AP-2α Transcription Factor Is Required for Early Morphogenesis of the Lens Vesicle

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INTRODUCTION

The eye, and in particular the lens, is an ideal model for studying embryonic induction and differentiation. Vertebrate eye development involves multiple inductive interactions and signals between the embryonic forebrain and surface ectoderm (reviews by Piatigorsky, 1981; Grainger, 1996; Fini et al., 1997). With closure of the neural tube, evaginations of the forebrain form the developing optic vesicles. The head ectoderm directly overlying the optic vesicle is then induced to thicken and form the lens placode, which subsequently invaginates into a lens pit that separates from the surface ectoderm to form a hollow lens vesicle. With invagination of the lens, the optic vesicle collapses inward and forms a bilayered optic cup, the inner layer of which will become the neural retina, while the outer layer develops into the retinal pigmented epithelium. Cells on the posterior side of the lens elongate into the primary lens fibers while the remaining surface ectoderm differentiates into the stratified epithelium of the cornea. Continued development of the neural retina involves the differentiation of a variety of distinct neural and glial cell types which are arranged in a layered pattern. Although eye development is one of the most extensively studied examples of proximate tissue interactions in the embryo the identity and specific role(s) of the regulatory molecules controlling ocular morphogenesis are largely unknown.

A number of transcription factors have recently been identified as candidate regulators of eye and lens development, including retinoic acid receptor transcription factors (RAR; RXR families) and homeobox genes including Six 3; Prox; Msx-1 and 2; eya-1, -2, and -3; and Pax6 (for complete list see reviews by Cvekl and Piatigorsky, 1996; Fini et al., 1997). Pax6, and its homologue in the fruit fly (Drosophila) eyeless (ey), have been shown to be essential for eye development. Homozygous mutations in Pax6 result in a complete lack of eyes (anophthalmia) in humans, mice, and flies (Walther and Gruss, 1991; Quiring et al., 1994). 

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erozygote mutations of this gene can lead to a number of ocular anomalies, including aniridia in humans, microphthalmia in small eye (Sey+/−) mice, and some cases of Peters’ anomaly, a congenital defect involving a physical connection between the lens and cornea (Hogan et al., 1986; Chalepakis et al., 1993; see review Fini et al., 1997). Misexpression of \( \text{Pax6} \) during development, as shown in Drosophila and other invertebrates, like squid, can result in ectopic eye formation (Hadler et al., 1995; Tomarev et al., 1997). However, recent studies in Drosophila have shown that misexpression of \( \text{sine oculis} \) (so) and \( \text{eyes absent} \) (eya) can also produce ectopic eye formation through an induction of \( \text{eyeless} \) (ey) (\( \text{Pax6} \) homologue) (Bonini et al., 1997; Chen et al., 1997a; Pignoni et al., 1997; review by Desplan, 1997). These findings demonstrate that the mechanisms regulating eye formation are complex and consist of a network of interactions among a number of genes, likely involving reciprocal feedback loops.

AP-2 transcription factors are a family of retinoic acid-responsive proteins, which have been identified as important regulators of gene expression during complex morphogenetic processes. Three related AP-2 genes comprise this family, AP-2\( \alpha \) (Williams et al., 1988), AP-2\( \beta \), and AP-2\( \gamma \) (or AP-2.2) (Moser et al., 1995; Bosher et al., 1996; Chazaud et al., 1996). During early stages of development all three AP-2 genes are coexpressed in cranial neural crest cells, whereas later in development they have both overlapping and distinct expression patterns in the developing epidermis, kidney, facial prominences, and limb bud (Mitchell et al., 1991; Moser et al., 1995; Chazaud et al., 1996; Moser et al., 1997a). AP-2 expression is also found in embryonic tissues which can contribute to the developing eye, including the

**TABLE 1**

Summary of Ocular Phenotypes in AP-2\( \alpha \) Null Embryos and Newborn Mice

<table>
<thead>
<tr>
<th>Stage</th>
<th>N. of eye regions examined (left and right sides)</th>
<th>N. with ocular phenotype</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>E9.5-10.5</td>
<td>6</td>
<td>6, placode or lens pit</td>
<td>100</td>
</tr>
<tr>
<td>E11.5</td>
<td>6</td>
<td>2, lens absent</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4, lens defect</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6, optic cup defect</td>
<td>100</td>
</tr>
<tr>
<td>E12.5-E15</td>
<td>14</td>
<td>2, lens absent</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6, lens defect</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6,(^a) eyes absent</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14, optic cup defect</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14, cornea absent</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14, eyelids absent</td>
<td>100</td>
</tr>
<tr>
<td>Newborn</td>
<td>12</td>
<td>2, lens absent</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6, lens defect</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4,(^a) eyes absent</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12, optic cup defect</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12, cornea absent</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12, eyelids absent</td>
<td>100</td>
</tr>
</tbody>
</table>

\(^a\) Overall % eyes absent in E12.5 and older embryos is 38%.

**FIG. 1.** Ocular defects in AP-2\( \alpha \) null mice. Key: (ey) eye, (ls) lens stalk, (nr) neural retina, (plf) primary lens fibers, (rpe) retinal pigmented epithelium. (A) Frontal section of E12.5 wild-type (+/+) embryo with eyes on the surface of the head. Mag. approx. 60×. (B) E12.5 AP-2\( \alpha \) null embryo with eyes embedded inside of the head. Mag. approx. 60×. (C) Wild-type (+/+ ) eye at E11.5. Mag. approx. 300×. (D) AP-2\( \alpha \) null eye at E11.5 in which the lens is absent and mesenchymal cells occupy the optic cup. Mag. approx. 260×. (E) AP-2\( \alpha \) null eye at E11.5 in which an abnormal lens pit has formed and the retinal pigmented epithelium is replaced by cells resembling the neural retina (arrowhead). Mag. approx. 260×. (F) Wild-type eye (+/+ ) at E12.5. Mag. approx. 300×. (G, H) AP-2\( \alpha \) null eyes at E12.5. In both cases the lens has failed to separate from the ectoderm and exhibits a lens stalk. Arrowhead indicates where rpe failed to form in H. Mag. approx. 280×. (I, J) AP-2\( \alpha \) null newborn eyes. Lenses lack an anterior epithelium. Anterior margins of the cup fail to form an iris and ciliary body (arrowhead). Mag. approx. 340×. (K) Higher magnification of mutant retina shown in J, demonstrating the lack of a defined ganglion cell layer (asterisks). Mag. approx. 900×.

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surface ectoderm and neural crest (Mitchell et al., 1991; Moser et al., 1997a), although an in depth analysis of AP-2 expression patterns in the embryonic eye has not been performed and existing data are inconsistent. A further link between AP-2 and eye development is suggested by previous in vitro studies which demonstrate that AP-2α regulates genes expressed in the eye, including the matrix metalloproteinase, gelatinase B (Fini et al., 1994), and keratin 3 (Chen et al., 1997). The AP-2α gene is a critical regulator of normal vertebrate embryogenesis: AP-2α null mice die perinatally and exhibit severe congenital abnormalities, including failure of closure of the anterior neural tube (exencephaly) and craniofacial defects including eye defects (Zhang et al., 1996; Schorle et al., 1996). Details regarding eye defects, however, were not pursued in these investigations. Thus, it remained unclear whether the ocular anomalies were caused by the lack of AP-2α in the tissues which form the eye or whether they were the result of severe disruptions in the tissue interactions in the head.

In this study, we investigated the hypothesis that disruption of AP-2α can directly disturb morphogenesis of the eye by examining the ocular defects and candidate downstream genes affected in both AP-2α null and chimeric mice. In order to understand the nature of the ocular defects in these mutants, we also determined the expression pattern of the three AP-2 proteins in the tissues which interact and contribute to the eye during normal development. We demonstrate that the three AP-2 proteins have unique and overlapping expression patterns in the developing eye with AP-2α uniquely expressed in the developing lens vesicle. Our findings revealed that corresponding ocular defects occur in AP-2α null and chimeric mice, including disruption of early morphogenesis of the lens vesicle. Importantly, chimeric mice exhibited ocular defects in the absence of head and face anomalies, demonstrating a direct role(s) for AP-2α in early development of the eye.

**MATERIALS AND METHODS**

**Mouse Embryos**

AP-2 expression studies were carried out on wild-type CD-1 embryonic and postnatal mice (Charles River Laboratories, Wilmington, MA). A range of developmental stages was obtained through timed matings. Nontime of the day on which a vaginal plug appeared was designated as embryonic day 0.5 (E0.5). The embryonic stage at which we began to collect was 9 days postconception, or E9, since at this stage initial evidence of eye development can be detected. Embryos were then collected at E9, E9.5, E10.5, E12.5, and E15 in order to examine specific stages of eye development. For each stage, the pregnant mother was euthanized by CO2, and the uterus was removed and placed in 1× phosphate-buffered saline (PBS). The embryos were dissected from the uterus, washed again in PBS, and then embedded in Tissue Tek II OCT compound (Lab Tech Products, Naperil, IL) and frozen in an ethanol/dry ice bath. Some of the embryos were also fixed in 0.2% paraformaldehyde prior to embedding. Mice at postnatal days 2, 7, 10, and 14 were also euthanized and eyes were dissected from the head and embedded as described above. Serial frozen (cryostat) sections, 5 to 7 μm thick, were made horizontally through the head of the embryos in order to obtain a cross-section of the developing eyes. Whole eyes from postnatal animals were also positioned such that cross-sections of each eye were achieved.

**AP-2α Null Embryonic and Newborn Mice**

Mice containing a disrupted AP-2α gene which were generated previously (Zhang et al., 1996) were bred in order to obtain homozygous null embryonic and newborn mice. In general, two developmental stages, E12.5 and newborn, were collected (Table 1). However, a smaller number of embryos at E9.5, E10.5, E11.5, and E15 were also selected for examination. The embryos were dissected from pregnant mothers as described above and fixed either for paraffin embedding in 10% PBS buffered formalin or for cryostat sectioning in 0.2% paraformaldehyde. Homozygous null embryos were easily distinguished from heterozygote offspring due to their severe craniofacial anomalies. Embryos to be embedded in paraffin were dehydrated in a graded series of ethanols to xylene and embedded in paraffin. Some of the embryos were also fixed in 0.2% paraformaldehyde for cryostat sectioning (0.2% paraformaldehyde). Homozygous null embryos were easily distinguished from heterozygote offspring due to their severe craniofacial anomalies. Embryos to be embedded in paraffin were dehydrated in a graded series of ethanols to xylene and embedded in paraffin. Sections were cut at 7 μm thick and stained with hematoxylin and eosin. Cryostat sections (2 to 7 μm) were used for immunohistochemical analyses (see below).

**Generation of AP-2α Chimeric Mice**

AP-2α-/- embryonic stem (ES) cells were generated from the embryonic stem cell line containing a disruption of one allele of the AP-2α gene (Zhang et al., 1996). A LacZ gene was inserted into the remaining wild-type allele of the AP-2α gene to produce a similar mutation and disrupt AP-2α function. The details of this procedure will be published elsewhere (Nottoli et al., 1998). C57BL/6 and Swiss Webster (SW) mice were purchased from Taconic. The ES cells were microinjected into 3.5 days postconception (dpc) embryos.

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**FIG. 2.** Expression pattern of Pax6 and MIP26 proteins in wild-type and AP-2α null eyes by immunohistochemistry. Key: (ce) corneal epithelium, (ey) eyelid, (le) lens epithelium, (m-lens) mutant lens, (lf) lens fiber cells, (ls) lens stalk, (nr) neural retina, (rpe) retinal pigmented epithelium. (A) Expression of MIP26 in wild-type eye (+/+ ) at E15. MIP26 is specifically expressed in the lens fiber cells. Some staining was also observed in the corneal epithelium; however, this appeared to be artifactual. Mag. approx. 480×. (B) Expression of MIP26 in the mutant newborn lens. Expression of MIP26 was detected over the entire lens including the lens stalk. Mag. approx. 440×. (C) Expression pattern of Pax6 in the wild-type eye (+/+ ) at E15. Pax6 was detected in the corneal epithelium, cells of the neural retina, and the anterior epithelium of the lens. Mag. approx. 220×. (D) Expression pattern of Pax6 in the mutant eye at E15. Pax6 was detected in the cell of the neural retina, but not in the mutant lens. Mag. approx. 220×. (E) Higher magnification of D demonstrating lack of Pax6 expression in the mutant lens. Mag. approx. 440×.

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C57BL/6 blastocysts using standard techniques (Hogan et al., 1994). Injected embryos were then transferred into pseudopregnant SW females, and mice were either allowed to go to term or sacrificed at E18. Mice which were allowed to go to term were euthanized at 3 weeks postnatal. Following dissection, the embryos and whole eyes from 3-week-old mice were fixed in 0.2% paraformaldehyde and embedded for frozen sectioning in OCT medium. Sections were cut at 7 μm and either stained with hematoxylin and eosin or for β-galactosidase (LacZ) activity in order to determine location of the ES cell progeny (Hogan et al., 1994).

### Immunohistochemistry

Indirect immunohistochemistry was used to detect AP-2 protein expression in the CD-1 embryos and whole eyes. Four different antibodies were used to recognize the three different AP-2 proteins: A commercially available polyclonal AP-2 antibody (SC-184) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used (1:250) which recognizes an AP-2α-specific epitope in the mouse (Zhang et al., 1996). Two rabbit polyclonal antibodies were used which recognize AP-2β (1:1000) (Bosher et al., 1996) or AP-2γ (1:250) (Turner et al., 1998) specific epitopes. All antibodies were separately incubated on frozen sections and the location of each endogenous AP-2 protein was revealed using an indirect biotin/avidin-immunoperoxidase system (Vector Laboratories, Burlingame, CA).

Indirect immunohistochemistry was also used to detect the expression of three proteins, Pax6, MIP26 (the lens main intrinsic polypeptide), and Brn-3b, in the developing wild-type and AP-2α mutant eyes. A rabbit polyclonal antibody recognizing the transcriptional activation domain of Pax6 (GM3) (from Dr. A. Cvekl, LMDB, NEI, NIH, Bethesda, MD) was applied (1:500) to frozen sections of both wild-type and mutant mice. A rabbit polyclonal antibody against a major intrinsic MIP26 (Bok et al., 1982) was applied (1:500) to both paraffin and frozen sections to detect MIP26 expression in the mutant lens (E12.5, E15, and newborn). A goat polyclonal antibody (SC-5026) (Santa Cruz Biotechnology Inc.) was used to detect Brn-3b in wild-type and mutant retinas. The locations of these antigens were then revealed using indirect biotin/avidin-immunoperoxidase systems (Vector Laboratories).

All sections were observed and photographed on a Nikon Labophot microscope. Photographs were then digitized and reproduced for publication using Adobe Photoshop 4.0 (Adobe System Incorporated).

### RESULTS

#### AP-2α Null Mice Exhibit Multiple Ocular Defects Including Failure of the Lens to Separate from the Surface Ectoderm

The ocular phenotypes of AP-2α mutant embryos were examined histologically at various developmental stages from E9.5 to newborn and results are summarized in Table 1. At early stages of embryogenesis (E9.5–10.5), AP-2α mutants had eyes which were on the surface of the head, similar to age-matched wild-type embryos. In contrast, all mutants examined at E12.5 and later did not exhibit eyes on their surface. Histological sectioning revealed that over one-third (38%) of these embryos completely lacked either one or both eyes (bottom of Table 1). The majority (62%) of the mutants, however, had eyes or eye rudiments which were embedded inside the head and surrounded by an overgrowth of mesenchymal and neural tissue (Figs. 1A and 1B).

In all of the mutants examined which had eyes, aberrant lens induction was evident. Lens induction had completely failed in some mutants (33% of E11.5 and 14% of E12.5) and in the place of surface ectoderm which typically invaginates to form the lens vesicle (wild-type mice ++/+; Figs. 1C and 1F) mesenchymal cells occupied the inner aspect of the optic cup (Fig. 1D). In the majority of the mutants, however (67% of E11.5; 43% of E12.5), the surface ectoderm (se) had been induced to invaginate into the optic cup (Figs. 1E and 1G–1J) but in comparison to wild-type ++/+ embryos (Figs. 1C and 1F) these lenses were abnormal. The mutant lenses were reduced in size and abnormally shaped. The mutant lens also remained connected to the overlying ectoderm via a lens stalk. As a result, an anterior lens epithelium, distinct from the surface ectoderm, as seen in wild-type embryos did not develop. Despite failure to separate from

### TABLE 2

<table>
<thead>
<tr>
<th>Stage</th>
<th>No. of eyes examined (left and right sides)</th>
<th>No. with ocular phenotype</th>
<th>%</th>
</tr>
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<tbody>
<tr>
<td>E18</td>
<td>8</td>
<td>4, eyelids open</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3, corneal-lens defect</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1, no obvious defect</td>
<td>12</td>
</tr>
<tr>
<td>3 weeks old</td>
<td>12</td>
<td>2, corneal epithelial defect</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6, additional corneal defects</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4, no obvious defect</td>
<td>33</td>
</tr>
</tbody>
</table>

Summary of Ocular Phenotypes in AP-2α Chimeric Embryos and Mice Which Lacked Craniofacial and Neural Tube Defects
FIG. 3. Corneal-lenticular defects in AP-2\alpha chimeric mice. Key: (cd) corneal defect, (cld) corneal-lenticular defect, (ce) corneal epithelium, (ir) iris, (le) lens epithelium, (ls) lens stalk, (s) corneal stroma. (A) Chimeric embryo at E18 with unfused eyelids and central corneal defect. Mag. approx. 20×. (B) Section of the chimeric embryo in A. The lens is misshapen with the bow region shifted downward (asterisk). The lens and corneal epithelium are contiguous via a lens stalk. Mag. approx. 240×. (C) Section similar to that in B, stained for β-galactosidase (LacZ) activity. Cells in the region of the lens defect stain positive for LacZ. Mag. approx. 240×. (D) Section of a corneal defect in a 3-week-old chimera. In the region of the defect, the corneal stroma is absent and the epithelium encircles pigmented cells. The anterior lens region (*) also appears dismorphic. Mag. approx. 340×. (E). Higher magnification of the corneal defect in D. Mag. approx. 880×.
the ectoderm, elongation of the primary lens fibers (plf) was observed in the mutant lens (Figs. 1G–1J).

**Misexpression of MIP26 and Pax6 in the Mutant Lens**

We surmised from our examinations of the morphology of the AP-2α mutant lens that an anterior lens epithelium was absent. In order to confirm which cell types, epithelial cells or fiber cells, were present in the mutant lens we examined the expression pattern of a fiber-specific protein MIP26, in both the mutant and the wild-type lens. MIP26 protein was specifically expressed in the fiber cells of the wild-type lens (E15), whereas the anterior lens epithelium lacked immunoreactivity (Fig. 2A). In contrast, in the mutants, intense immunoreactivity with the MIP26 antibody was found over the entire lens (Fig. 2B). A region resembling an anterior lens epithelium, which lacked immunoreactivity to the MIP26 antibody was not observed, with the only exception that immunoreactivity was occasionally absent in some cells lining the lens stalk.

The expression pattern of Pax6 protein was also examined since Pax6 is known to be expressed in the lens epithelium and to have a critical role in eye and lens development (Cvekl and Piatigorsky, 1996; Quinn et al., 1996). In the embryonic wild-type eye at E15, Pax6 was expressed in the nuclei of the anterior lens epithelium, as well as in the corneal epithelium and in the majority of the cells in the developing neural retina (Fig. 2C). This pattern of expression is consistent with earlier reports of Pax6 protein immunolocalization in the developing mouse eye (Davis and Reed, 1996). In comparison, in the mutant eye (E15), Pax6 was detected in all cells of the developing neural retina; however, its expression could not be detected in any section of the mutant lens (Figs. 2D and 2E). It is important to note that some cells which lined the mutant lens stalk were not reactive to either the MIP26 or the Pax6 antibody, suggesting that these cells were not typical lens epithelial cells nor fiber cells (data not shown).

**Optic Cup Defects in the AP-2α Null Mice**

Additional defects were evident in the AP-2α mutant eye, including the absence of a developing cornea and eyelids (Figs. 1G–1J). Defects in the developing optic cup were also common. Although a bilayered optic cup could form in the mutant eye, as in wild-type animals (Figs. 1C and 1F), a retinal pigmented epithelium (RPE) failed to develop on the dorsal side (Figs. 1E, 1H, and 1J). In the place of the RPE, a pseudostratified neuroepithelial layer had formed which resembled that of the inner neural retinal layer (Figs. 1E, 1H, and 1J). In addition, the optic stalk was frequently misplaced, with the choroidal fissure either delayed or failing to close. Additional optic cup defects in the null mutant eyes included the lack of formation of the iris and ciliary body at the anterior margins of the developing optic cup (Figs. 1I and 1J). In nearly all of the mutant retinas examined, distinct ganglion cell and inner plexiform layers were absent (Figs. 1I, 1J, and 1K). Instead, there appeared to be nonuniform packing and density of cells throughout (Fig. 1K). We confirmed whether ganglion cells were absent in the mutant retina by immunolocalizing Brn-3b protein, a POU domain transcription factor which is exclusively expressed within a subpopulation of ganglion cells in the developing retina (Xiang et al., 1993). In contrast to wild-type retinas at E15 we did not detect Brn-3b in age-matched null mutant retinas (not shown) suggesting that loss of AP-2α expression has disrupted differentiation of this subset of cells.

**AP-2α Chimeric Mice Exhibit Ocular Defects Independent of Neural Tube and Craniofacial Defects**

Homozygous disruption of the AP-2α gene in mice resulted in compound defects suggesting that multiple morphogenetic processes had been disturbed (Zhang et al., 1996; Schorle et al., 1996). Derivation of chimeric mice comprised of AP-2α+/− and AP-2α−/− cells has revealed that a number of defects observed in the knockout embryos can occur independently, including eye defects (Nottoli et al., 1998). The chimeric embryos and postnatal animals which specifically displayed ocular defects, in the absence of craniofacial defects and exencephaly, provided us with a mechanism by which to isolate the role of AP-2α in eye development. Descriptions of these defects are summarized in Table 2.

Gross inspection revealed obvious ocular anomalies in several of the E18 embryos. Over one-third (38%) of chimeric eyes exhibited a small central corneal defect consisting of pigmented cells, which was evident through unfused eyelids (Fig. 3A). Histological examination of the chimeric eyes revealed that unlike wild-type animals in which the lens has a spherical shape, the lenses in chimeric mice were

![FIG. 4.](image-url)
misshapen, conforming to an unusually shaped optic cup (Fig. 3B). As a result, the bow region of the lens, where epithelial cells transition into fibers, was not in the equatorial region as in wild-type mice but was shifted posteriorly. The most striking ocular defect found in these chimeras was a corneal-lenticular adhesion anomaly (Figs. 3B–3E) in which the central portion of the lens epithelium had protruded through the corneal stroma and appeared contiguous with the overlying central corneal epithelium via a lens stalk (Figs. 3B and 3C). In the region in which the lens and corneal epithelium appeared associated, darkly pigmented cells were found (Figs. 3B, 3D, and 3E), which corresponded to the gross corneal defect observed prior to sectioning (Fig. 3A).

As indicated under Materials and Methods, a β-galactosidase (LacZ) gene was used to disrupt the remaining AP-2α allele. The LacZ gene enabled us to identify null cells which contributed to the developing mutant eye. AP-2α+/– cells, as revealed by staining for LacZ, were detected in the region of the lens stalk, demonstrating contribution of the AP-2α-null cells to the genesis of the corneal-lenticular adhesion (Fig. 3C). Further experiments using the AP-2α-specific antibody confirmed that these LacZ-positive cells, which normally express AP-2α, are not immunoreactive to the antibody (not shown). Several 3-week-old chimeras also exhibited central corneal defects (17%) similar to those found in E18 embryos (Figs. 3D and 3E). In the region of the defect a corneal stroma is absent and the corneal epithelium encircles pigment granules. Although the central lens and corneal epithelium were not associated, the central-anterior lens morphology appeared altered, in a manner that was suggestive of a prior adhesion with the cornea.

In 50% of the chimeras examined at E18, eyelids were found to be fully or partially open, unlike wild-type embryos at the same stage in which eyelids are typically fused (Fig. 4A; Table 2). Sectioning and staining for LacZ revealed a substantial contribution of AP-2α-null cells to the epidermis and leading periderm of the eyelid (Fig. 4B). We also noted that mice heterozygous for the AP-2α gene showed partial penetrance (25%) for eyelid defects which presented as partially closed lids that were swollen and purulent. In these cases, the eyelid margins were unusually thickened and exhibited a hyperproliferative epidermal layer and an underlying stroma containing an increased blood vasculature (not shown).

Defects were also observed in the developing retina of E18 and postnatal chimeras. These retinas contained round, multicelled clusters or foci of cell bodies (Figs. 4C–4E). In addition, the embryonic chimera retinas exhibited regions in which there appeared to be a loss of ganglion cells, as well as a disrupted inner plexiform layer, similar to that which was observed in the null mutants. In some regions of the postnatal chimeric retinas there appeared to be an unequal contribution of cells to the various retinal layers, as well as considerable folding of the retina (Fig. 4E).

Expression Pattern of AP-2 Proteins during Morphogenesis of the Eye

In order to understand the nature of the ocular defects in the mutants we determined the expression pattern of AP-2α and additional AP-2 proteins (AP-2β; AP-2γ) in the developing eye. Initially we used a pan-specific antibody which appeared to recognize all three AP-2 proteins. AP-2α was first detected during early ocular embryogenesis (E9.5) in the developing lens: staining was detected in the enlarged nuclei of the lens placode, a region of thickened ectoderm overlying the developing optic cup (Fig. 5A). AP-2α protein was also detected in neural crest-derived mesenchymal cells located between the evaginating optic cup and forebrain and those adjacent to the lens placode (Fig. 5A). To determine which of the three AP-2 proteins were expressed in these populations of cells, antisera specific for each of the AP-2 proteins were used. These studies revealed that AP-2α was expressed in cells of the developing lens placode (Figs. 5A and 5B), whereas AP-2β was expressed in the lens placode, as well as the surrounding mesenchymal cells (not shown). AP-2γ was not detected in the eye at this stage of development (not shown).

At embryonic day E10.5, AP-2α and AP-2β proteins were expressed in the cuboidal epithelial cells lining the lens pit and in cells of the adjoining surface ectoderm (AP-2α, Fig. 5C; and AP-2β, not shown). Following separation from the surface ectoderm, and formation of a hollow lens vesicle at E12.5, AP-2 expression became confined to the anterior lens epithelium (Fig. 5D) and was not expressed in cells on the posterior side of the lens which had elongated into the primary lens fiber cells. Immunolocalization with the specific antisera at E12.5 and E15 demonstrated that AP-2α becomes the only AP-2 protein to be expressed in the developing lens vesicle, following separation from the ectoderm, whereas AP-2α and β were coexpressed in the remaining overlying surface ectoderm destined to differentiate into the corneal epithelium (E15, Figs. 5E–5H). The neural crest-derived mesenchymal cells reacted to both the pan-specific AP-2 antibody (Fig. 5E) and the AP-2β antibody (Fig. 5G), but not to the AP-2α-specific antibody. Thus AP-2β appears to be uniquely expressed in the neural crest-derived mesenchymal cells, which contribute to both the corneal stroma and the endothelium.

At embryonic day 15 AP-2γ protein expression was first detected in the eye, in the mesenchymal cells of the developing eyelids (Figs. 6A and 6B). Pan-specific antibody staining revealed that AP-2 proteins were expressed in a subset of cells within cornea, the structures of the uvea, including the iris stroma and the anterior angle, retina (Fig. 6C), lens and eyelid stroma, and epithelium. At postnatal day 10, all three tissue layers of the cornea expressed AP-2, including the anterior epithelium, collagenous stroma, and underlying endothelium (Fig. 6D). However, by day 14 expression of AP-2 was found mainly in the basal cell layer of the epithelium, and this was specifically AP-2α (Fig. 6F). AP-2α and β proteins were also first detected in the devel-
apoptosis in normal and \( \alpha \)-deficient neural retina at E15 (Figs. 5E–5G) specifically in a subpopulation of cells of the presumptive inner nuclear layer. By postnatal day 14, \( \alpha \)-AP-2 (Fig. 6E) and \( \beta \) (not shown) were coexpressed in two populations of cells, a subset of cells in the ganglion cell layer, and also in cells of the inner nuclear layer.

In order to confirm our immunolocalization findings we made use of the \( \alpha \)-AP-2-deficient eye in order to determine the location of the remaining \( \alpha \)-AP-2 proteins. Using the pan-specific antibody it was found that \( \alpha \)-AP-2 protein was expressed in cells of the inner layer of the developing retina and in the mesenchymal cells surrounding the lens (Fig. 6G). \( \alpha \)-AP-2 expression was not, however, detected in the developing mutant lens. These results confirm our earlier findings that \( \alpha \)-AP-2 is uniquely expressed in the developing lens, whereas additional \( \alpha \)-isoforms are expressed in the remaining ocular tissues, including the retina and neural crest-derived mesenchymal component of the eye.

**DISCUSSION**

We have shown that \( \alpha \)-AP-2 null mutants have a number of early and specific defects in the eye and lens, which correlate with the pattern and time at which \( \alpha \)-AP-2 is expressed in the eye during normal ocular development. In the majority of \( \alpha \)-AP-2 null mutants examined, the lens placode had been induced to invaginate; however, further stages of lens development and differentiation appeared disrupted. In particular, \( \alpha \)-AP-2 null embryos exhibited a defect in which the developing lens vesicle did not detach from the overlying ectoderm. Our findings suggest that additional tissue defects in the head were not the principal cause of the eye defects in the null mutants since \( \alpha \)-AP-2 chimeric embryos which lacked neural tube and craniofacial defects exhibited a similar lens anomaly. In these chimeras, \( \alpha \)-AP-2 null cells, as marked with LacZ, were found to be associated with the region of the corneal-lenticular defect. Together these data suggest \( \alpha \)-AP-2 has a direct role in early morphogenesis of the lens vesicle.

Immunolocalization findings for \( \alpha \)-AP-2 expression during normal lens development also suggest that \( \alpha \)-AP-2 is required for lens development. During separation of the lens ectoderm from the surface, \( \beta \)-AP-2 expression was lost and \( \alpha \)-AP-2 became the only \( \alpha \)-AP-2 protein expressed in the lens, and this expression was confined to the anterior lens epithelium. Separation of the lens is the developmental event disrupted in the mutants. Thus, it is intriguing to propose that the unique expression of \( \alpha \)-AP-2 in this anterior region is critical for the morpho genetic movement of lens detachment, the lack of which cannot be compensated for by another \( \alpha \)-AP-2 member. Collectively, these findings support the hypothesis that \( \alpha \)-AP-2 acts directly to control invagination and separation of the lens vesicle during embryogenesis.

Eye and lens development were not completely abolished in all \( \alpha \)-AP-2 null mutants, and at least a rudimentary lens had formed in the majority of the mutants examined. This suggests that \( \alpha \)-AP-2 may not be required in the very early embryonic stages of lens induction during which competence of the ectoderm is established. However, since \( \beta \)-AP-2 is also expressed in the lens placode redundant function with \( \beta \)-AP-2 could mask an early role for \( \alpha \)-AP-2. The evidence from this investigation suggests that \( \alpha \)-AP-2 is required in later stages of lens specification and differentiation including invagination and separation of the lens ectoderm from the surface. The molecular factors which control detachment of the lens from the surface ectoderm have not been identified. However, ultrastructural studies, mainly on the developing chick lens rudiment, suggest that two main mechanisms are responsible for obliteration of the lens stalk including cell death and alterations in cell adhesion (Silver and Hughes, 1973; Schook, 1980; Garcia-Porron et al., 1984). \( \alpha \)-AP-2 has been implicated in apoptosis. For example, in vitro studies have shown that \( \alpha \)-AP-2 can suppress c-myc-induced apoptosis and increased amounts of apoptotic death have been observed in regions of both \( \beta \)-AP-2 and \( \alpha \)-AP-2-deficient mice (Moser et al., 1997b; Zhang et al., 1996; Schorle et al., 1996). However, the role of \( \alpha \)-AP-2 as a survival factor during lens development is not conceivable since an increased amount of cell death, as determined by the histological appearance of apoptotic cells, was not observed in the lens of the \( \alpha \)-AP-2-deficient mice. Furthermore, if apoptosis were increased in the AP-

**FIG. 5.** Expression of \( \alpha \)-AP-2 protein during early morphogenesis of the murine eye. Key: (ae) anterior lens epithelium, (conj) conjunctiva, (fb) forebrain, (le) lens epithelium, (lf) lens fibers, (lp) lens placode, (lp) lens pit, (lv) lens vesicle, (mc) mesenchymal cells, (oc) optic cup, (ov) optic vesicle, (se) surface ectoderm. (A) E9.5. \( \alpha \)-AP-2 expression was detected using a pan-specific antibody (pan) in the lens placode and neural crest-derived mesenchymal cells. Mag. approx. 380×. (B) E9.5. \( \alpha \)-AP-2 expression was restricted to the developing lens placode. Mag. approx. 380×. (C) E10.5. \( \alpha \)-AP-2 expression was detected in the cells of the lens pit and adjacent surface ectoderm. Mag. approx. 380×. (D) E12.5. \( \alpha \)-AP-2 expression (pan) was detected in the surface ectoderm, the anterior epithelium of the lens vesicle, and mesenchymal cells. Mag. approx. 380×. (E) E15. \( \alpha \)-AP-2 expression (pan) was detected in the developing corneal epithelium, conjunctiva, eyelid, mesenchymal cells, epithelium of the lens, and subpopulation of cells in the neural layer of the developing optic cup (arrowheads). Mag. approx. 560×. (F) E15. \( \alpha \)-AP-2 was expressed in the corneal epithelium, conjunctiva, epithelium of the lens, and subpopulation of cells of the optic cup (arrowheads). Mag. approx. 560×. (G) E15. \( \beta \)-AP-2 was expressed in corneal epithelium, conjunctiva, eyelid, mesenchymal cells, and subpopulation of cells of the optic cup (arrowheads). Mag. approx. 560×. (H) E15. \( \alpha \)-AP-2 expression (pan) in the developing lens epithelium. Mag. approx. 560×.
2α-deficient lens we would not expect to find the persistent adhesion between the lens and overlying ectoderm since cell death is thought to positively influence this morphogenetic event. A more likely role for AP-2α in early morphogenesis of the lens is in the regulation of cell adhesion. Genes involved in cell-cell and cell-matrix adhesion are expressed in the eye and have also been shown to be regulated by AP-2 in vitro, including the extracellular matrix proteinase gelatinase B (Fini et al., 1994; Chen et al., 1997b). We have observed that the expression pattern of AP-2α in the developing lens and cornea parallels the cell-cell adhesion molecule E-cadherin before and after separation of the lens vesicle. Interestingly, AP-2 binding has been shown to be required for activation of the E-cadherin promoter in epithelial cell cultures (Behrens et al., 1991; Hennig et al., 1996).

Our findings demonstrated that Pax6 expression was absent while MIP expression was expanded in the AP-2α mutant lens. We propose two possible explanations for these findings. First, misexpression of these proteins may be due to the lack of a true epithelial cell component in the mutant lens. Histological evidence revealed that the mutant lens was lacking a typical anterior lens epithelium compared to wild-type lenses, suggesting that the lens may be composed entirely of lens fiber cells. However, some of the cells within the mutant lens stalk at E15 did not have a fiber cell appearance and were not immunoreactive to either the MIP or Pax6 antibody suggesting that epithelial cells are not simply replaced by fiber cells in the mutant lens. Another possible cause for the misexpression of MIP and Pax6 in the AP-2α-deficient lens may be that AP-2α is a required regulator of MIP and/or Pax6 expression. In support of this hypothesis, recent in vitro findings indicate that AP-2α is one of the transcription factors that interact with several domains of the human MIP gene promoter element (Ohtaka-Maruyama et al., 1998). Likewise, a number of possible binding sites for AP-2 have been identified in the Pax6 promoter (Plaza et al., 1995). Interestingly, the expression pattern of Pax6 in the developing eye (Koroma et al., 1997) closely parallels that of AP-2α. Similar to the AP-2α null mutants, Pax6 (Sey) mutants also exhibit a small lens which often fails to detach completely from the cornea (Hanson et al., 1994). Such similarities may indicate that these genes cross-regulate each other or that they interact biochemically to regulate downstream genes which are involved in development of ocular tissues. In this regard, Pax6 and AP-2 have both been shown to control the expression of genes involved in cell adhesion (Chalepakis et al., 1994; Holst et al., 1997).

In addition to defects in the developing lens, we observed anomalies in a number of other ocular tissues in the AP-2α null mutants and chimeras including the eyelids and developing optic cup. A normal eyelid formation has been reported in other mutant mouse strains and is often associated with inappropriate growth factor signaling (Juriloff et al., 1996). Normal development of the eyelid involves migration of the leading periderm cells and the more posterior neural crest-derived mesenchyme, over the developing corneal epithelium, three tissues that express AP-2α. Thus, the lack of AP-2α in one or more of these tissues could disrupt the signaling pathways involved in eyelid formation.

Defects in the optic cup were observed in the null mutants by E12.5 of development and included the absence of a RPE on the dorsal side of the optic cup, with a duplicated retina in its place. This phenotype could have resulted from either a lack of specification of the RPE on this side of the developing optic cup or transdifferentiation of the RPE into neural retina. Transdifferentiation of RPE to neural retina has been observed both in vitro (reviewed by Zhao et al., 1995, 1997) and in vivo, in amphibian species that exhibit regenerative capacities, as well as in the chick (Park and Hollenberg, 1991; Sakaguchi et al., 1997). Defects in the normal patterning of the optic cup along the dorsal-ventral retinal axis have also been observed in RXR null mice (Kastner et al., 1994). In addition, exogenously added retinoic acid causes induction of retinal duplications in zebrafish (Hyatt et al., 1992) which resemble the phenotypes in the AP-2α null mutants. Since the activity and expression of AP-2α has been shown to be responsive to retinoids (Williams et al., 1988; Luscher et al., 1989; Shen et al., 1997), it is possible that AP-2α is involved in the retinoic acid signaling pathway(s) which influences patterning of the eye.

We did not observe AP-2α expression in the RPE during development, nor was AP-2α expressed in the neural retina at these early stages of optic cup development suggesting that this phenotype may be a secondary defect caused by

**FIG. 6.** Expression of AP-2 protein in the embryonic and postnatal eye using immunohistochemistry. Key: (ce) corneal epithelium, (ey) eyelid, (en) corneal endothelium, (g) ganglion cell layer, (in) inner nuclear layer, (ir) iris, (me) mesenchymal cells, (re) retina, (s) corneal stroma. (A) E15. AP-2γ expression was detected in the mesenchymal cells of the eyelid (arrowhead). Mag. approx. 150×. (B) Higher magnification of A demonstrating AP-2γ staining in the eyelid (arrowhead). Mag. approx. 560×. (C) Postnatal day 10. AP-2 expression (pan) is demonstrated in the corneal epithelium, corneal stroma, and endothelium, iris, and retina. Mag. approx. 280×. (D) Higher magnification of the cornea shown in C. Mag. approx. 560×. (E) Postnatal day 14. Expression of AP-2α in the retina is confined to cells in the ganglion cell layer and the inner nuclear layer. Mag. approx. 560×. (F) Postnatal day 14. Expression of AP-2α in the cornea is specific to the basal cells of the epithelium. Mag. approx. 560×. (G) Expression pattern of additional AP-2 proteins in the AP-2α-deficient mutant eye at E15 using the pan-specific antibody (pan). AP-2 protein is expressed in the neural retina (unlabeled arrowheads), and mesenchymal cells near the lens. Mag. approx. 220×.
earlier disruptions in the proximate tissue interactions involved in ocular development. The developing lens and neural crest derived mesenchymal cells surrounding the developing optic cup are known to influence development of the optic cup (Reneker et al., 1995). One possibility is that loss of AP-2α expression in the lens placode may have altered the signals required for specification of RPE on the dorsal side of the cup. Lack of differentiation of the iris and ciliary body from the anterior margins of the optic cup in the mutant eye may also have occurred due to alterations in the signals normally provided by the lens. It is equally possible, however, that the mutant optic cup lacks the competence to differentiate into the iris and ciliary body.

The retinal defects in the older knockout embryos, as well as in the chimeras, including the lack of ganglion cell and inner plexiform layers, suggests an intrinsic role for AP-2α during normal histogenesis of the retina. Our inability to detect Brn-3b expression in the mutant retina confirmed our observations that a distinct ganglion cell layer was absent. The lack of Brn-3b expression could be due to a delay in differentiation of a subset of ganglion cells or loss of neurons contributing to the ganglion cell layer altogether. Since AP-2 is expressed with the onset of differentiation in the ganglion cell layer and neuroblastic zone of the presumptive inner nuclear layer, it may participate in the signaling pathways which determine the final differentiation outcome of the inner retinal layers. AP-2α appears to be expressed in a subset of retinal cells after they complete mitosis, suggesting that it is involved in the differentiation pathways rather than earlier cell fate decisions, however this remains to be tested.

In summary, we have shown that each of the three AP-2 proteins (α, β, and γ) have dynamic spatial and temporal expression patterns during development of the murine eye, with AP-2α expressed in a number of ocular tissues which exhibited developmental defects in the AP-2α null mutants and chimeras. These data support a direct requirement for AP-2α in early morphogenesis of the eye, and in particular for the ocular structures in the anterior segment, including the lens and cornea. The human AP-2α gene, TFAP2α, has been mapped cytogenetically to human chromosome 6p24 (Davies et al., 1995), which lies within a region that has been associated with a diverse array of human ocular disorders (Palmer et al., 1991). Among these disorders is Peters’ anomaly and Rieger’s syndrome (Fitch and Kayback, 1978) which have phenotypes resembling those observed in the AP-2α mouse mutants, including a corneal-lenticular adhesion anomaly. Further analysis of the specific role(s) that AP-2α has in morphogenesis of the eye may help to determine the candidate disease genes and developmental mechanisms responsible for these disorders.

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