Differential Expression of \( \alpha \text{A-} \) and \( \alpha \text{B-} \) Crystallin During Murine Ocular Development

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**Purpose.** To compare the temporal and spatial expression patterns of \( \alpha \text{A-} \) and \( \alpha \text{B-} \) crystallin mRNA during ocular development.

**Methods.** Tissue samples from embryonic day 9.5 (E9.5) through postnatal day 14 were collected from FVB/N strain mice. The specimens were fixed in paraformaldehyde, histologically processed, and assayed for \( \alpha \text{A-} \) and \( \alpha \text{B-} \) crystallin mRNA expression by in situ hybridization.

**Results.** During ocular development, \( \alpha \text{B-} \) crystallin transcripts are present in the lens placode at E9.5. Transcripts of \( \alpha \text{A-} \) crystallin are first observed in the lens cup at E10 to 10.5. During subsequent development of the lens, \( \alpha \text{A-} \) crystallin transcripts are most abundant in the fiber cells, and \( \alpha \text{B-} \) crystallin mRNA is preferentially expressed in epithelial cells. Transcripts of \( \alpha \text{A-} \) crystallin were detected only in the lens. In contrast, \( \alpha \text{B-} \) crystallin transcripts are present in retinal pigment epithelium, optic nerve, extraocular muscle, iris, ciliary body, cornea, and several nonocular sites, such as heart and nasal epithelium.

**Conclusions.** Transcription of \( \alpha \text{B-} \) crystallin precedes the expression of \( \alpha \text{A-} \) crystallin during murine ocular development. Furthermore, the patterns of \( \alpha \text{A-} \) and \( \alpha \text{B-} \) crystallin expression in the lens are distinctive: \( \alpha \text{A-} \) is upregulated and \( \alpha \text{B-} \) is downregulated during prenatal fiber cell differentiation. These results indicate that the \( \alpha \)-crystallin genes are not identically regulated either within or outside the lens.

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The vertebrate lens expresses two \( \alpha \)-crystallin proteins encoded by the \( \alpha \text{A-} \) and \( \alpha \text{B-} \) crystallin genes. Traditionally, the \( \alpha \)-crystallins have been thought to function as lens-specific structural proteins. Both \( \alpha \)-crystallins are evolutionarily related to the small heat-shock proteins of *Drosophila melanogaster*. The discovery that, like heat shock proteins, \( \alpha \)-crystallins can function as molecular chaperones suggests that \( \alpha \)-crystallins may have specific biochemical functions beyond that of purely structural proteins.

The \( \alpha \text{A-} \) and \( \alpha \text{B-} \) crystallin proteins are approximately 60% identical in amino acid sequence and are encoded by two unlinked genes thought to have arisen by gene duplication. Even though \( \alpha \text{A-} \) crystallin is largely a lens-specific protein, trace amounts (up to 17 ng/mg protein in rat spleen) have been found in other tissues. Significant amounts of \( \alpha \text{B-} \) crystallin (up to 2% of total soluble protein in rat soleus muscle) have been reported in several nonocular sites, including heart, skeletal muscle, and kidney. Additionally, \( \alpha \text{B-} \) crystallin has been detected in skin, lung, placenta, and brain. \( \alpha \text{B-} \) crystallin accumulation is also a feature of some astrocytic tumors and several degenerative disease processes. In contrast to \( \alpha \text{A-} \) crystallin, which is not stress inducible, \( \alpha \text{B-} \) crystallin transcription is induced by heat and osmotic shock in cultured cells.

Although some studies have reported little difference in the onset or pattern of mammalian \( \alpha \text{A-} \) and \( \alpha \text{B-} \) crystallin expression in the lens, the reports suggest temporal and spatial differences for the developmental expression of these genes. To date, no detailed studies comparing the expression patterns of \( \alpha \text{A-} \) and \( \alpha \text{B-} \) crystallin mRNA throughout murine ocular development have been carried out. To analyze the regulation of \( \alpha \text{A-} \) and \( \alpha \text{B-} \) crystallin expression between embryonic day 9.5 (E9.5) and postnatal day 14 (P14) of mouse development, mRNAs for these two molecules were localized by in situ hybridization. The in situ hybridizations revealed that \( \alpha \text{B-} \) crystallin is ex-
pressed by E9.5 in the lens placode, whereas αA-crystallin transcripts are detected at E10.5. Within the prenatal lens, αB-crystallin is expressed primarily by the epithelial cells, and αA-crystallin is transcribed preferentially in the fiber cells.

METHODS

Collection of Tissues
Embryos were collected from timed matings of albino inbred (FVB/N) mice. Noon on the date of identification of a vaginal copulation plug was considered to be 0.5 days of pregnancy (E0.5). Embryos were collected each day from E9.5 through E15.5. Embryos were dissected free from extraembryonic membranes in RNase-free phosphate-buffered saline and immediately fixed overnight in 4% phosphate-buffered paraformaldehyde, pH 7.3, at 4°C. Eyes were removed from postnatal mice at birth and at 9 and 14 days after birth and were fixed overnight in 4% paraformaldehyde. All animals were used in accordance to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of Riboprobes
The plasmid pMzAcR2 containing a cDNA clone from murine αA-crystallin was provided by Dr. Joram Piatigorsky (National Institutes of Health, Bethesda, MD). An 880 bp PstI fragment of pMzAcR2 encoding amino acids 10 to 173 and 388 bp of 3' untranslated sequence of αA-crystallin was subcloned into the PstI site of pBluescript KS (Stratagene, La Jolla, CA) to create pBSαARPV. The plasmid pCry1, containing a 694 bp polymerase chain reaction-amplified cDNA clone of murine αB-crystallin11 inserted into the Smal site of pBluescript KS, was provided by Dr. Roman Klemenz (University Hospital, Zurich, Switzerland). The polymerase chain reaction-amplified cDNA in pCry1 included 27 bp of 5' untranslated sequence, as well as the entire coding and 3' untranslated sequences of αB-crystallin mRNA. For αA-crystallin, pBSαARPV was cut with EcoRI and transcribed with T7 RNA polymerase (Pharmacia, Piscataway, NJ), or cut with BamHI and transcribed with T3 RNA polymerase (Stratagene) to generate sense and antisense riboprobes, respectively. For αB-crystallin, pCry1 was cut with XbaI and transcribed with T3 RNA polymerase (Stratagene) to generate sense and antisense riboprobes, respectively. In vitro transcriptions were performed using 35S-labeled UTP (Amersham, Arlington Heights, IL) as described previously.22

In Situ Hybridization
Paraformaldehyde-fixed tissues were processed and hybridized as described previously.22 Hybridized slides were air dried, dipped in Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY), and exposed in the dark for 48 hours before development with Kodak D-19 developer. Slides were counterstained with Harris hematoxylin.

RESULTS

Expression of α-Crystallins in Early Ocular Development
By E9.5 in the mouse, the optic vesicle has come into contact with the surface ectoderm, and the lens placode has formed. At this stage, no transcripts of αA-crystallin were detected in the lens placode or elsewhere (Figs. 1A, 1C, 1E). In contrast, αB-crystallin transcripts were detected in the surface ectoderm corresponding to the lens placode (Figs. 1B, 1D, 1F). In addition, an intense signal for αB-crystallin at E9.5 was
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Figure 2. Cardiac expression of αB-crystallin at embryonic day 9.5 (E9.5). Darkfield (A) and brightfield (B) images of a transverse section of an E9.5 mouse labeled by in situ hybridization with a 35S-labeled antisense riboprobe to αB-crystallin. The anterior part of the embryo is to the right. The most intense hybridization is seen in the developing heart (ht). t = tail; ph = pharynx; ot = otic vesicle. Calibration bar = 240 μm.

present in the developing heart (Fig. 2). In fact, αB-crystallin transcripts were found in the heart at all embryonic stages tested, consistent with previous reports of expression in prenatal and postnatal heart tissue. We also detected αB-crystallin hybridization in cells of the amnion (not shown). By E10.5, the lens placode had invaginated to form a lens cup. At this point in development, the patterns of αA- and αB-crystallin mRNA expression in the eye were similar—both were detected only in the cells of the lens cup (Fig. 3). The anterior-most cells of the lens cup, which connect to the surface ectoderm, remained unlabeled with either α-crystallin antisense riboprobe.

At E11.5, the lens cup closes to form a lens vesicle. At this stage, the expression patterns of αA- and αB-crystallin were divergent (Fig. 4). αA-crystallin transcripts, though expressed throughout the lens vesicle (Fig. 4A), were most highly concentrated in the cells of the posterior lens vesicle (Fig. 4C). These cells elongate to form the primary lens fiber cells. In contrast, αB-crystallin transcripts were more abundant in the anterior cells than the posterior cells of the lens vesicle (Figs. 4B, 4D). In addition, at E11.5, transcripts of αB-crystallin were detected in the cells of the posterior optic cup (Fig. 4B, arrow), which differentiate into the retinal pigment epithelium (RPE). Furthermore, αB-crystallin transcripts were detected in the RPE in all subsequent prenatal and postnatal stages examined.

By E12.5, the posterior cells of the lens vesicle elongate into the lumen to form the primary lens fiber cells, and the patterns of αA- and αB-crystallin expression within the eye become more distinctive (Fig. 5). Transcripts of αA-crystallin were lens specific and much more abundant in the fiber cells than in the epithelial cells (Figs. 5A, 5C). In the lens, αB-crystallin transcripts exhibited a reciprocal pattern of expression compared to those of αA-crystallin and were found more abundantly in the lens epithelium than in the lens fibers (Figs. 5B, 5D). In fact, the number of silver grains corresponding to αB-crystallin transcripts in the central primary fiber cells were only slightly above background at E12.5. Positive hybridization for αB-crystallin mRNA also was detected in the RPE and the optic stalk, a precursor of the optic nerve, as well as in the extraocular premuscle mass (Fig. 5B). In addition, αB-crystallin transcripts were detected in the developing olfactory epithelium from E12.5 through the latest stage examined at E15.5 (Fig. 6).

At E13.5, the primary fiber cells have elongated to fill the lumen of the lens vesicle, and secondary

Figure 3. α-Crystallin expression at the lens cup stage. Darkfield (A,B) and brightfield (C,D) images of transverse sections of embryonic day 10.5 (E10.5) mouse eyes labeled by in situ hybridization with antisense riboprobes to αA-crystallin (A,C) or αB-crystallin (B,D). Hybridization signals are evident in the posterior cells of the lens cup (lc) for both α-crystallin genes. At this stage, no hybridization was detected in the cells of the surface ectoderm (se) or in the cells of the optic cup, which will differentiate into neural retina (nr) and retinal pigment epithelium (rp). Calibration bar = 60 μm.
Developmental Expression of α-Crystallin Genes

2279

FIGURE 4. α-Crystallin expression at embryonic day 11.5 (E11.5) (the lens vesicle stage). Darkfield (A,B) and brightfield (C,D) images of transverse sections through E11.5 mouse eyes labeled by in situ hybridization with antisense riboprobes to αA-crystallin (A,C) or αB-crystallin (B,D). The brightfield image for αA-crystallin (C) shows the intense hybridization in the primary fiber cells of the lens vesicle (lv). The silver grains are so concentrated that the signal appears artifactually dark (indicated by +) in the darkfield image (A). The hybridization signal for αB-crystallin is concentrated over the anterior epithelial cells of the lens vesicle and is also seen in the developing retinal pigment epithelium (rp), indicated by the arrow (B). Neither probe hybridizes to the neural retina (nr) at this stage of development. Calibration bar = 115 μm.

fiber cells are beginning to differentiate from lens epithelial cells at the lens equator. Transcripts of αA-crystallin, though still detected above background in the lens epithelium, were concentrated in the lens fiber cells (Figs. 7A, 7C). This pattern of αA-crystallin mRNA distribution remained consistent through E14.5 (Fig. 8) and E15.5 (not shown). Hybridization to the αB-crystallin riboprobe increased in lens fiber cells from E12.5 to E15.5, but the concentration of transcripts remained highest in the lens epithelium (Figs. 5, 7, 8). Outside the lens, αB-crystallin mRNA was detected in the optic nerve, the extrinsic ocular muscles, and the cornea. In the optic nerve, αB-crystallin expression remained localized to the neuroepithelium of the optic stalk, and this pattern of expression continued through E15.5. No αB-crystallin expression was detected in the axons of the retinal ganglion cells (Fig. 8), which begin invading the optic nerve by E12.5. The extrinsic ocular muscles expressed αB-crystallin mRNA at all stages examined (Figs. 5B, 7B, 8B). In the cornea, αB-crystallin was first detected in the putative epithelium at E13.5 (Fig. 7B). Both the corneal epithelium and the inner surface of the cornea, corresponding to the position of the future endothelium, demonstrated hybridization above background levels at E14.5 (Fig. 8B, arrows) and at E15.5 (not shown). No specific hybridization was detected using sense riboprobes to either αA- or αB-crystallin at any stage examined (see Fig. 8).

Expression of β-Crystallins During Postnatal Development

After birth, the expression patterns of αA- and αB-crystallin within the lens were similar (Fig. 9). Transcripts from both genes were detected in the epithelium, but both transcripts were substantially higher at the lens equator, where secondary fiber cell differenti-
FIGURE 6. αB-Crystallin expression in nasal epithelium. Dark-field (A) and brightfield (B) images of transverse sections through an embryonic day 14.5 mouse head labeled by in situ hybridization with an antisense riboprobe to αB-crystallin. Transcripts of αB-crystallin are present in the nasal epithelium (arrows) lining the developing nasal passages. Calibration bar = 480 μm.

FIGURE 7. α-Crystallin expression at embryonic day 13.5 (E13.5). Darkfield (A,B) and brightfield (C,D) images of transverse sections through E13.5 mouse eyes labeled by in situ hybridization with antisense riboprobes to αA-crystallin (A,C) or αB-crystallin (B,D). The hybridization signal for αA-crystallin is intense in the lens fibers (lf, C), which appear artifactually dark in the darkfield image (+). Transcripts of αA-crystallin are also present in the lens epithelium (le). Hybridization to αB-crystallin is more intense in the epithelium than in the fiber cells. Transcripts of αB-crystallin also are detected in the retinal pigment epithelium (rp), optic muscles (om), optic nerve (on), and developing cornea (c; arrows, B). No hybridization is evident in the neural retina (nr). Calibration bar = 120 μm.

DISCUSSION

The α-crystallins are synthesized as major components of all vertebrate lenses. These proteins are thought to be essential for the maintenance of lens transparency. In addition, the pattern of expression of αB-crystallin suggests that it may have an important function in other normal tissues and in cellular responses...
Developmental Expression of α-Crystallin Genes

FIGURE 8. α-Crystallin expression at embryonic day 14.5 (E14.5). Darkfield (A,B,E,F) and brightfield (C,D) images of transverse sections through E14.5 mouse eyes labeled by in situ hybridization with antisense (A-D) or sense (E,F) riboprobes to αA-crystallin (A,C,E) or αB-crystallin (B,D,F). Hybridization to the antisense αA-crystallin riboprobe is limited to the lens, where it is most abundant in the lens fiber cells (lf). In the lens, hybridization to the antisense αB-crystallin riboprobe is most intense in the lens epithelium (le). In addition, the antisense αB-crystallin riboprobe labeled extrinsic ocular muscles (om), the retinal pigment epithelium (rp), and the epithelial, as well as endothelial surfaces (arrows, B) of the cornea (c). Hybridization to the antisense αB-crystallin riboprobe was not detected in the neural retina (nr) or in the ganglion cell axons present in the optic nerve (on). No specific hybridization was detected with sense riboprobes to either αA-crystallin (E) or αB-crystallin (F). Calibration bar = 240 μm.

FIGURE 9. Postnatal expression of α-crystallin in the lens. Brightfield images of transverse sections at birth (P0) (A,B) and at postnatal day 9 (P9) (C,D) and P14 (E,F) mouse eyes labeled by in situ hybridization with antisense riboprobes to αA-crystallin (A,C,E) or αB-crystallin (B,D,F). Hybridization is indicated by the black appearance of the silver grains. As the lens ages, transcripts for both αA- and αB-crystallin become progressively more restricted to the young cortical fiber cells and more sparse in the older nuclear fiber cells. Calibration bar = 240 μm (A,B), = 480 μm (C to F).

Interestingly, these investigators found no α-crystallin immunoreactivity in the rat lens placode but did find αA-crystallin expression in the lens cup, followed by αB-crystallin expression 2 days later at E14.19 Also in the rat, Northern blot analyses have indicated that αB-crystallin expression begins several days after αA-crystallin expression.18 In the amphibian, Rana temporaria, neither α-crystallin subunit was detected immunohistochemically in the lens epithelium of developing or young froglets.30 These observations suggest that there may be species-specific variations among vertebrates in the expression patterns of αA- and αB-crystallins.

In our studies, expression of αA-crystallin mRNA was first detected in the invaginating lens cup at E10.5. This is slightly earlier than that reported by Tréton et al.,27 who also used in situ hybridization to mouse embryos, and detected αA-crystallin mRNA in the elongating posterior cells of the lens vesicle at E11.5. Brahma and Sanyal18 reported that both αA- and αB-crystallin immunoreactivity first appear in the mouse lens vesicle at E11.25.

The patterns of αA- and αB-crystallin mRNA expression within the mouse lens are strikingly diver-

to certain chronic diseases.10–13 Although no other lens proteins have been studied more intensively, questions about the temporal and spatial patterns of expression of the two α-crystallin genes during mammalian ocular development have remained unanswered. In this study of the developing mouse eye, we have demonstrated that αB-crystallin mRNA is expressed in the lens placode and that it precedes the expression of αA-crystallin. This finding is in agreement with that reported by Oguni et al.,19 in which they found αB-crystallin, but not αA-crystallin, in the human lens placode by immunohistochemistry.
Cornea (c) (C,D) and ciliary body (cb) (E,F). High magnification antisense riboprobes to αB-crystallin. In addition to the lens gent. Although both α-crystallin mRNAs can be detected in all parts of the lens after the lens cup stage, αA-crystallin mRNA was consistently more abundant in fiber cells than in epithelial cells, and αB-crystallin transcripts displayed a reciprocal expression pattern. The observed pattern of α-crystallin mRNA accumulation is consistent with the findings of several groups who have reported quantitative increases in the αA-crystallin:αB-crystallin ratios as bovine lens cells differentiate from epithelial cells to fiber cells.

In contrast to αA-crystallin, αB-crystallin expression was detected in many ocular sites outside the lens, including RPE, ciliary body, iris, cornea, and optic stalk–nerve. Expression of αB-crystallin in the RPE was first detected at E11.5 and was present throughout embryogenesis. At P0, P9, and P14, the density of silver grains over the RPE was only slightly above background levels. αB-crystallin expression in the postnatal rat RPE and in human RPE cultures has been reported. αB-crystallin transcripts were present in the developing ciliary body at E15.5 and in the postnatal iris epithelium, consistent with reported immunolocalization studies. In the cornea, αB-crystallin has been reported only in endothelium, where it was localized immunohistochemically in postnatal bovine, human, and monkey eyes. We first observed corneal expression of αB-crystallin on the anterior surface of the cornea at E13.5. At E14.5 and E15.5, both the anterior (epithelial) and the posterior (endothelial) surfaces of the cornea exhibited hybridization signals for αB-crystallin. At P0, αB-crystallin hybridization was decreased in the corneal epithelium, but no transcripts were detected in the corneal endothelium. By P9 and P14, strong hybridization to αB-crystallin was detected in the corneal endothelium, with no signals detected elsewhere in the cornea. At this point, it is unclear whether the transient appearance, disappearance, and reappearance of αB-crystallin transcripts on the endothelial surface of the cornea represents temporal mRNA expression patterns from the same population of cells or whether different populations of cells express αB-crystallin in this location at different time points.

αB-crystallin transcripts also displayed an interesting pattern in the developing optic nerve. Although no hybridization was detected in the optic stalk at E10.5, the optic stalk neuroepithelium was heavily labeled at E12.5, when the axons from the retinal ganglion cells begin to invade the optic stalk lumen. At later embryonic time points, the intensity of hybridization signal in the optic nerve decreased, but αB-crystallin transcripts continued to be localized to the optic stalk cells through at least E15.5. It is interesting to note that the cells of the optic stalk give rise to glial cells of the optic nerve. Retinal glia, astrocytes, and Müller cells were among the first extralenticular cells shown to express α-crystallin. Perhaps these optic stalk-derived glial cells are responsible for the αB-crystallin hybridization observed in the postnatal optic nerve.

At present, it is unclear why αB-crystallin is found in diverse ocular sites whereas αA-crystallin remains lens specific. Although the αA-crystallin promoter has been shown to bind the transcription factor Pax-6 in vitro, it is possible that in vivo αB-crystallin expression is regulated more directly by Pax-6 than αA-crystallin. Pax-6 is expressed in many ocular tissues, including the lens, cornea, optic nerve, and retina, as well as in several nonocular sites, such as the developing central nervous system. The expression of αB-crystallin in the snout may be regulated by Pax-6 because Pax-6 is expressed in the prenatal nasal epithelium. However, the expression of αB-crystallin in the muscle and heart is not dependent on Pax-6. In addition, there are sites of Pax-6 expression, such as the brain and neural retina, at which no αB-crystallin transcripts are detected. In fact, no α-crystallin transcripts were detected in the neural retina at any stage.
examined. Therefore, although Pax-6 may be important for αB-crystallin expression in certain tissues, particularly in lens and nasal epithelium, Pax-6 expression alone is not sufficient to activate αB-crystallin transcription in some tissues, such as neural retina, and appears unnecessary to induce αB-crystallin in other tissues, such as heart. We did not detect any αA-crystallin transcripts outside the lens, but our search for transcripts outside the developing eye was not exhaustive.

The divergent patterns of αA- and αB-crystallin transcript accumulation within and outside the lens are consistent with the previous finding that these proteins exist independently in both the heart and lens. These results suggest that αB-crystallin, in particular, may have a biologic role completely independent of αA-crystallin. The αA- and αB-crystallin promoters have been studied in some detail, and many of the elements required for lens and nonocular expression have been identified. Future studies will define more precisely the regulatory regions responsible for the expression of αA- and αB-crystallin mRNA in the lens and in the rest of the developing eye. Because both genes are expressed in the lens, there may be some overlapping regulatory features. However, the reciprocal patterns of mRNA accumulation in the lens suggest that the two α-crystallin genes have evolved independent mechanisms of regulation within, as well as outside, the lens.

Key Words

crystallins, development, gene expression, in situ hybridization, lens

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