Aldose Reductase mRNA Is an Epithelial Cell-Specific Gene Transcript in Both Normal and Cataractous Rat Lens

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Aldose reductase (AR) is implicated in the development of sugar cataracts by its reduction of galactose or glucose to polyols. The authors' recent work suggested that AR mRNA is found to be expressed in high concentrations in rat-lens epithelial cells after exposure of the animal to a diet containing 50% galactose. They localized the AR mRNA in the lens cells by in situ hybridization with a previously described AR clone. The data establish that AR mRNA is apparently an epithelial and not a fiber-cell gene transcript. It accumulates in the epithelial cell, then it is carried into the newly differentiated fiber cell, and finally it concentrates in the posterior region of the matured fiber cell. The AR mRNA is found in all of the anterior epithelial cells including the equatorial and central epithelia. It is present at highest concentrations in the elongating epithelial cells, and it is distributed equally throughout the secondary fiber cells at the bow, with no indication of a preferential buildup of AR mRNA in any of the nucleated fiber cells in the cortex. This differs from what the authors reported to occur with MP26 mRNA, a fiber cell-specific gene transcript. They found that MP26 mRNA was absent from the epithelial cells but was preferentially found in the secondary fiber cells. Present data suggest that the increase in AR mRNA concentration observed to occur in cataractous lenses is a result of epithelial cell proliferation, where every cell appears to be competent in expressing AR mRNA. The results of this research imply that AR mRNA is a lens epithelial cell-specific gene transcript in both normal and cataractous lenses. Invest Ophthalmol Vis Sci 31:1876-1885, 1990

Localization of aldose reductase (AR) mRNA in normal and cataractous rat lens by in situ hybridization is expected to yield information on its preferential distribution and level of its expression in those cells. This enzyme has been shown to belong to the same protein superfamily as aldehyde reductase, prostaglandin F synthase, and α-crystallin.1 The AR is a monomer of α subunits, and aldehyde reductase is a dimer of α and β or δ subunits.2 In particular AR is thought to be responsible for accumulation of polyols in lenses exposed to high doses of glucose or galactose.3,4 Therefore, quantitation of AR mRNA, including its nuclear RNA precursor, by in situ hybridization methods can provide an estimate of the level of a cell-specific gene response, if any, to the pathology of sugar cataracts. In this report we show that AR mRNA is apparently a lens epithelial-cell and not a fiber-cell gene transcript. It is expressed in both the monolayered and multilayered epithelial cells. Its distribution suggests dynamic behavior, ie, it is long lived and appears to migrate along and within the differentiated fiber cell to collect finally in the posterior region of mature fiber cells.

Materials and Methods

AR 10Q Clone

The AR 10Q clone was isolated from a λgt11 rat lens cDNA library as described.3 The AR cDNA insert is 1206 base pairs in length, corresponding to about 85% of the full size expected for AR, and it contains virtually the entire 3′-untranslated region. The insert was subcloned5 into a Bluescribe plasmid (Stratagene, San Diego, CA) for generating RNA transcripts used in the hybridization experiments.

Preparation of Sense and Antisense AR RNA Probes

For synthesis of 35S-labeled antisense RNA we used a 20-μl reaction mixture containing6 400 μCi (19 μM) of 35S-labeled uridine triphosphate (specific activity, > 1000 Ci/mM), evaporated to dryness, 4 μl of

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5 × transcription buffer (40 mM Tris HCl, pH 7.9, 10 mM NaCl, 6 mM MgCl₂, and 2 mM spermidine), 2 μl of 100 mM dithiothreitol, 1 μl of 10 mM solution each of adenosine triphosphate, cytidine triphosphate, guanosine triphosphate, 1 μg recombinant Bluescribe plasmid restricted with the appropriate restriction enzyme—for the AR 10Q clone; restricted with PvuII for synthesis of full-length antisense cRNA with T3 RNA polymerase, and Smal for synthesis of full-length sense cRNA with T7 RNA polymerase—for the MP26 clone; restricted with PvuII for synthesis of sense RNA with T3 RNA polymerase, and Smal for synthesis of antisense RNA with T7 RNA polymerase—40 units RNasin, and 15–20 units T7 or T3 RNA polymerase. The reaction mixture was incubated at 37°C for 30 min. An additional 15–20 units of T7 or T3 RNA polymerase were added, and incubation was continued for an additional 30 min at 37°C. The DNA template was later digested with 2 units of RNase-free DNase per μg DNA template at 37°C for 15 min in the presence of an additional 40 units of RNasin. After digestion with DNase, 50 μg of yeast tRNA were added, and the mixture was diluted to 100 μl with 1 × saline sodium citrate (0.15 M NaCl, 0.015 M Na citrate) and immediately deproteinized with phenol and chloroform-isooamyl alcohol by standard methods. The RNA was precipitated overnight by the addition of 4 M Na acetate, pH 6.0, to 0.3 M, and 2.5 volumes of -20°C ethanol. The precipitated RNA was collected by centrifugation, dried under a vacuum at room temperature, and dissolved in 50 μl of sterile diethylpyrocarbonate-treated distilled water (DEPC-water). Low molecular-weight materials were removed by passage through a Select-D (RF) column (5 Prime-3 Prime, West Chester, PA). The 35S-RNA was recovered in a final volume of 50 μl.

In Situ Hybridization

Glass slides were coated with poly-L-lysine hydrobromide (molecular weight, 150,000–300,000; Sigma, St. Louis, MO) to enhance tissue-section adhesion. Tissue sections of 5 μm were prepared from fresh lens enucleated from 50 g female Sprague Dawley rats (3–4 weeks of age) fed either Purina chow alone or in combination with 50% galactose for up to 20 days, resulting in sections representing either normal or cataractous lens of different stages. These studies conformed to the ARVO Resolution on the Use of Animals in Research. Lenses were fixed in 4% paraformaldehyde, and tissue sections were prepared for in situ hybridization analysis. The sections were deparaffinized and hydrated in DEPC-water by standard procedures, washed twice in phosphate-buffered saline (PBS), and incubated in 0.1 M Tris-HCl/50 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, containing 1 μg of proteinase K (Boehringer Mannheim, Indianapolis, IN) per ml for 30 min at 37°C. After incubation with proteinase K, slides were rinsed in 4% paraformaldehyde for 20 min, washed once for 5 min in 3 × PBS, twice for 5 min in PBS, rinsed in DEPC-water, dehydrated in graded ethanol:DEPC-water (30%, 60%, 80%, and 95%) for 15 sec each, and finally air dried. The slides were rinsed in 100 mM triethanolamine, pH 8.0, and acetylated in the same buffer containing 0.25% acetic anhydride by incubation for 10 min at room temperature. Slides were then removed and allowed to air dry for about 15 min at room temperature. A volume of 50 μl of hybridization reaction mixture (50% deionized formamide/300 mM NaCl/20 mM Tris-HCl, pH 7.5, with 5 mM EDTA, pH 8.0, with 1 × Denhardt’s solution/10% dextran sulfate/100 mM dithiothreitol, and 1–2 × 10⁶ cpm of 100°C preheated probe per 10-μl reaction volume) was placed on each slide per 22 × 60-mm coverslips. Slides were covered with silicconized coverslips, edges sealed with rubber cement, and incubated in a sealed slide box for 17–20 hr at 45–50°C. After incubation, coverslips were removed by immersion in washing buffer (50% deionized formamide/2 × SSC/100 mM dithiothreitol, pH 7.5), and the slides were washed in the same buffer by incubation for 20 min at 54°C, rinsed twice in 2 × SSC/10 mM dithiothreitol, incubated in 2 × SSC/1 mM EDTA containing 20 μg per ml RNase A plus 1.0 unit per ml RNase T1 for 30 min at 37°C, transferred to washing buffer, and again incubated for 20 min at 54°C. The latter step was repeated twice, then rinsed twice in 2 × SSC. The slides were dehydrated in graded ethanol (30%, 60%, 80%, and 95%) containing 400 mM ammonium acetate for 15 sec each, air dried, immersed in NTB2 (Eastman Kodak, Rochester, NY) nuclear track emulsion diluted 1:1 with 600 mM ammonium acetate in the dark, air dried in a sealed box, exposed at 4°C for 5–6 days, developed, stained with hematoxylin and eosin, and finally mounted with coverslips for microscopy.

Results

In situ hybridization of tissue sections from normal and cataractous rat lens with 35S-labeled sense AR RNA probe is shown in Figure 1. The results demonstrate that insignificant hybridization occurred with RNA transcribed with T7 RNA polymerase from the AR clone both for normal lens (shown at a magnification of 790X) and for the 20-day cataractous lens (shown at a magnification of 200X). On the other hand, antisense RNA transcripts from the AR clone, catalyzed by T3 RNA polymerase, show positive hy-
Fig. 1. In situ hybridization of lens sections with $^{35}$S-labeled sense RNA transcripts from aldose reductase 10Q clone as described under Materials and Methods. (A) Bow of normal rat lens, magnification ×790; (B) multilayered epithelial cells from 20-day cataractous lens, magnification ×200.

Hybridization with the lens sections as evidenced by the high density of grains seen in Figure 2. The following data quantitate the distribution of $^{35}$S-labeled AR RNA probe in the normal lens (Fig. 2B). We compared our previous results on the distribution of MP26 mRNA, a fiber cell-specific gene transcript, in an equivalent lens section processed under the same experimental conditions (Fig. 2A). The data strongly indicate that AR RNA grains appear in a cluster at the zone of initiation of epithelial cell elongation, while the number of those grains is diminished significantly in the differentiated fiber cell (Fig. 2B).
Fig. 2. Overall view of bow and cortex extending to the nucleus of normal rat lens sections hybridized with (A) 35S-labeled anti-sense MP26 RNA transcripts prepared as described in our previous communication, and (B) 35S-labeled anti-sense aldose reductase RNA transcripts prepared as described under Materials and Methods. c, cortex; e, epithelium; solid arrows, high grain density areas; open arrows, low grain density areas. MP26 grains are found in secondary fibers at significantly high levels but appear to be at insignificant levels in the epithelia (A). AR grains appear to be most intensive in elongating epithelia, to a lesser extent in the remaining epithelia, but at insignificant levels in secondary fibers. Magnification ×240.

data on the MP26 RNA-grain distribution (Fig. 2A), on the other hand, illustrates the differences in the distribution of grains for a fiber cell-specific gene transcript versus one that appears to be epithelial cell specific. The MP26 RNA-grain assembly begins at the first differentiated fiber cell, and none is found in
Fig. 3. Closer examination of AR grain density at the bow and in the elongating epithelium in normal lens. Open arrows point to the high grain density area. Secondary fibers appear to maintain a significantly lower number of grains around the fiber cell nucleus, and their density does not appear to be particularly intensive about the nucleus, probably signifying lack of active mRNA buildup in those cells, verified by examination of both AR grains about the epithelium nucleus and MP26 grains about secondary fiber cell nuclei. Magnification X790.

epithelial cells, although more AR RNA grains were found in epithelial cells located in the elongation zone (Fig. 2B). This quantity diminishes significantly in the differentiated fiber cell (Fig. 2B). Maturing fiber cells in the cortex continue to have high activity for MP26 RNA (Fig. 2A), and these same cells have a lower activity for AR RNA (Fig. 2B). Closer examination of the elongating cells (Fig. 3) suggests a similar conclusion: newly formed fiber cells lose their ability to accumulate a high density of AR RNA grains, although this activity is evident in the elongating epithelial cells (Fig. 3, open arrows). Further evidence suggesting that nucleated fiber cells in the cortex do not accumulate new AR RNA is shown in Figure 4. There is a comparable distribution of grains between nuclei and cytoplasm that is not significantly higher than the background (Figs. 1, 4). Since the epithelial cells appeared to be highly active in concentrating AR RNA grains, we then examined various epithelial-cell sections spanning from the bow towards the center of the anterior surface. These results (Fig. 5) show that the highest activity occurs in those cells neighboring the elongating cells (Fig. 5A, upper left); however this activity, although of a lower magnitude, continues in all of the epithelial-cell sec-

Fig. 4. Normal rat lens secondary fiber cells nuclei showing lack of retention of high density grains, possibly suggesting lack of new AR mRNA buildup in those cells. There is equal distribution of grains between fiber cell nuclei and cytoplasm. Original magnification X790.
Fig. 5. High AR grain density is found in all of the normal lens epithelial cells examined. (A) Epithelial cells next to bow on left; (B, C, D) examination of other anterior epithelial cells spanning across the anterior surface of the lens to the central epithelial cells shown in (E). Grain density appears to be highest in areas neighboring the elongating epithelial cells in (A), then decreases gradually from sections (A) to (E), the central epithelia zone. Magnification ×790.

The data strongly suggest that AR RNA is a specific product of the lens anterior epithelial cells and not a product of the fiber cells. That it is not a product of the nucleated fiber cells is shown in Figures 2 and 4. Figure 6 displays the density of AR RNA grains found in a normal lens nucleus; this
The current data pertain to the distribution of AR mRNA only and not to its translational product, AR. Table 1 shows a summary of estimated grain count per about 12.5 μm² of normal and cataractous lens sections.

**Discussion**

Our recent data have shown that AR mRNA is increased in rat lens epithelial cells in developing cataracts. This was demonstrated by lens epithelial and cortical cytosol dot-blot hybridization methods using 35S-labeled uridine triphosphate antisense AR RNA transcripts from AR 10Q clone. We wanted to visualize the actual distribution of AR mRNA in both normal lenses and lenses undergoing development of galactose-cataracts. The current data establish that AR RNA grains, a visual measure for the presence of AR mRNA and its nuclear RNA precursor in cells,

![Fig. 6. AR grain density in the normal lens nucleus. Grain density appears to be at level as found in Figure 1 with sense AR RNA probe. Original magnification x790.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933580/)

occurs at a low level as found in the presence of the sense AR RNA probe (Fig. 1).

Further examination of the distribution of AR RNA grains in the normal lens revealed that these grains appear to compartmentalize in the posterior region of the fiber cells (Fig. 7). Such a high density was also observed with the MP26 RNA grains. It is possible that the high number of grains found in these cells reflects active synthesis of AR mRNA accumulating posteriorly during fiber-cell maturation.

Examination of a lens undergoing development of osmotic cataracts confirms that AR RNA grains are confined to the epithelial cells only, further supporting the conclusion that AR mRNA is a product of the epithelial cells and not of the fiber cells, unlike the transcript MP26 mRNA. We did not find any evidence to suggest that in a cataractous lens AR mRNA synthesis may be supported by fiber cells. A representative of AR RNA-grain distribution in a 20-day cataractous lens is shown in Figure 8. A relatively high density of AR RNA grains is found in epithelial cells adjacent to the bow (Fig. 8A, upper left) and in areas harboring multilayered epithelial cells (Fig. 8A, upper right). Figure 8B shows a closer examination of the density of the AR RNA grains in the multilayered epithelial cells. There was significant activity for AR mRNA or its nuclear RNA precursor. Figure 8C shows a similar high activity in proliferating epithelial cells in areas between those examined in Figures 8A and 8B. This supports our previous report on increased AR mRNA concentration in lens epithelial cells undergoing development of cataracts.

![Fig. 7. A concentration gradient in AR grain density is found in normal rat lens sections spanning from nucleated cortical fiber cells to a section of the posterior capsule. A high AR grain density is found to concentrate along the posterior zone of the lens. A similar distribution of MP26 grains along the posterior zone of the lens was found, as we have shown in a previous communication. Original magnification x790.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933580/)

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Fig. 8. Distribution of AR positive grains in rat lens made cataractous by feeding of a Purina Chow diet containing 50% galactose for a period of 20 days. (A) Shows a section of the lens extending from the bow to about the central epithelium, magnification ×200. (B) The multilayered epithelium shown in (A) at right, magnification ×600. (C) Multilayered epithelial cells situated between the bow (A, left) and the triangular multilayered epithelial cells (A, right), magnification ×600. All epithelial cells appear to contain a high density of grains, although a lower level than what is found in the elongating epithelial cells.
are present at high densities in lens epithelial cells (Figs. 2, 3, 5, 8) and in posterior regions of fiber cells (Fig. 7). The data further show that this density decreases significantly and abruptly in elongating fiber cells (Figs. 2, 3) and in cells which first begin to express MP26 mRNA in the lens bow.6 Our comparison of the distribution of AR mRNA versus MP26 mRNA in the lens (Fig. 2) supports the conclusion that, although we found AR RNA grains in fiber cells, these grains probably represent AR mRNA accumulated in elongating epithelial cells (Figs. 2, 3) and not necessarily new fiber-cell gene transcripts. It is apparent that: (1) MP26 mRNA is a product of the differentiated fiber cell, as shown by in situ hybridization6 (Fig. 2); and (2) AR mRNA is a product of the lens epithelial cell, where accumulation is accelerated in elongating epithelial cells (Figs. 2, 3), lens cells that are negative for MP26 RNA grains.6 Elongation of the equatorial epithelial cells is generally correlated with the initiation of secondary fiber-cell differentiation.10 Based on these data we propose that the observed high concentration of AR mRNA in the elongating epithelial cells of normal lens may signify a specific enhancement of gene activity for AR mRNA in these cells. The data further suggest that there is some preference for collecting AR mRNA in fiber cells probably during the process of maturation (Fig. 7). Like MP26 mRNA,6 AR mRNA appears to concentrate in fiber cells in areas neighboring the vitreous humor.10 That AR mRNA is synthesized largely in elongating epithelial cells may imply that the AR gene is destined to become suppressed in differentiated fiber cells, a hypothesis that remains to be confirmed.

Recent studies by Bondy and Lightman11 showed that AR mRNA grains increased in number from the central epithelium to the equatorial region, in agreement with our data (Fig. 5). However, they also reported that the AR mRNA grains were absent from terminally differentiated fiber cells. We found that mature fiber cells appear to retain a high number of grains in their posterior regions (Fig. 7). It is unlikely that these grains represent newly synthesized AR mRNA because the cells appear to lack nuclei. An increase in stability of lens mRNA might account for the survival of AR mRNA in mature fiber cells.12 Since the hybrids are assumed to represent specifically an association between the AR antisense cRNA and the AR mRNA,5,9 the data do not then suggest that AR mRNA fully disappears from mature fiber cells as has been suggested.11

The enzyme, AR, has been repeatedly implicated as a common factor which initiates the cataractous process in both diabetic and galactosemic cataracts.3-4 Various AR inhibitors have been shown to delay the onset of sugar cataracts.13-16 Thus, localization of the enzyme is important. Akagi et al17 localized AR in the human lens to the epithelial cells and the secondary fiber cells. Based on our results on AR mRNA distribution in the rat lens (Figs. 2-8), we showed that AR mRNA concentrates in the epithelial cells, diminishes in the secondary fiber cells, and collects in the posterior region of the lens. Therefore, AR should also be localized in these regions. It is not clear at present why the enzyme was not found in the posterior fiberplasm.17 It is unlikely that all of the AR RNA grains represent hybridization to fragmentary AR mRNA sequences. Liu et al18 reported that relatively high levels of AR were found in the bovine lens epithelium, cortex, and nucleus. It is possible, however, that: (1) the posterior AR mRNA remains inactive in the absence of the enzyme substrate, (2) the enzyme may be found at concentrations that could not be detected by immunofluorescence, (3) the mRNA may be temporally active depending on the state of the nutritional environment of the lens because the mRNA concentrates in a region of the lens that is in contact with the vitreous humor, or (4) in cells containing nuclei high RNA grain density there may be active synthesis of AR mRNA, and in cells which lack nuclei, a high number of RNA grains might represent long-lived RNA either stored for later use or specifically serving a metabolic activity yet unidentified in the posterior fiberplasm. MP26 mRNA distribution6 also shows a preference to collect in posterior regions of mature fiber cells. Analysis of the distribution of specific α-, β-, and γ-crystallin mRNAs does not show preference for concentrating at the posterior fiberplasm (data not shown). So far, preferential accumulation of mRNA in the posterior region of the lens is a property for both AR and MP26 mRNAs. In all probability AR and MP26 mRNAs may have a function associated with the physiologic state of the cell and its membrane.11

Table 1. Summary of estimated grain count per cm² (at a magnification of X790) of normal and cataractous lens sections (all normalized to a magnification of X790)

<table>
<thead>
<tr>
<th>Location</th>
<th>AR probe</th>
<th>Grain no.</th>
<th>Figure no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elongating epithelia</td>
<td>sense</td>
<td>0–10</td>
<td>1A</td>
</tr>
<tr>
<td>Elongating epithelia</td>
<td>anti-sense</td>
<td>&gt;200</td>
<td>3</td>
</tr>
<tr>
<td>Fiber cells (bow)</td>
<td>anti-sense</td>
<td>&lt;50</td>
<td>3</td>
</tr>
<tr>
<td>Distant fiber cells</td>
<td>anti-sense</td>
<td>5–10</td>
<td>4</td>
</tr>
<tr>
<td>Anterior epithelia</td>
<td>anti-sense</td>
<td>&gt;200</td>
<td>5A</td>
</tr>
<tr>
<td>Anterior epithelia</td>
<td>anti-sense</td>
<td>5–10</td>
<td>5B</td>
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<td>Anterior epithelia</td>
<td>anti-sense</td>
<td>&gt;50</td>
<td>5C</td>
</tr>
<tr>
<td>Central epithelia</td>
<td>anti-sense</td>
<td>30–50</td>
<td>5D</td>
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<tr>
<td>Central epithelia</td>
<td>anti-sense</td>
<td>30–50</td>
<td>5E</td>
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<tr>
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<td>7</td>
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<td>Cataractous epithelia</td>
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<td>100–500</td>
<td>8B</td>
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<td>Cataractous epithelia</td>
<td>anti-sense</td>
<td>&gt;100</td>
<td>8C</td>
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</table>
Key words: aldose reductase mRNA, sugar cataracts, in situ hybridization, lens epithelium, MP26 mRNA, lens cortex

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References