulombre, 1964).

The first developmental defects of *Sey/Sey* embryos that we detect are the abnormal shape of the brain and optic vesicle. The failure of the optic vesicles to constrict proximally occurs before the time of normal lens development and may reflect a requirement for Pax-6 in the neural ectoderm. The subsequent absence of lens may contribute to the later distal distortion of *Sey/Sey* optic vesicles, but this phenotype might also result

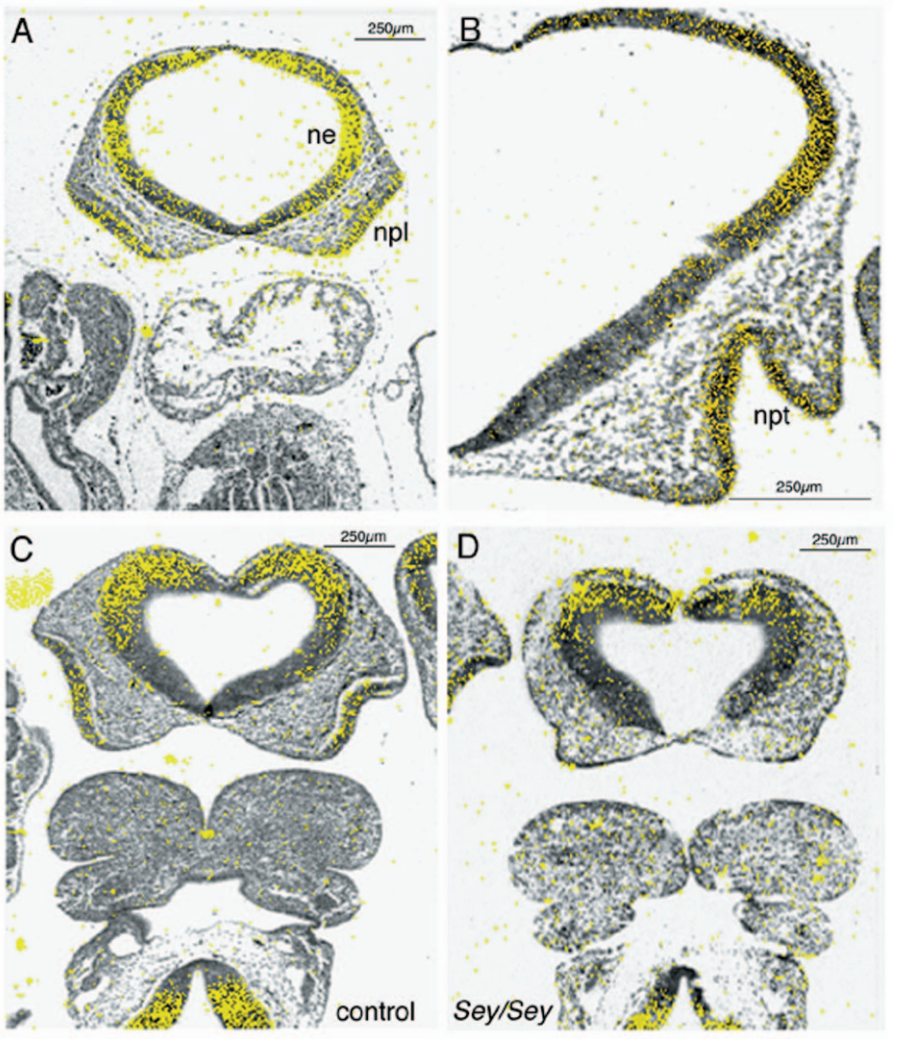

Fig. 6. Frontal sections through the nasal region showing *Pax-6* mRNA expression, yellow, in normal embryos at (A) E9.5 and (B) E10.5. Absence of nasal placode and nasal region expression at E9.75 from (D) *Sey/Sey* embryo, compare with (C) control embryo. Nasal placode (npl), nasal pit (npt), neuroepithelium of prosencephalon (ne).

from a lack of functional Pax-6 in the distal optic vesicle, where *Pax-6* mRNA is most strongly expressed.

Compared with littermates, optic vesicles of *Sey/Sey* embryos from E9.5 to E11.5 have a more uniform lumen width along their proximal-distal axis. *Sey/Sey* optic vesicles (E11.5 to E15.5) do eventually form structures reminiscent of both optic cup and optic stalk. These *Sey/Sey* structures vesicles retain some features of normal patterning, despite morphological abnormalities. *Tyrp2*, which in normal eyes is expressed only in the most distal optic stalk and the RPE layer of the optic cup, retains this distally restricted expression in *Sey/Sey* optic vesicles. As found in normal eyes, *Pax-6* transcripts in *Sey/Sey* optic vesicles are also most abundant in distal structures and are present in more extensive domains than those of *Tyrp2*.

Tyrp2 and *Pax-6* expression also highlight some abnormal features of *Sey/Sey* optic vesicles. Schmahl et al. (1993) suggested that the optic vesicle abnormalities could result from a disruption of proliferation or differentiation choices in the distal optic vesicle. Our findings support this suggestion. The small size of the expression domains of *Tyrp2* and *Pax-6* in *Sey/Sey* embryos may indicate that growth of the distal optic vesicle fails to keep pace with the development of other structures. This may explain the increasing separation of optic vesicle from the surface ectoderm. The two layers of the cup-like structure in *Sey/Sey* optic vesicles are morphologically similar and have similar expression characteristics, suggesting that differentiation of the developing cup into distinct layers is disrupted. Since there is no clear equivalent of the RPE, differentiation towards this cell type might be particularly affected in *Sey/Sey* mice.

Whilst the absence of lens in *Sey/Sey* mice complicates the identification of roles for *Pax-6* within the optic vesicle, it also allows us to use *Sey/Sey* mice to study the influence of the lens on optic vesicle development. Thus, those aspects of proximodistal patterning of the optic vesicle that are retained by *Sey/Sey* embryos do not depend upon the presence of a lens adjacent to the distal end of the optic vesicle.

Timing of Pax-6 action in lens formation

Lens 'induction' is a multi-step process (Grainger et al., 1988). The initiation of the lens formation pathway involves a series of inductive interactions prior to the contact between the surface ectoderm and the optic vesicle. Surface ectoderm passes through a progression of states of competence to respond to the different lens-inducing signals (Karlinen-Jääskeläinen, 1978; Henry and Grainger, 1987, 1990; Serventnick and Grainger, 1991; reviewed by Jacobson and Sater, 1988; Grainger, 1992). Although contact with the optic vesicle was long thought to be both necessary and sufficient to induce surface ectoderm to form lens (reviewed by Saha et al., 1989), it has been shown that the optic vesicle is not essential for initial formation of lens but can nevertheless induce lenses from ectoderm with lens-forming potential (Henry and Grainger, 1990) and is important for maintenance and growth of the lens (Saha et al., 1989).

Within the process of lens development, Pax-6 must act prior to, or at, the time of placode formation, i.e. before E9.5 in the mouse. Studies in amphibians suggest that the very earliest stages of lens induction occur around the time of gastrulation (Jacobson, 1966; Henry and Grainger, 1987). If comparable processes occur in mammalian eye development they are unlikely to involve Pax-6, since *Pax-6* is only expressed from the time (E8.0 in mouse) when the optic pits first appear. (Walther and Gruss, 1991; Krauss et al., 1991a,b; Puschel et al., 1992). Since surface ectoderm in *Sey/Sey* embryos fails to attain the earliest recognisable stage of lens formation, use of these animals does not address possible roles for *Pax-6* later in lens development. Nevertheless, Pax-6 is necessary for normal lens formation during the time, E8.0-E9.5, between the

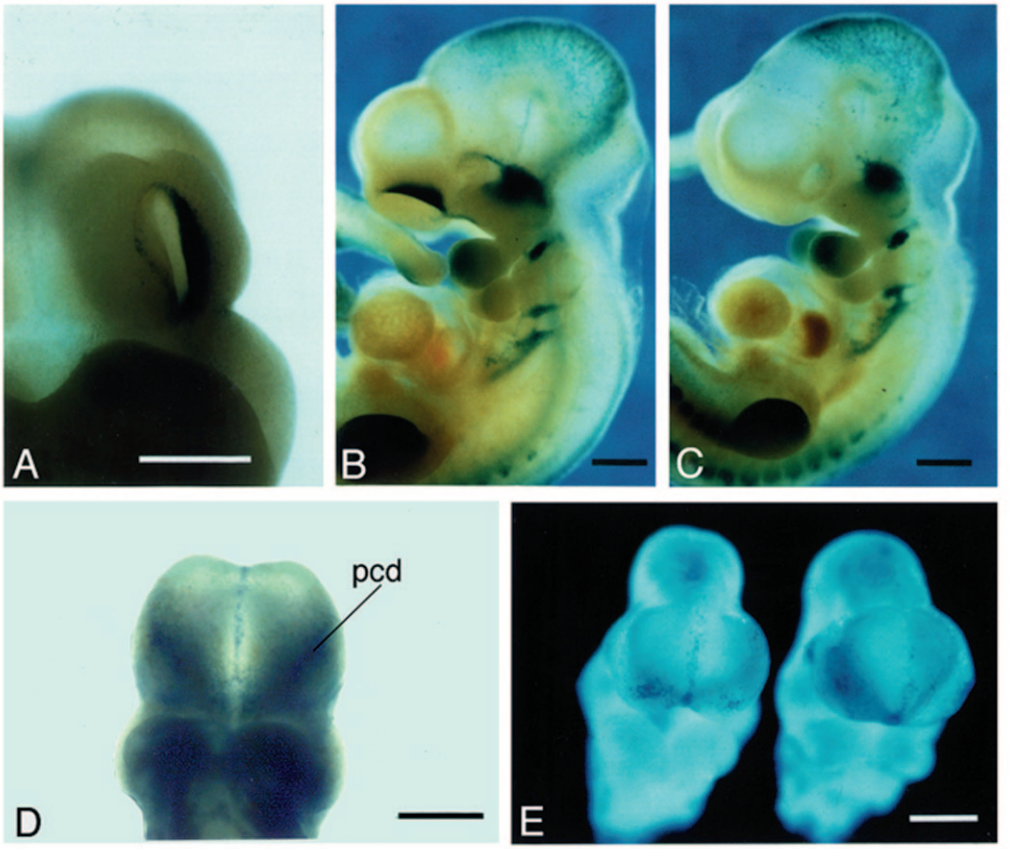

Fig. 7. (A-C) Expression of the *Msx-1* transgene ΔH6 at E10.5. Normal lateral placodal epithelium expression (A); control embryo (B); *Sey/Sey* embryo (C). (D-E) Nile Blue Sulphate (NBS) staining for programmed cell death. Arc of NBS-staining, programmed cell death (pcd), around rim of normal placode in D. (E) NBS staining in *Sey/Sey* embryo (left) and control embryo (right). All scale bars represent 1 mm.

first appearance of *Pax-6* expression and lens placode formation.

Pax-6 and lens determination

In normal development of the mouse eye, broad domains of *Pax-6* mRNA expression in head surface ectoderm are down-regulated, with expression being maintained in the developing lens placode. In the chick, the ability of isolated ectoderm to differentiate into lens in culture is initially a property of head ectoderm over a broad domain which, with time, also becomes restricted to the lateral regions adjacent to the optic vesicles (Barabanov and Fedtsova, 1982). Therefore, the surface ectoderm expression of the *Pax-6* gene and the *Sey/Sey* mutant phenotype are consistent with a role for Pax-6 in lens determination.

Tissue recombination experiments using the rat *Small eye* (Fujiwara et al., 1994) found that lens formation depended upon the genotype of the surface ectoderm used and was independent of the genotype of the optic vesicle. Thus *rSey/rSey* surface ectoderm never formed lens, whereas +/+ and *rSey*/+ surface ectoderm could form lens, even when cultured with *rSey/rSey* optic vesicle. It is possible that these results reflect a failure of signalling in vivo prior to the 20-somite stage when the recombinations were performed, but they are equally consistent with a requirement for functional Pax-6 within the surface ectoderm in order to form a lens placode and lens. Moreover, they further limit the role of the optic vesicle in the development of the homozygous mutant phenotype, as *rSey/rSey* optic vesicles can support the later development of the lens.

Pax-6 expression and ability to transdifferentiate to lens

Some non-lens tissues that express *Pax-6* are able to transdifferentiate into lens. The prospective pineal gland expresses *Pax-6* (Walther and Gruss, 1991), is multipotent and has the capacity to form lens (Watanabe et al., 1992). Similarly embryonic retina, iris and pigmented retinal epithelium at the anterior of the eye all express *Pax-6* mRNA and all have the ability to transdifferentiate into lens (reviewed by Okada, 1991). *Pax-6* expression may be a prerequisite for lens formation and, in non-lens tissue, may reflect a lens-forming ability that is normally overridden by diversion to other pathways.

We cannot rule out the possibility that the failure of lens formation in *Sey/Sey* embryos results entirely from a disruption of an interaction with the optic vesicle. An alternative explanation, Pax-6 involvement in lens determination, is supported by the association of *Pax-6* expression with ability of surface ectoderm to differentiate into lens, by the rat *Small eye* tissue recombination results and by the relationship between *Pax-6* expression and ability to transdifferentiate into lens. In addition, an activity of Pax-6 independent of the influence of the optic vesicle is revealed by down-regulation of *Pax-6* mRNA in *Sey/Sey* surface ectoderm.

Down-regulation of *Pax-6* mRNA in *Sey/Sey* surface ectoderm

Activation of *Pax-6* mRNA in *Sey/Sey* embryos is normal, but at the time when most surface ectoderm normally ceases to express *Pax-6*, the turn-off mechanism appears to extend to the whole surface ectoderm.

This extended down-regulation could be explained in a number of ways, but the observation that *Pax-6* mRNA continues to be expressed in normal chick surface ectoderm in the absence of an optic vesicle (Li et al., 1994) argues against failed signalling from optic vesicle and against a negative influence on *Pax-6* expression from mesenchymal-like cells gaining access to placode region surface ectoderm. We suggest a role for Pax-6 as an effector of its own expression in placode-forming ectoderm. Plaza et al. (1993) found a Pax-6-binding site in the quail *Pax-6* promoter through which Pax-6 up-regulated its own transcription in transfection assays, so the Pax-6-dependent regulation that we observe may reflect direct autoregulation.

Nasal placode formation and nasal processes

Both nasal cavities and lens develop from ectodermal placodes. We have found extensive similarities between the nasal lens placodes in their *Pax-6* expression and *Sey/Sey* mutant phenotype. Like the lens placode, the nasal placode normally expresses *Pax-6* mRNA and fails to form in *Sey/Sey* mutants. Similarly *Pax-6* mRNA expression turns off throughout the nasal region in *Sey/Sey* embryos. These results are consistent with the suggestion of Hogan et al. (1986) that the eye and nasal phenotype of *Sey/Sey* mice might stem from a common defect in the formation or early differentiation of these placodes.

The absence of nasal region *Msx-1* transgene expression in *Sey/Sey* embryos allows us to speculate that *Msx-1*, or genes regulating it, could be targets for Pax-6 in the nasal region. More concretely, absence of this expression shows that ectoderm in the nasal region of *Sey/Sey* embryos not only fails to attain the morphology characteristic of a placode, but also lacks gene expression normally associated with the nasal placode. Characteristic domains of morphological cell death, detected by Nile Blue Sulphate staining, are present in the nasal region of *Sey/Sey* embryos and control animals but, in *Sey/Sey* embryos, the domains are disorganised. Presence of these domains indicates that nasal region ectoderm is distinct from other head ectoderm, and so suggests that localisation of the nasal territory need not involve Pax-6 expression. Instead, Pax-6 may play a role in the transition from ectoderm to placode. From the similarity between lens and nasal placodes in terms of their normal development, *Sey/Sey* phenotype and *Pax-6* expression, we expect the requirement for Pax-6 in lens placode development to also be in the transition from ectoderm to placode.

We would particularly like to thank Richard Baldock for facilitating the computer presentation of expression data, Andrew Ross for help with the electron microscopy, Norman Davidson, Sandy Bruce and Douglas Stuart for photographic work, and the staff at the Biomedical Research Facility, Western General Hospital, Edinburgh for expert technical assistance. Ian Jackson and Albert E.Chung generously provided probes, and we thank Veronica van Heyningen for guidance and support of this work and Isobel Hanson for critical reading of the manuscript.

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(Accepted 31 January 1995)