Spatial distribution of 3-hydroxy-3-methylglutaryl coenzyme A reductase messenger RNA in the ocular lens: relationship to cholesterologenesis

Richard J. Cenedella and Honglan Shi

Department of Biochemistry, Kirksville College of Osteopathic Medicine, 800 West Jefferson, Kirksville, MO 63501

Abstract This study probes the regulation of cholesterol biosynthesis in the ocular lens by estimating the concentration and distribution of the messenger RNA for the rate-controlling enzyme for sterol synthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR). Because the lens is dependent on biosynthesis for cholesterol, HMGR activity is crucial for the life-long growth of this organ. Young rat lenses were serially divided into several fractions by dissolution in an SDS-containing buffer and each fraction was equated to a percent of the lens radius based upon its protein content. HMGR enzyme activity and cholesterol synthesis has been shown to disappear from the lens cortex over a narrow arc of radius due to loss of enzyme protein. Using a published competitive reverse transcriptase-polymerase chain reaction method for amplifying HMGR mRNA (Powell, E. E., and P. A. Kroon. 1992. *J. Lipid Res.* **33:** 609–614), an average of about 46,000 copies of this mRNA was estimated per lens at all rat ages examined (5-day-old to adult). However, copies/μg total RNA decreased with aging. The distribution of HMGR mRNA across 95–60% of the lens radius was essentially uniform at 2000–3000 copies/mm^3 tissue. But the very superficial cortex contained 5- to 7-times this concentration and accounted for about 35% of the total copies/lens. We estimated that cells in this region each contained 1 to 2 copies of message, a value similar to the estimated copy number of HMGR message in human lymphocytes (Powell and Kroon, ibid). This suggests that the translational efficiency and stability of lens HMGR mRNA must be very high. We hypothesize that transcription of the HMGR gene occurs only in the outer 5% of the lens radius and that concentrations of message in deeper lens regions are inadequate to support enzyme synthesis. A halt in enzyme synthesis with even slow steady state proteolysis could result in rapid loss of HMGR protein and, thus, cholesterol synthesis from lens cells.—**Cenedella, R. J., and H. Shi.** Spatial distribution of 3-hydroxy-3-methylglutaryl coenzyme A reductase messenger RNA in the ocular lens: relationship to cholesterologenesis. *J. Lipid Res.* 1994. **35:** 2232–2240.

Supplementary key words competitive RT-PCR • mRNA distribution • cholesterol synthesis

The ocular lens grows throughout life by the terminal differentiation of proliferative epithelial cells into greatly elongated fiber cells. With the exception of the monolayer of epithelial cells that cover the anterior surface, the lens is composed solely of fiber cells. Fiber cell elongation requires formation of vast amounts of plasma membrane (1) that can possess cholesterol to phospholipid molar ratios of as high as 4 to 1 (2). As the lens must obtain this cholesterol by biosynthesis (3, 4), the activity of the rate-controlling enzyme in cholesterologenesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is crucial for the life-long growth of this organ. The capacity of HMGR inhibitors, such as lovastatin, to produce cataracts in animals emphasizes this importance (5).

Our laboratory has been interested in the regulation of cholesterol biosynthesis in the lens. We recently demonstrated that cholesterol synthesis in the lens is spatially coordinated with the synthesis of membrane phospholipid and intrinsic protein (6). Peak synthesis of cholesterol, phospholipid, and intrinsic protein occurred in the outer 3–6% of the lens radius and synthesis of all three halted at about the outer 10% radius mark. The distribution of cholesterol synthesis paralleled the distribution of HMGR enzyme activity and protein mass (7). These findings indicated that the abrupt cessation of plasma membrane formation in the ocular lens involved loss of HMGR activity over a narrow arc of the radius, and this activity loss was due to disappearance of enzyme protein. In the present study, we have attempted to map the spatial distribution in lens of the mRNA for HMGR in order to determine whether the loss of enzyme protein could be related to cessation of enzyme synthesis. A recently reported method for quantitating HMGR mRNA by a competitive reverse transcription (RT)-polymerase chain reaction (PCR) system (8) provided the opportunity to estimate absolute copy numbers of this rare lens message.

Abbreviations: HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; RT-PCR, reverse transcriptase-polymerase chain reaction; DEPC, diethyl pyrocarbonate.

The competitive RT-PCR approach has also been used to quantitate the lens concentration and distribution of rare messages for the c-jun and c-fos protooncogenes and the p53 antioncogene (9, 10). A preliminary account of this work has been presented (11).

MATERIAL AND METHODS

Lens fractionation and RNA extraction

Decapsulated lenses (20–30) from 19- to 20-day-old rats (Hilltop Lab Animals, Inc., Scottdale, PA) killed by carbon dioxide inhalation were divided into several uniform fractions by gradual dissolution in a sodium dodecylsulfate (SDS)-containing buffer as described recently (6). Based upon the protein content of each fraction and information on the radial distribution of protein in the young rat lens (12), the individual fractions were equated to specific percentages of the lens radius. Each of 8 to 10 fractions (3–4 ml/fraction) was flash frozen and lyophilized. The residue was dissolved in 0.2 ml of diethyl pyrocarbonate (DEPC)-treated water and homogenized for 1 min (Tekmar Homogenizer, Tekmar Co., Cincinnati, OH) in 2.4 ml of TRI REAGENT™ and the RNA was extracted according to the manufacturer's instructions (Molecular Research Center, Inc., Cincinnati, OH). In some experiments, fresh intact lenses, decapsulated lenses, or lens epithelia (capsules) from rats of various ages were directly homogenized in the TRI REAGENT™ (1.2 ml/100 mg tissue). The quantity of RNA recovered per lens by direct homogenization versus extraction of the individual solubilized fractions was similar. For example, the RNA content of directly homogenized 26-day-old lenses was 2.01 μg RNA/mg lens (wet) versus 2.09 μg and 2.11 μg/mg of 19- and 20-day-old fractionated lenses, respectively. We assume that the quality of the RNA isolated by the two extraction techniques was similar. The TRI REAGENT™-RNA isolation method provides for the rapid recovery of total RNA of high purity. The RNA content of each sample was estimated from absorbance at 260 nm (1.0 O.D. unit for 40 μg RNA/ml) after dissolving the samples in 70–150 μl of sterile DEPC-treated water. Samples were divided into 10–20 μl aliquots and frozen and stored at −70°C. The RNA samples were subsequently either subjected to electrophoresis through agarose–formaldehyde gels according to Maniatis, Fritsch, and Sambrook (13) or analyzed for their HMGR mRNA copy number as described below.

RT-PCR estimation of HMGR mRNA copy number

HMGR mRNA copy number (c) was estimated by slight modification of the RT-PCR procedure recently described by Powell and Kroon (8). The identified 3' and 5' primers (8) spanning a 246-bp segment of the HMGR cDNA, 2904 bp total (14), were synthesized (Genosys Biotech Inc., The Woodlands, TX). Both the 3' and 5' HMGR primers code for sequences on either side of introns in the genomic DNA; therefore, because they span introns, genomic HMGR DNA would be excluded from amplification. The RNA transcript of plasmid pAW109 (Perkin-Elmer Cetus, Norwalk, CT) yields a 303-bp cDNA product with these primers and, thus, provides a positive internal control for the competitive PCR analysis.

Reverse-transcription and PCR amplification were conducted essentially as described by the Gene Amp RNA PCR kit instructions (Perkin-Elmer Cetus). All reactions were conducted in sterile-capped microfuge tubes (600 μl). The RT master-mix contained 6.35 mM $MgCl_2$, 1.27 × PCR buffer II, 1.27 mM dATP, dGTP, dCTP, and dTTP, 1.27 U/μl of RNase inhibitor, 3.17 U/μl reverse transcriptase, and 1.9 pmol/μl of 3' primer. The master-mix (15.75 μl) was added to 500 to 2400 copies of pAW109 in 1 μl and 400 to 1600 ng of total RNA in 3.25 μl. After overlaying with 40 μl of light mineral oil (Fisher Scientific Co., Fair Lawn, NJ) reverse transcription was conducted for 15 min at 42°C in a water bath. The mixture was then heated at 95°C for 5 min and transferred to an 80°C heated block. Eighty μl of PCR master-mix (at 80°C) was immediately underlayered into each RT-solution and mixed three times by gentle aspiration. The final PCR reaction solution (100 μl) contained 3 mM $MgCl_2$, 1.2 × PCR buffer II, 0.3 μM 3' and 5' primers, 2.1 U Thermus aquaticus DNA polymerase (Perkin-Elmer Cetus) and 14 μM digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN). The cDNA amplification cycle was denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min. Amplification was conducted for 30 to 39 cycles using a Hybaid thermal cycler (National Labnet Co., Woodbridge, NJ).

PCR products were detected by chemiluminescence as described by Powell and Kroon (8). Briefly, 10 μl of each PCR reaction mixture was electrophoresed at 100 v for 3–3.5 h in 3% agarose gels (Bio-Rad DNA Sub-Cell, Bio-Rad Labs, Inc., Hercules, CA) and transferred by overnight blotting to 10 × 15 cm positively charged nylon membranes (Boehringer Mannheim). After baking and exposure to blocking buffer, the membranes were incubated for 30 min with antidigoxigenin-IgG conjugated to alkaline phosphatase (Boehringer Mannheim) diluted 1 to 3500 in blocking buffer. Detection by chemiluminescence was conducted by 5 min treatment of the membrane with 15 ml of CSPD, 0.1 mg/ml (Tropix Inc., Bedford, MA). The moist membrane was placed on a glass plate and covered with an airtight layer of Saran Wrap (Dow Chemical, Co., Indianapolis, IN) and exposure to X-OMAT-AR imaging film (Eastman Kodak Co., Rochester, NY) for 1–30 min. Several exposures were made with each membrane so that bands of a range of in-

tensities could be analyzed by densitometric scanning (GS 300 scanning densitometer, Hoefer Scientific Instruments, San Francisco, CA). The areas under the scan curves of the 303-bp internal control product and the 246-bp HMGR cDNA target product were measured and the copies of HMGR mRNA were estimated by comparison of the relative areas. The control signal corresponded to a known number of pAW109 molecules (500–2400) added to the RT reaction. No PCR products were obtained when reverse transcriptase was omitted from the RT master-mix (under conditions where both the pAW109 and HMGR mRNA products were obtained in its presence). And no products were obtained when RNA was omitted from the otherwise complete RT-PCR reactions.

Assay of HMGR enzyme activity and estimation of protein mass

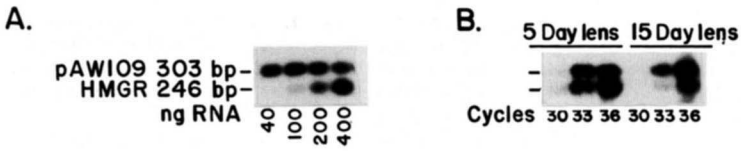
Whole lenses, decapsulated lenses, and lens capsules (epithelial cells) were homogenized in buffer A (5 mM Tris, 1 mM EDTA, 5 mM 2-mercaptoethanol, pH 8) using a Dounce homogenizer and assayed for HMGR enzyme activity by a modification of the method of Panini, Sexton, and Rudney (15) which we recently described in detail (7). One unit of enzyme activity is defined as 1 pmol of mevalonate formed per min at 37°C.

Twenty four lenses from 7- and 14-day-old rats, 30 lenses from 21-day-old rats, and 20 lenses from 50-day-old rats were decapsulated. The outer cortex was dissolved by gentle stirring for 15 min in a tergitol (Type 15-s-9, Sigma Co., St. Louis, MO) containing buffer (5 mM Tris, 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.1 mM leupeptin, 0.25%, w/v, tergitol, pH 8.0). Tergitol, in contrast to SDS, did not inhibit HMGR enzyme activity. Essentially all of the lens HMGR enzyme activity and protein mass is present in the outer cortex (7). The capsules were recombined with the 15-min dissolution fraction of its lens group and Dounce-homogenized. Protein was assayed as before (7) and 0.1% of the total protein (44–52 μg) was subjected to SDS-PAGE. Aliquots of each homogenate were also assayed for HMGR enzyme activity (U/lens). Relative HMGR protein mass was estimated by an enhanced chemiluminescence detection system (Amersham Corp. Arlington Heights, IL) after Western blotting with the A9 monoclonal antibody to HMGR and exposure to peroxidase-conjugated goat anti-mouse IgG as previously described (7).

RESULTS

Selection of RNA mass and control copy number for RT-PCR amplification

Amplification of the target HMGR cDNA in the presence of the pAW109 is dependent on the balance between the copies of control and target in the reaction system. In general, a marked excess of either control or target can competitively block the PCR amplification of the other (16). Thus, for each lens RNA sample, a concentration range of RNA and a copy number of pAW109 that would permit expression of both cDNA products was empirically selected by trial and error. Generally the copy number of control was held constant and RNA mass was varied. Apparently there is a fairly broad range of RNA concentrations over which the estimated target number will be constant. For example, amplification of 100 ng to 400 ng of lens capsule RNA in the presence of 1200 c of internal control all gave an estimated 4000–5000 c of HMGR mRNA per μg of total RNA (**Fig. 1A**). Powell and Kroon (8) similarly observed that the copy number estimated for HMGR mRNA from human liver was



ng RNA	cHMGR mRNA per sample	cHMGR mRNA per μg RNA	Cycles	cHMGR/μg Total RNA 5 Days	15 Days
40	-	-	30	5400[a]	-
100	426	4260	33	2900	1200
200	1002	5010	36	3300	1800
400	1563	3908			

Fig. 1. Reverse transcription and PCR amplification of lens HMGR mRNA. A: Influence of RNA concentration. Reverse transcription reaction mixtures (20 μl) contained varying concentrations of total RNA (20-day rat lens capsules) plus 1200 c of pAW109. Eighty μl of PCR master-mix was added and the RT products were amplified for 33 cycles. B: Influence of cycle number. RT reaction mixtures (20 μl) contained 2000 c of pAW109 and 800 ng of total RNA from 5-day-old lenses or 1000 c of internal control and 400 ng of total RNA from 15-day-old lenses. The RNA was extracted from intact lenses. [a]Error for this estimated value could be higher due to the low density of the control and target products signals.

proportional to the total RNA mass in the RT-PCR system over a 4-fold increase in RNA mass and with a constant copy number of pAW109. They also report that the estimated target copy number remained constant over six cycles of amplification using a given mass of RNA and copies of internal control. Usually 33 cycles of amplification was needed to detect the lens HMGR cDNA product when 400–800 ng of RNA was RT-PCR-amplified in the presence of 1000–2000 c of pAW109. The target to control signal ratio resulted in similar HMGR mRNA copy numbers at 33 and 36 cycles; however, there was a tendency for the target copy number to increase with increasing cycles (Fig. 1B). The copy number estimated after 30 cycles for the 5-day lens sample is questioned due to the low intensity of both control and target signals.

Lens concentration and content of HMGR mRNA with aging

Total RNA was recovered from lenses of 5-, 15-, 19-, and 26-day-old rats plus adult females. The RT-PCR amplification systems contained from 400 ng (5 days old) to 1600 ng (adult) of total RNA and from 500 to 2400 copies of pAW109. RNA samples from the 5- and 15-day-old lenses and adult lenses were PCR-amplified on two different occasions (separate days). In addition, RNA extracted from a second pool of adult rat lenses was assayed for HMGR mRNA copy number. Results of the duplicate assays were in good agreement (**Fig. 2A**). In an experiment with adult lenses, RNA was separately extracted from the lens capsule (epithelial cells) and fiber cell mass. The lens concentration of HMGR mRNA sharply decreased with aging from about 3000 c/μg RNA at 5 days of age to about 1000 c/μg at 26 days, and remained constant thereafter (Fig. 2A). The decreasing concentration likely reflected the slowing of rat lens growth by 3 weeks of age (see Fig. 6 insert), and thus a lessening need of cholesterol for membrane formation, plus dilution of the HMGR mRNA by the increased RNAs needed to support massive synthesis of the crystallin proteins. In contrast with concentration, the lens content of HMGR mRNA remained surprisingly constant over the entire age range examined at between 31,000 to 57,000 copies per lens, with a mean of about 46,000 copies (Fig. 2B). This finding suggests that lens HMGR mRNA possesses a relatively long half-life, a possibility supported by the presence of large amounts of RNA in the nucleus, the oldest lens region (**Fig. 3**).

Lenses from 20-day-old rats were fractionated by gradual dissolution in an SDS-containing buffer, RNA recovered, and each fraction was equated to a percent-arc of the radius based upon its protein content. An average radius was calculated from the mean lens volume (mm^3) by equating volume with mass (14.67 mg/lens). Although the outer cortex possessed the highest concentration of RNA (Fig. 3A), the inner two lens fractions together accounted for about 40% of the total lens RNA (Fig. 3B). The presence of 28S and 18S ribosomal RNA bands in all fractions indicates a high stability of at least these lens RNAs with aging (**Fig. 4**).

Copy number distribution of HMGR mRNA in the lens

RNA recovered from fractionated 19-day-old lenses was assayed for HMGR mRNA copy number. Each RT-PCR amplification reaction contained 1200 c of the internal control and 800 ng of total RNA from one of eight lens fractions. RT products were amplified from 30 to 36 cycles. The outer 3% of the lens radius contained about 7400 c of the message/μg of total RNA (**Fig. 5A**) or 15000 c/mm^3 of tissue (Fig. 5B). The outermost fraction ac-

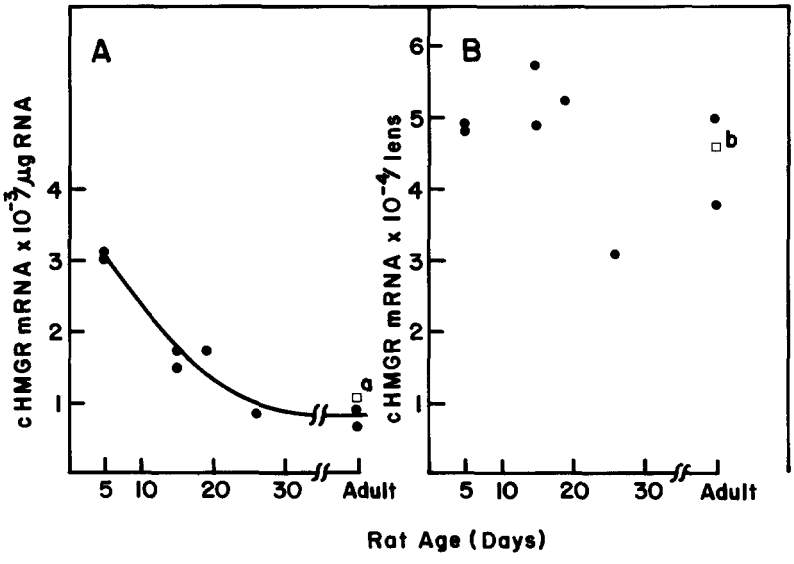


Fig. 2. Lens HMGR mRNA copy number with aging. RT reaction mixtures (20 μl) contained from 500 to 2400 c of the internal control, pAW109, and from 400 to 1600 ng of total RNA. RNA was extracted from pools of intact lenses except for the 19-day-old rats where RNA was extracted from lenses dissoluted into eight fractions by the SDS-containing buffer. These are the same lenses used to generate the data presented in Fig. 5. RT products were amplified from 30 to 39 cycles. Points at given ages represent duplicate RT-PCR assay results obtained with the same RNA extract but run on different days. Total RNA was also extracted and assayed from two different pools of adult rat lens (●, □). [a]Lens epithelium from adult rat lens contained 4200 c/μg RNA versus 920 c/μg RNA in the total fiber cell mass. [b]The epithelium contained 4700 c out of a total 45,700 c/adult rat lens. This is similar to HMGR enzyme distribution where the epithelium (capsule) contained 13% of total lens enzyme activity.

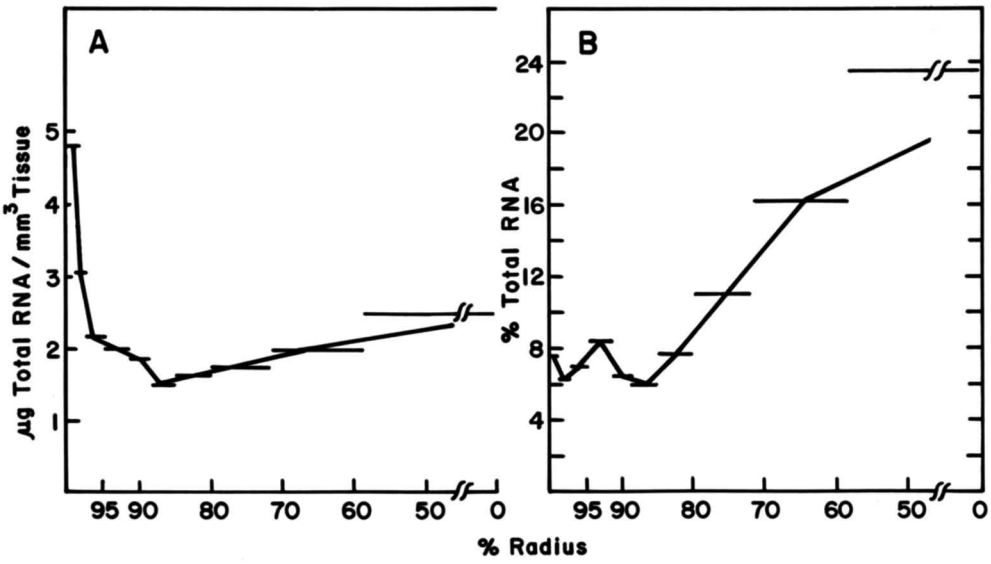


Fig. 3. Radial distribution of total RNA in the rat lens. Thirty decapsulated lenses from 20-day-old rats were separated into 10 fractions by gradual dissolution in 3-ml aliquots of buffer containing 0.2% (w/v) SDS. Each fraction was equated to a percent of the lens radius based upon its concentration estimated from absorbance at 260 nm. The horizontal bars indicate the HMGR mRNA copy number for specific arcs of lens radius.

counted for about 35% of the total copies of HMGR mRNA in the whole lens (18,400 out of 52,400 copies). With the exception of this very superficial cortex, the distribution of HMGR mRNA was surprisingly uniform across the cortex, with 2000–3000 c/mm³ of tissue in fractions spanning from 95% to 60% of the lens radius. Even the nucleus contained about 1200 c/mm³. Similar results were obtained in a second experiment with fractionated 26-day-old rat lenses. The most superficial fraction (100–99% of the radius) contained about 9000 c/mm³ of tissue. The copy number decreased in deeper cortex to a constant 2000–3000 c/mm³ across the cortex (data not shown).

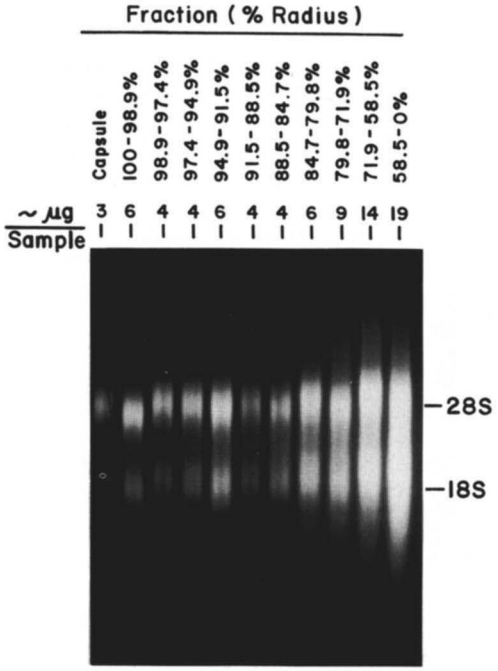


Fig. 4. Electrophoresis of total RNA extracted from 20-day-old rat lenses. Ten percent of each RNA fraction identified in Fig. 3 was separated on 1% agarose gels and stained with ethidium bromide. The mass estimates of RNA applied to each gel lane was based upon absorbance at 260 nm.

Aging-related changes in lens HMGR enzyme activity and protein mass

In contrast to the stable HMGR mRNA content of the lens with aging (Fig. 2B), there was a sharp decrease in the HMGR enzyme activity per lens with aging from 8 to 40–50 days of age (**Fig. 6**). Thereafter, units of activity per lens remained constant. This age-related difference between the messenger RNA and enzyme activity content of the lens could be explained by the possibility that all of the HMGR enzyme protein is functionally active; it is almost entirely located in the outer 10% of the lens radius (7), but much of the HMGR mRNA is not functional because it is present in deeper lens regions where the endoplasmic reticulum required for its translation has disappeared and where mRNA copy numbers could be inadequate to support translation. Aging-related changes in HMGR protein mass paralleled the changes in enzyme activity (**Fig. 7**).

DISCUSSION

Although competitive RT-PCR amplification is probably the most accurate method for quantitating mRNA

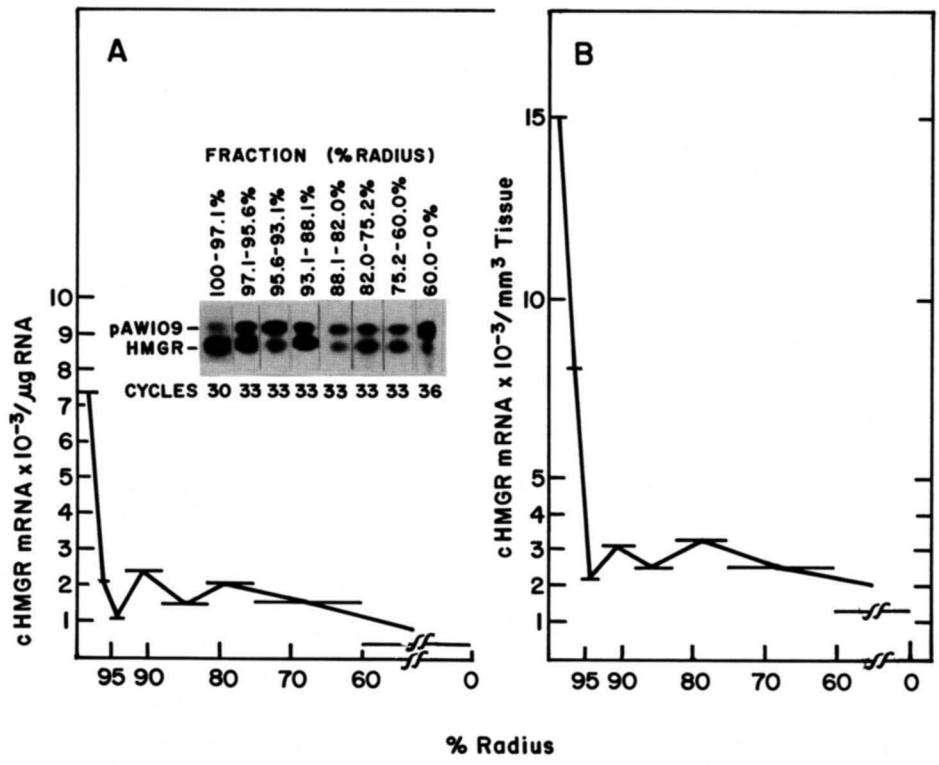


Fig. 5. Spatial distribution of HMGR mRNA copy number in the lens. Twenty decapsulated lenses from 19-day-old rats were fractionated by gradual dissolution in an SDS-containing buffer. RNA was extracted with TRI REAGENT™ after lyophilization of each fraction. HMGR mRNA copy number was estimated by the RT-PCR amplification. Each RT reaction (20 μl) contained 1200 c of internal control, pAW109, and 800 ng of total RNA. After adding 80 μl of PCR master-mix, the RT products were amplified 30 to 36 cycles. The HMGR copy number was estimated for each fraction by comparison with the internal control signal. The horizontal bars indicate the HMGR mRNA copy number for specific arcs of lens radius.

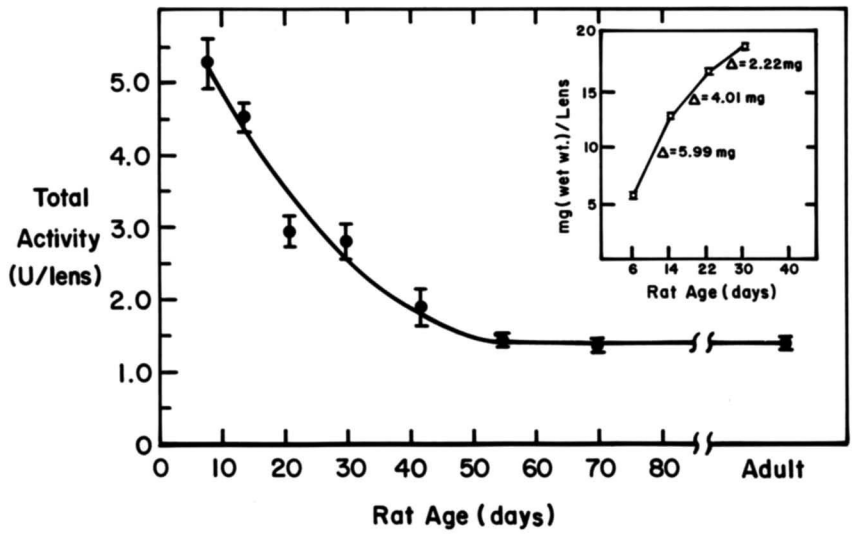


Fig. 6. Age-related changes in lens HMGR enzyme activity. Aliquots of whole lens homogenates were assayed for HMGR enzyme activity as described in the text. Each value is the mean ± SEM (bars) of four separate pools of lens at each age. One unit of reductase activity equals 1 pmol of mevalonate formed per min at 37°C. The insert figure is adapted from Fig. 1 of Cenedella, R. J. 1989. *Invest. Ophthalmol. Vis. Sci.* **30:** 575–579 with permission of the publisher.

Age (Days)	Relative Enzyme mass per lens	Relative Enzyme Activity per lens
7	1.00	1.00
14	0.93	0.86
21	0.64	0.55
50	0.31	0.28

Fig. 7. Age-related changes in lens HMGR protein mass. The outer cortex of lenses from 7-, 14-, 21-, and 50-day-old rats was removed by dissolution in a tergitol containing buffer. Aliquots of each solution were assayed for HMGR enzyme activity as described in the text; 0.1% of the total protein (44 to 52 μg) in each sample was subjected to SDS-PAGE. This represented 0.1% of the protein from 24 lenses in the case of the 7- and 14-day-old rats and 0.1% of 30 and 20 lenses in the case of the 21- and 50-day-old rats, respectively. HMGR protein was detected by enhanced chemiluminescence detection after Western blotting with the A9 monoclonal antibody (Mab) to HMGR. Relative HMGR mass was estimated by scanning exposed X-ray film and comparing the areas under the scan curves. Relative enzyme mass and activity are expressed on a per lens basis. L, rat liver microsomal protein (<1 μg).

levels, significant errors are possible. For example, differences in the efficiency of cDNA synthesis from the target and internal control in the reverse transcription reaction is a potential source of error. Wadhwani et al. (17) recently estimated a 28% efficiency of the RT reaction (ng cDNA synthesized/ng RNA) when a control RNA was reverse-transcribed in the absence of other RNA. Differences in efficiency for a target and its control RNA simultaneously reverse-transcribed could be possible. If reverse transcription of the pAW109 RNA was more efficient than that of the HMGR mRNA, our estimations of HMGR mRNA copy number would be low.

Both HMGR enzyme activity and protein mass were shown earlier to abruptly disappear from the lens cortex at a location corresponding to about the outer 10% mark of the radius (7). Lens sterol synthesis also halted at this approximate location (6). In contrast, the distribution of the HMGR mRNA remained essentially uniform across the lens cortex (95–60% of the radius) at about 2000–3000 c/mm³ of tissue. Even the nucleus, inner 60% of the radius, contained about 1200 c/mm³. Messenger RNAs for the crystallin proteins (18) and membrane intrinsic protein, MP26, (19) have also been identified in the nucleus. Lens RNA is reported to be relatively stable (20, 21), a concept supported by the current finding that the rat lens nucleus contains much intact 28S and 18S ribosomal RNA. The extent to which nuclear messages are translationally active is unknown; however, several groups have demonstrated that poly A⁺ RNA extracted from the nucleus can support synthesis of most lens proteins when incubated with a rabbit reticulocyte lysate (22–24). These mRNAs might not support nuclear protein synthesis in vivo due to inadequate cell concentrations or to lack of endoplasmic reticulum.

The near uniform distribution of HMGR mRNA across most of the lens cortex could suggest that the rapid disappearance of HMGR enzyme activity and protein mass from the cortex are not related to changes in mRNA concentrations. Nevertheless, inadequate HMGR mRNA concentrations could contribute to the observed changes in enzyme distribution. The concentration of HMGR mRNA in the very superficial cortex was 5- to 7-times greater than that in even slightly deeper layers. We estimate that 15,000 c of message/mm³ of outer cortex could provide 1 to 2 copies per fiber cell in this region. This estimation assumes that the outer cortex contains between 7000 and 14,000 fiber cells/mm³ (see footnote 1 for the calculation). At first inspection, a mRNA copy number of 1 or 2 per cell seems unrealistically low to support enzyme synthesis; however, based upon the data of Powell and Kroon (8), human lymphocytes and hepatocytes are calculated to contain only 4 and 19 copies of HMGR mRNA per cell, respectively (see footnote 2 for calculation). Us-

[1]Estimated volume of rat lens cortical fiber cells. The radius of the 19-day-old lenses was calculated from lens weight (14.42 mg) assuming that 1 mg of tissue equals 1 mm³:

$$r = \sqrt[3]{\frac{14.42\ \text{mm}^3\ (\text{mg})}{4.189}} = 1.51\ \text{mm}$$

The radius at the mid point of the outer lens layer examined (100–97.1% of r) would be 1.49 mm. The cross-sectional area of a cortical lens fiber cell is taken as 30 μ² (3μ × 10μ) (25). The length of a fiber cell in this layer was calculated from the lens circumference, C = πD/2 = 4.67 mm. This assumed that the cells extend from pole to pole; i.e., they spanned one-half of the circumference at this point. Fiber cells of the frog lens are stated to reach the anterior and posterior poles at 13 cell layers below the anterior epithelium (26). Thus, the estimated volume of a fiber cell in this layer is about 140,000 μ³ (4670 μ × 30 μ²). Fiber cells per mm³ of lens would be 10^9 μ³/mm³ ÷ 140,000 μ³/cell = 7140. If the fiber cells were only one-half the estimated length, the cell number per mm³ would be doubled.

[2]Estimated copies of HMGR mRNA in human lymphocytes and hepatocytes. Powell and Kroon (8) determined that human lymphocytes and hepatocytes contained about 830,000 and 240,000 copies of HMGR mRNA/μg of total RNA, respectively. Liver contains about 10 μg of RNA/mg (mm³) (Molecular Research Center Technical Bulletin, 1992; Molecular Research Center, Inc., Cincinnati, OH). Taking a hepatocyte volume at 8000 μ³ (20 μ × 20 μ × 20 μ), 1 mm³ of liver should contain 125,000 cells. Thus, 12,500 cells should contain about 1 μg of RNA and 240,000 copies of HMGR mRNA or 19.2 copies/cell. Assuming white cells contain 5 μg of RNA per 10^6 cells (P. Chomczynski, Molecular Research Center, personal communication), they should contain about 4 copies of HMGR mRNA per cell (830,000 copies/μg RNA ÷ 200,000 cells/μg RNA).

ing Northern blotting with cRNA probes, Rudling (27) also found mouse liver to contain about 16 copies of HMGR mRNA per cell. These estimations imply that the translational potential of only a few copies of the message per cell can provide for adequate synthesis of this enzyme protein. Yet, there must obviously be a mRNA level below which HMGR protein synthesis will halt or at least will be inadequate to compensate for steady state proteolysis. The lens could function very close to that level. The average copy number of HMGR mRNA in cortex deeper than 95% of the radius could be on average much less than one per cell. Although mRNAs may be long-lived in the lens relative to other tissues, degradation of HMGR mRNA with fiber cell aging must occur as the total copies of the message per lens did not increase with aging from 5-day-old rats to adults. The half-life of HMGR mRNA in other tissues could be very short. Under conditions of steady state transcription of HMGR mRNA in hamster kidney cells, Choi, Lundquist, and Peffley (28) observed that block of mRNA degradation produced a 10-fold increase of the mRNA within 2 h.

Based upon these considerations, we hypothesize that transcription of the HMGR gene occurs in only the outer 5% of the lens radius. This is also the location of peak levels of HMGR enzyme activity and protein mass and cholesterol synthesis (6, 7). The possibility that HMGR mRNA molecules are also imported into the fiber cell from differentiating epithelial cells is not discounted. Without continuing transcription, the fiber cell concentrations of this message eventually decrease to stable but nonfunctional levels. A halt in enzyme synthesis with even a slow steady state proteolysis would eventually result in disappearance of HMGR protein from fiber cells. The half-life of HMGR protein in lens is unknown, but because of the general slow turnover of lens proteins, the half-life could be significantly longer than in hepatocytes, $t_{1/2} \cong 2$ h (29), or cultured cells, $t_{1/2} \cong 10$–13 h (30).

We also recognize that additional factors could contribute to the rapid loss of HMGR enzyme activity and protein mass from the lens cortex. Both inhibition of translation and stimulation of enzyme proteolysis have been shown to regulate HMGR activity in other cell types (29–31). Although these mechanisms could contribute to the loss of HMGR from the lens, destruction of the fiber cell endoplasmic reticulum could also be important. As fiber cells age, they eventually lose all subcellular organelles (26). Translation of functional HMGR requires targeting of a specific 39 amino acid signal peptide to the endoplasmic reticulum (32). This peptide is not cleaved and eventually constitutes the amino terminal 39 residues of the intact enzyme. Without endoplasmic reticulum, HMGR cannot be glycosylated and protected from proteolysis. The current observation that the copy number of HMGR mRNA decreases abruptly from the outermost cortical layer suggests to us that HMGR protein synthesis is essentially confined to the outer 5% of the lens radius. Cessation of enzyme synthesis, due to decreasing concentration of HMGR mRNA, might occur prior to loss of the endoplasmic reticulum.

Although cholesterol is a major component of lens plasma membrane (2), its concentration in rat lens is only about 2 mg/g wet weight (33). By contrast, soluble crystallin proteins account for 400 mg/g wet weight (34). Thus, one can reasonably assume that the copy number of mRNA molecules per lens cell needed to support translation of the cholesterol synthesizing enzyme is orders of magnitude less than copy numbers of messages for the structural-crystallin proteins. Relative but not absolute concentrations of lens crystallin mRNAs have been measured by Northern blotting, in situ hybridizations, and RT-PCR amplification (18, 35, 36).

Finally, the current results might have relevance to potential effects of the HMGR inhibitor class of hypocholesterolemic drugs on human lens. Vastatins can inhibit cholesterol synthesis (37, 38) and halt cholesterol accumulation (4) in rat lenses. There has been concern over the ocular safety of lovastatin, as it can produce cataracts in dogs at high dosage (5). Withdrawal from lovastatin therapy might also effect the lens if this drug markedly increased HMGR mRNA levels in the lens as it does in liver (39). The present results suggest that lens HMGR mRNA is very long lived. An increase in this message might lead to long-term enhancement of lens cholesterol synthesis. In fact, Kalinowski et al. (38) demonstrated that withdrawal from lovastatin treatment led to increased cholesterol synthesis by the rat lens due to increased HMGR enzyme activity. As the lens cannot lose cholesterol, any increase should result in a permanent compositional change of at least some fiber cell membranes.

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