Visual sensitivity and interphotoreceptor retinoid binding protein in the mouse: regulation by vitamin A

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ABSTRACT Interphotoreceptor retinoid binding protein (IRBP) is a retinoid and fatty acid binding glycoprotein secreted by rod and cone photoreceptors in all vertebrates. IRBP is believed to serve as a carrier for retinoids in the bleaching and regeneration cycle of rhodopsin. IRBP protein has been found to be decreased in vitamin A-deprived rats; it is rapidly recovered after retinol repletion. To understand the mechanism for this recovery, we determined whether vitamin A affects transcription and translation of the IRBP gene. Wild-type and transgenic mice harboring the IRBP promoter-CAT reporter fusion gene were maintained on a retinol-deficient diet supplemented with retinoic acid ($-$A) or on a control diet ($+$A) for up to 60 wk postweaning. Some of the $-$A mice were given retinol repletion for 7 days ($-$A$+$A). Electroretinography analysis revealed alterations in waveform and a 2 log unit decrease in b-wave sensitivity in the $-$A mice over a broad range of stimulus wavelengths. Retinol repletion effected a full recovery. Immunochemistry showed a significant decrease in the immunogold-labeled IRBP between the retinal pigment epithelium and the outer segments of the $-$A mice compared with $+$A and $-$A$+$A mice. Northern blots showed no differences in the amounts of IRBP or CAT mRNA between these three treatment groups. These results suggest that the regulation of IRBP by retinol is not transcriptional.—Liou, G. I., Matragoon, S., Chen, D.-M., Gao, C.-L., Zhang, L., Fei, Y., Katz, M. L., Stark, W. S. Visual sensitivity and interphotoreceptor retinoid binding protein in the mouse: Regulation by vitamin A. *FASEB J.* **12,** 129–138 (1998)

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IN THE VERTEBRATE RETINA, the bleaching and regeneration cycle of rhodopsin depends critically on the transfer of all-*trans* retinol and 11-*cis* retinal between the outer segment of the photoreceptor and the retinal pigment epithelium (RPE)[2] through the interphotoreceptor matrix (IPM) (1). The transfer of these retinoids in the IPM is believed to be mediated by interphotoreceptor retinoid binding protein (IRBP) (2–7), a photoreceptor secretory glycoprotein that binds these retinoids as well as fatty acids (8–10). IRBP is expressed during early retinal development before the formation of photoreceptor outer segments (11–15). It has thus been postulated that IRBP may be required for photoreceptor development (12) and maintenance.

Some factors influence the level of IRBP mRNA. When developing or adult mice were deprived of normal light, there was a decrease in IRBP mRNA, but no alteration in the retinal morphology or the amount and distribution of IRBP (16). By contrast, light/dark conditions altered mRNAs in unison with their proteins for rhodopsin, α-transducin, and arrestin in the adult rat retina (17, 18). These observations suggested that the relatively constant level of IRBP throughout adulthood is critical for photoreceptor maintenance (16) and that regulation of IRBP protein in the retina is not coupled to IRBP mRNA control.

If a constant level of IRBP is critical for photoreceptor health, factors that diminish the IRBP level may lead to impairment. This notion is supported by recent studies in vitamin A-deprived rats. Weaned rats maintained on a retinol-deficient diet supplemented with retinoic acid for 23 wk suffered from losses in rhodopsin, outer segment size, visual sensitivity, and IRBP. While all losses were recovered after vitamin A repletion, IRBP recovery was the fastest (19, 20). Thus, IRBP may be a key regulatory factor in the production of photoreceptor

proteins and the subsequent renewal of the outer segment.

In rats, both the protein and mRNA levels of another vitamin A binding protein, the cellular retinol binding protein (CRBP), were reduced as a result of total retinoid (retinol and retinoic acid) deprivation (21). The reduced mRNA level could be recovered rapidly during vitamin A replenishment. In the fly, opsin gene transcription is also regulated by retinoids (22–24), and it has been proposed that in vertebrates retinoids may regulate the expression of genes involved in vision (25). Based on the rapid recovery of IRBP during retinol replenishment, it was hypothesized that IRBP gene transcription may be regulated directly by retinol (20). To test this hypothesis and to understand the mechanism of IRBP recovery by vitamin A repletion, we first established that the IRBP content and visual function in mice are both retinol-dependent and then showed that this retinol-regulated IRBP content change is not at the level of transcription (26).

MATERIALS AND METHODS

Animals and diets

Wild-type mice (C57BL/6, Jackson Lab, Bar Harbor, Maine) and transgenic mice harboring a human IRBP promoter-CAT (chloramphenicol acetyltransferase) reporter fusion gene (family 101, hybrid between FVB and C57BL/6) (27) were maintained on a synthetic, retinol-deficient (retinoic acid-sufficient) diet (−A) (Harlan/Teklad, Madison, Wis.) or a retinol-sufficient control diet (+A). These diets were prepared according to the formula previously used for rats (28). Mice were housed under 12 h/12 h light cycle at an average light level of 7–10 lx measured at the middle of the cage compartment with a portable radiometer/photometer (Model 27–5479 Ealing Electro-Optics, Inc., Holliston, Mass.). This level of illumination was low enough so that no apparent light-induced damage to the retinas occurred during the course of the experiments. To avoid the potential effects of circadian rhythms, mice for IRBP and mRNA measurements were killed approximately 4 h after light onset in their light cycle.

Investigations were performed in accordance with the guidelines established by the Committee on Animal Use for Research and Education at the Medical College of Georgia and with the ARVO Resolution on the Use of Animals in Research. For electroretinography (ERG) studies, mice were used according to approved protocol 1082 from the Saint Louis University Animal Care Committee.

Vitamin A deprivation and repletion

Weaned mice were maintained on the synthetic +A and −A diets for 30 to 60 wk. Vitamin A decrease was determined biochemically by a reduction in testicular CRBP mRNA (data not shown). This method of vitamin A status determination is based on a recent report that retinoic acid transfer from circulation to the testis in rat is limited; the required retinoic acid in this organ must be supplied by retinol (29; Dr. William S. Blaner, personal communication). Apparently no photoreceptor cell death had occurred after 60 wk of −A feeding,

as indicated by the full or nearly full recovery of ERG after retinol repletion (see below).

Approximately half of the −A mice were given a single intramuscular injection of all-*trans* retinol (Sigma, St. Louis, Mo.) after light CO_2 or light ether anesthesia and returned to the +A diet for up to 7 days. The preparation of retinol solution and the amount for injection were based on those used for rats, in proportion to their body weight (19). Each animal received 30 µl of the retinol solution of 300 µg/ml in a hind leg muscle.

IRBP immunocytochemistry

The concentration and distribution of IRBP in the mouse retina were determined by immunocytochemical labeling of IRBP according to the previously published method for rats (20). Monoclonal antibody MAbH3-B5, specific for human IRBP amino acids AASEDPR between position 361 and 367, was a gift from Dr. Larry A. Donoso at Wills Eye Hospital, Philadelphia (30). For quantitative electron microscopic immunochemistry, the number of immunogold particles was determined in a region extending from the bases of the RPE apical microvilli 6 µm into the outer segment area of the retina. Data were obtained from a minimum of a 25 µm length of each retina measured along a line parallel to the apical surface of the RPE cells. The portion of each retina analyzed was taken from the superior–inferior meridian and centered on a point approximately 500 µm superior to the optic nerve head. The specificity of the immunolabel was confirmed by the absence of bound protein A-gold in sections incubated with buffer instead of the monoclonal antibody.

Electroretinogram measurements

For ERG measurements, wild-type mice were dark-adapted for 12 h and anesthetized by intraperitoneal injection with a combination of 100 µg ketamine, 100 µg xylazine, and 4 mg of urethane per 10 g body weight. The pupils were maximally dilated with 1% Cyclogyl and 2.5% Mydfrin. For direct-coupled (DC) ERG recording, the mice were placed on a metal platform and shielded with a cover of aluminum. Dark adaptation was maintained throughout the ERG recording sessions. ERGs were recorded from the right eye, unless otherwise mentioned, of each animal by using a cotton wick electrode soaked in 0.9% NaCl. Electrical signals were amplified with a Getting microelectrode amplifier (Model 5, Iowa City, Iowa), viewed, stored, and analyzed with a MacLab/2e (Milford, Mass.) MacIntosh LC computer system. A dual beam optic system was used to deliver stimuli via beam splitter and lenses. Intensity (adjusted by neutral density filters) and duration of stimuli and other conditions were according to previous procedures (19), except that stimuli were monochromatic. A 150-W xenon arc lamp (XBO 150 W/CR, Osram, Germany) operated by a power supply (Model 1600, Opti-Quip, Highland Mills, N.Y.) and a Bausch-Lomb 500 mm monochromator (Rochester, N.Y.) provided monochromatic stimuli from 350 to 650 nm.

Northern blot analysis

Individual retinas homogenized by treated sand (31) were used for isolation of total RNA by using the selective binding properties of silica gel-based membrane with the microspin technique (RNeasy kit, QIAGEN, Chatsworth, Calif.). Equal amounts (4 µg) of total RNA, determined by absorbance at 260 nm, from individual retinas were electrophoresed in 1% agarose gel in formaldehyde (32), blotted to Zeta-Probe GT Blotting Membrane (Bio-Rad, Richmond, Calif.) by capillary
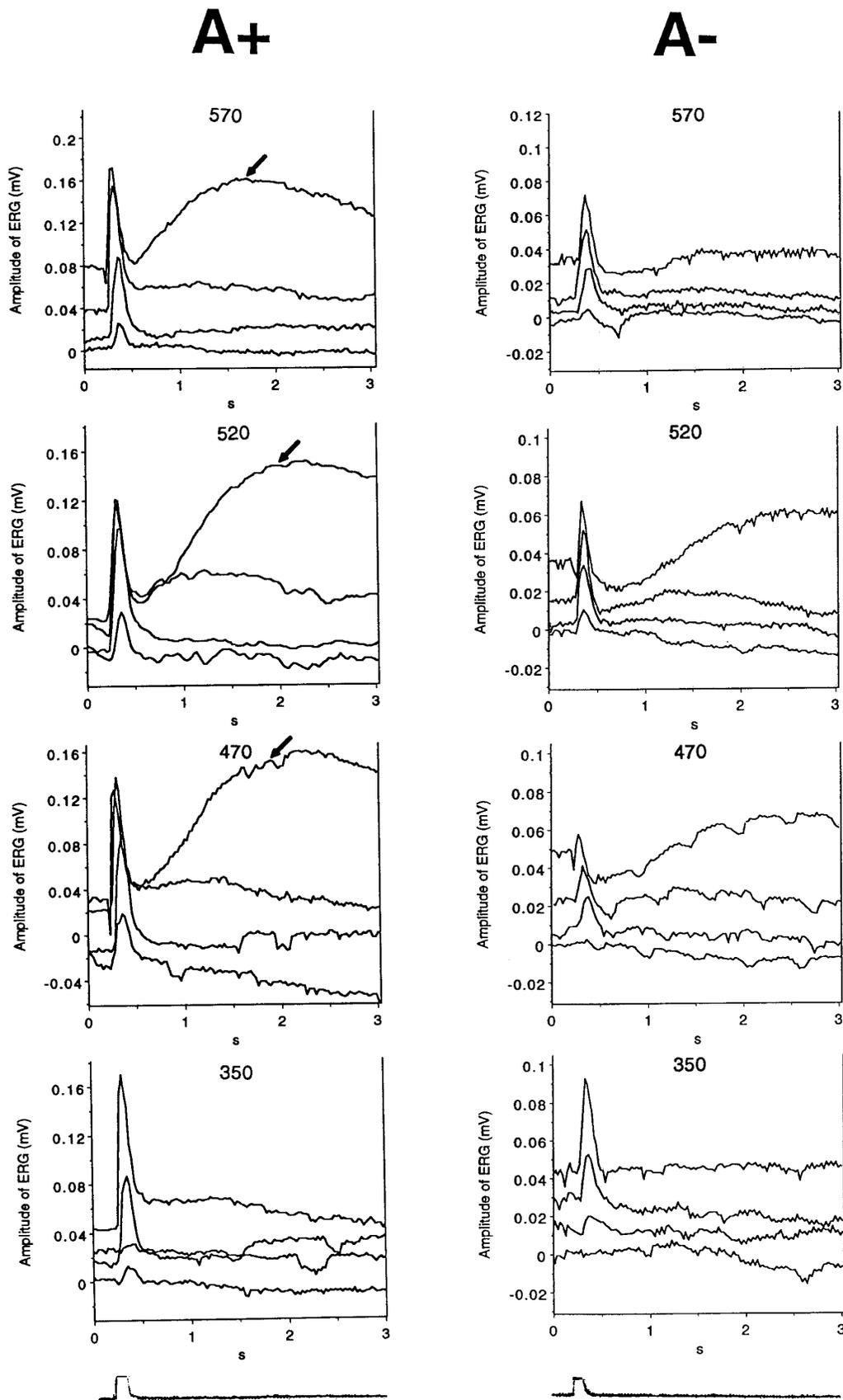
Figure 1. Representative ERG waveforms and intensity responsivities from wild-type mice fed +A and −A diets for 60 wk. The upward deflection in the trace under the ERG recordings indicates timing and duration (100 ms) of the stimulus used to elicit the ERG response. Monochromatic stimuli at 570, 520, 470, and 350 nm were used as labeled. The log units of stimulus intensity (quanta cm^{-2} s^{-1}) are shown as decreasing from top to bottom in +A mice: *(continued at bottom of following page)*

transfer, and hybridized with probes for the mouse IRBP gene (a cDNA fragment encompassing 720 bp of the coding region at the 3′ end) (15), the CAT gene (27), or the mouse gene encoding glyceraldehyde-3-phosphate dehydrogenase (G3PDH: glyceraldehyde-3-phosphate dehydrogenase, an internal standard) (33). Before blotting, the gel was treated with 10 mM NaOH for 15 min to facilitate the transfer of large RNA to membrane. Blotting, hybridization, and washing were all performed according to the manufacturer's instructions (Zeta-Probe GT blotting membrane, Bio-Rad).

The effects of retinol deficiency and repletion on retinal IRBP mRNA were quantitatively determined by Northern blot analysis using equal amounts of total RNA and normalized to the internal standard. Densitometric analysis was performed on an EC 910 Densitometer (E-C Apparatus Corp., St. Petersburg, Fla.) with an HP 3395 Integrator.

Statistical analyses

Determination of whether dietary retinol had significant influences on retinal IRBP immunoreactivity by quantitative electron microscopic immunochemistry and on IRBP mRNA by Northern blot was accomplished using analysis of variance. Comparisons among individual treatment groups were performed with the Newman-Keuls procedure (34).

RESULTS

Effect of vitamin A deficiency and repletion on visual sensitivity

After the mice were maintained on synthetic diets for 60 wk, the ERG amplitudes in −A mice were significantly lower than in +A mice at all wavelengths of stimuli tested (**Fig. 1**). In +A mice, the c-wave, as indicated by the arrows in Fig. 1, can be recorded with longer wavelengths. This c-wave was much smaller in −A mice. Comparing spectral sensitivities (**Fig. 2**), the stimulus intensities required to produce criterion (10 μV) b-waves in the ERG was nearly 2 log units higher in −A mice than in +A mice. This reduction in visual sensitivity was shown to be mediated by a corresponding reduction of rhodopsin levels in rats (35). Recovery of visual sensitivity, as determined by the ERG, was observed 7 days after retinol repletion. The visual sensitivity of the −A mice was preferentially reduced at ultraviolet (UV) wavelengths. The spectral sensitivity function measured for control (black C57BL/6, 12 month-old, raised on standard lab mouse diet) mice was identical to that of the +A mice (data not shown).

Effect of vitamin A deficiency and repletion on the distribution of IRBP immunostaining

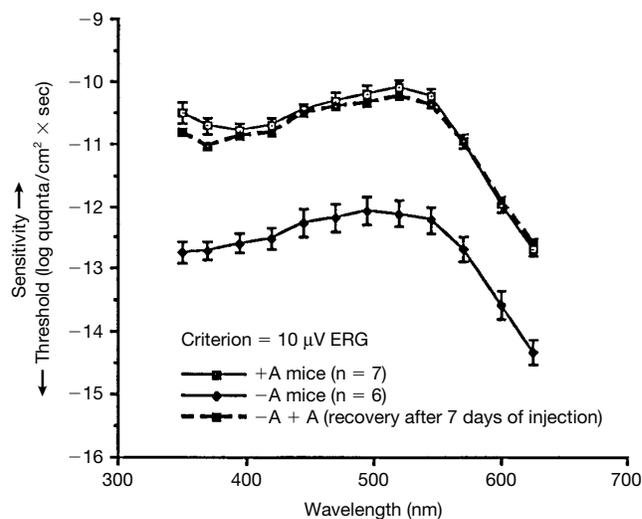A substantial amount of immunogold-labeled IRBP was identified over the RPE apical microvilli and be-

Figure 2. Recovery of ERG after retinol repletion. Spectral sensitivities were measured for +A, −A, and −A + A (7 days after retinol repletion) mice between 350 and 650 nm. The +A and −A mice were maintained on the respective diets for 60 wk. The criterion was a 10 μV b-wave.

tween the outer segments in the retina of the +A mice (**Fig. 3A** and **Fig. 4**). In the retinas of the −A mice, the amount of immunolabel was significantly reduced (by approximately 80%) (Figs. 3B and Fig. 4). One week after repletion of −A mice with retinol (−A+A), the amount and location of the IRBP immunolabel were similar to levels observed in the +A animals (Fig. 3C and Fig. 4). No label was identified in the retina samples not incubated with IRBP antibody (Fig. 3D).

Quantitative influence of vitamin A on IRBP mRNA

The major goal of this study was to determine whether the mRNA level of the IRBP gene is affected by dietary vitamin A. In our transgenic mouse lines harboring the IRBP-CAT fusion gene, the expression of the CAT gene under the direction of the IRBP promoter mimics that of the endogenous gene (27, 36). To determine whether the mRNA of the transgene was also affected by vitamin A, one of the transgenic lines (family 101) was used along with the wild-type mice in this study. Northern analysis was used to quantify the influence of vitamin A on IRBP mRNA, using constant and equal amounts of total RNA determined by absorbance at 260 (260/280 absorbance ratio is around 1.9), confirmed by 18S and 28S ribosomal RNA (visualized under UV light) and an internal standard, mouse G3PDH gene. The tran-

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Figure 1. (*continued*) 570 nm: 16.52, 14.58, 12.92, 11.60; 520 nm: 16.34, 14.38, 12.90, 11.43; 470 nm: 16.19, 14.23, 12.56, 11.28; 350 nm: 13.86, 12.09, 10.76, 10.05. Nearly identical units of intensity are also shown as dcreasing from top to bottom in −A mice: 570 nm: 16.40, 14.49, 12.83, 11.52; 520 nm: 16.24, 14.28, 12.80, 11.33; 470 nm: 11.16, 12.44, 14.11,16.07; 350 nm: 13.95, 12.24, 10.85, 10.14.
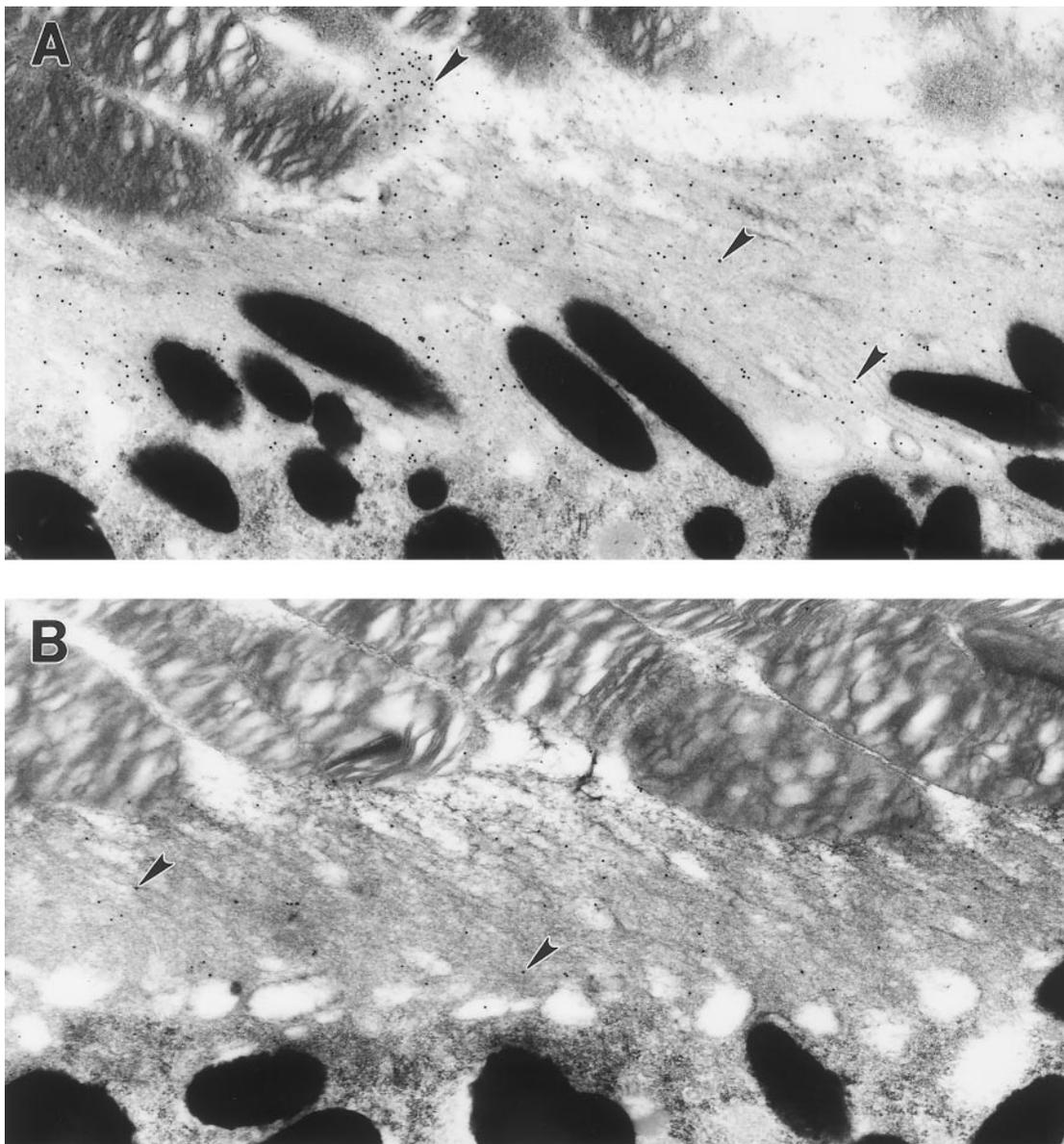
Figure 3. Electron micrographs of immunogold stained sections of the apical retinal pigment epithelium and adjacent retina from mice that had been subjected to +A (*A*), −A (*B*), and −A + A (*C*) treatments. The number of bound gold particles (arrowheads) was substantially lower in −A animals (*B*) than in either of the other treatment groups. *D*) Electron micrograph of a section of the apical retinal pigment epithelium and adjacent retina from a mouse fed the +A diet. The section was stained using the same immunogold procedure as that used to stain the other sections shown in Fig. 3, except that buffer was substituted for the IRBP antibody. Almost no bound gold particles were observed. Bars (1 µm) indicate magnification for all four micrographs (*see next page for C, D*).

script of mouse G3PDH, as determined by its cDNA probe, was not affected by the vitamin A status of these mice (**Fig. 5A**). Results of the Northern analysis showed that there were no apparent differences in the IRBP and CAT mRNA levels between any of the dietary groups (Fig. 5*A*). Quantification of multiple Northern analyses by densitometric analysis again showed no significant differences in the mean relative hybridization intensity in any treatments (Fig. 5*A*). In Fig. 5*B*, only the results of the wild-type mice were used for the calculation and statistics. Autoradiograms of the Northern blot were scanned at dif-

ferent band intensities to ensure that densitometric analysis was performed in the linear response region of the photographic emulsion.

The transgenic mice used in this study, family 101, did not show an altered endogenous IRBP transcription by integration of the transgene construct (27). The different ratio of IRBP to G3PDH in the wild-type vs. transgenic mice (Fig. 5) was probably due to multiple reprobing and difference in exposure time. However, this does not affect the bottom line result of Northern analysis: the level of IRBP or CAT transcript is not altered by vitamin A deprivation.
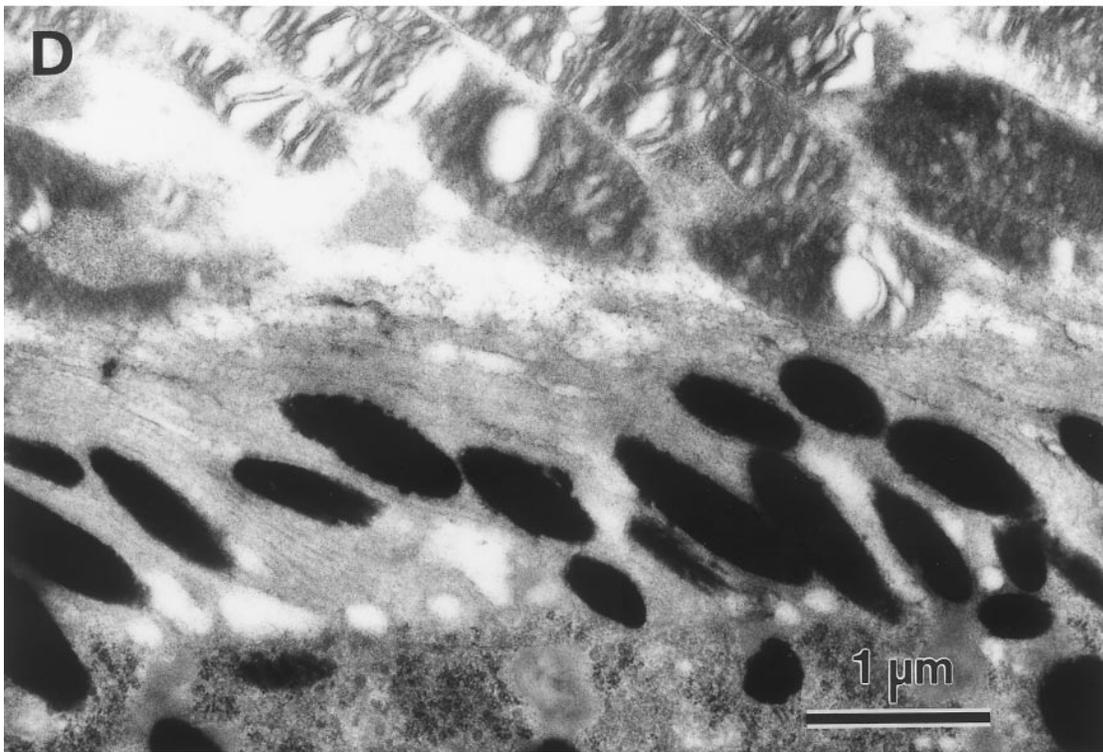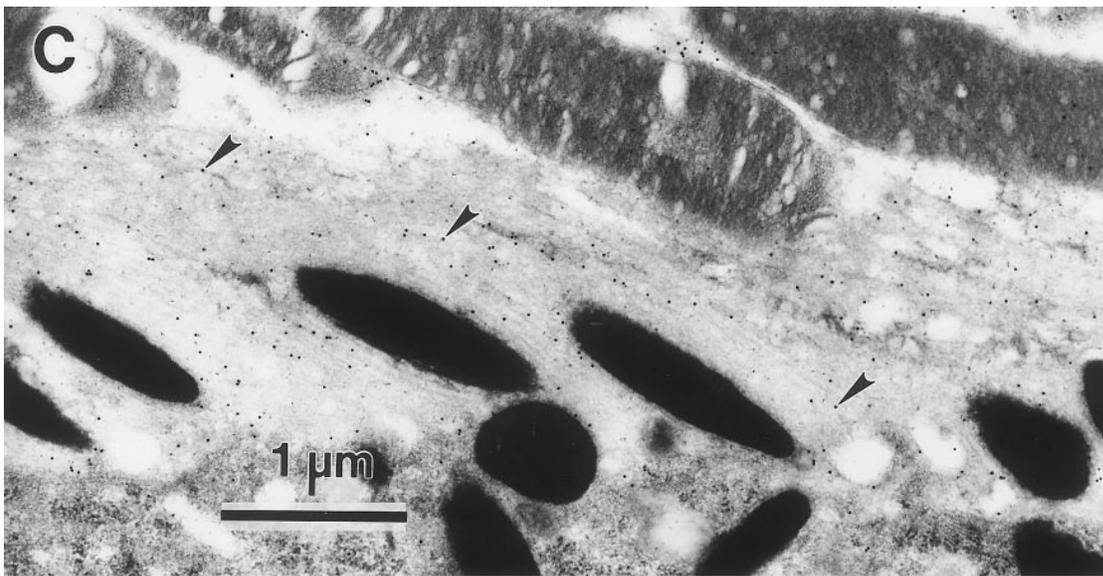
Figure 3. (*continued*)

DISCUSSION

Vitamin A deprivation decreases visual sensitivity, which is reversed by vitamin A repletion in the mouse

As early as 1960 (37) it was shown that dietary deficiency in retinoids resulted in a selective diminution of the photoreceptor outer segments and eventual photoreceptor cell death. Vitamin A deprivation in studies of vertebrate vision requires that the animals be maintained on retinoic acid-supplemented diets since retinoic acid is essential for development in vertebrates and for normal function in numerous cell types. On the other hand, retinol or retinal is usually considered to be required only by a few cell types, including photoreceptors (38), while also serving as the metabolic precursor of retinoic acid. Retinoic acid is not metabolized back to either retinol or retinal. Therefore, continuous feeding of a retinol-deficient but retinoic acid-supplemented diet leads to visual loss while maintaining the general health of the animals.
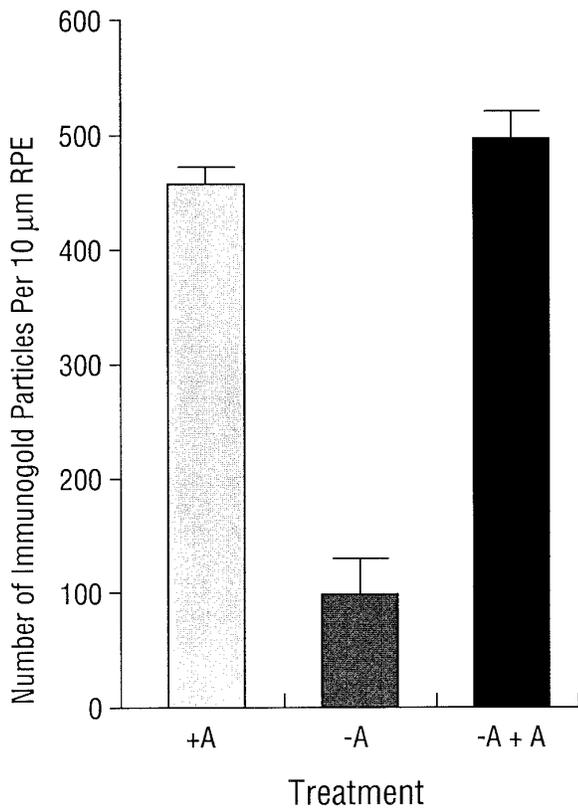
Figure 4. Quantitative electron microscopic immunochemistry. Data in the graph represent means ± SD.

Recent studies of retinol-deficient but retinoic acid-sufficient rats supported this conclusion because they suffered from decreases in outer segment size and retinal rhodopsin content (35). Moreover, it was established that these visual losses were completely recovered upon vitamin A replenishment as measured by visual sensitivity, rhodopsin levels, and photoreceptor morphology (19). This indicates that retinol deficiency did not result in photoceptor cell loss.

The increasing utilization of transgenic mice in vision research justified our reexamination of the rat findings in the mouse. Our present study showed that the visual sensitivity was also retinol dependent in the mouse and could be fully recovered upon retinol repletion if repletion was initiated before retinal degeneration occurred (Figs. 1 and 2). In both the mouse (present study) and rat (19), vitamin A deprivation caused several orders of magnitude of visual sensitivity loss, which was reversed quickly upon repletion.

Vitamin A deprivation preferentially decreases UV sensitivity.

An unexpected finding from the ERG analyses was that UV visual sensitivity was decreased up to half a log unit relative to the visible wavelength sensitivity

by vitamin A deprivation (Fig. 2). This seemed a remarkable coincidence since the same had been shown in *Drosophila* (39). In the fly, the inability to selectively decrease UV sensitivity by UV adaptation suggested that a UV-absorbing retinoid sensitized a blue-absorbing rhodopsin (39). It is now thought that rodents have UV visual sensitivity mediated by UV cones (40). In separate experiments (data not shown), short wavelength adaptation was shown to selectively depress the UV peak in the +A and control (normal lab mouse diet) black mice. This suggests that UV sensitivity is mediated by the UV cone mechanism and that the UV cone's contribution to overall spectral sensitivity is preferentially decreased by retinol deprivation. In summary, the mechanisms of UV sensitivity loss in mice and flies are different.

Regulation of IRBP content of the retina is not related to mRNA levels

Because of IRBP's pivotal role in retinoid transport, the recovery of photoreceptor function by retinol repletion necessarily depends on normal IRBP function. This notion is supported by previous data from studies of rats (19, 20) in that the visual losses caused by retinol deprivation were recovered after retinol repletion. Notably, IRBP recovery occurred more rapidly than that of other factors (19, 20). This suggests that during the recovery from retinol deficiency, IRBP may be primary in regulating the production of photoreceptor proteins and the subsequent renewal of the outer segment and recovery of sensitivity.

Our Northern blot analysis indicates, for the first time, that steady-state levels of the IRBP mRNA are not altered by the retinol status in the mice. If the turnover rate of IRBP mRNA is unaltered by the retinol status, this result suggests that the transcription rate of the IRBP gene is not regulated by retinol. It has been well established that retinoic acid, in the form of all-*trans* and 9-*cis* isomers, is involved in regulating cell differentiation and development through the action of specific nuclear transcription factors: retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (41). Some genes, including the CRBP gene are regulated by RARs (42). Whether the IRBP gene is regulated by the RARs is not easily tested because mice cannot be deprived of retinoic acid. In *Drosophila*, which can be totally deprived of retinoid, retinoic acid activates transcription of the opsin gene (23, 24) and the gene for a retinoid binding protein in the compound eye (43). It has been shown that retinoic acid supplementation increases the arrestin mRNA in the mouse (44).

The question of potential regulation of IRBP gene expression by retinoic acid acting through nuclear receptors and putative response elements in the promoter region of the IRBP gene has been addressed by gel shift and functional analyses (45). In the gel
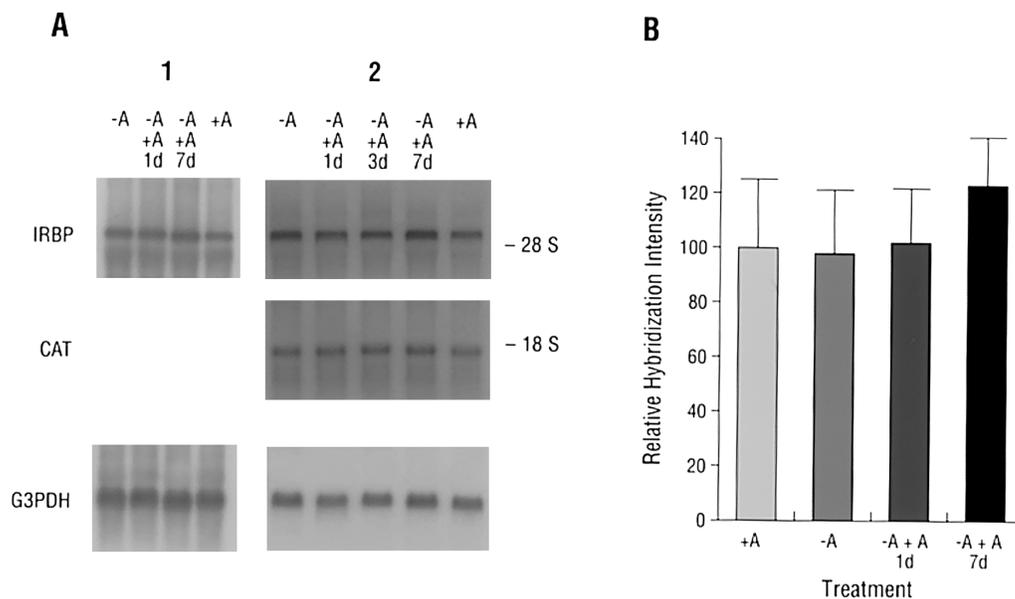
Figure 5. *A*) Northern blot analysis of the IRBP gene and IRBP promoter-directed CAT transgene of one representative set of retina samples from control, retinol deficiency, and repletion groups. Mice were maintained on +A or −A diets for 48 (wild-type mice, panel 1) or 60 wk (transgenic mice, panel 2). Equal amounts (see Methods and Results) of total RNA from individual retinas were analyzed for mouse IRBP, CAT, or mouse G3PDH. Positions for the 18S and 28S ribosomal RNAs, visualized under UV light, are indicated. *B*) Quantitative densitometric analysis of the effects of retinol deficiency and repletion on IRBP transcripts from individual retinas as determined by Northern blot analysis. Mice were maintained on +A or −A diets for 48 or 60 wk. Equal amounts of total RNA from individual retinas were analyzed. Relative hybridization densities, normalized for total RNA or G3PDH, are: +A, 598 ± 155; −A, 586 ± 145; −A + A, 1 day, 613 ± 119; and −A + A, 7 day, 740 ± 115. These values are expressed relative to the value of +A, which was assigned a value of 100. Each bar represents the mean $\pm$ SD of three to five determinations from each treatment group.

shift analysis, the IRBP promoter showed specific bindings with RARα and RXRγ. To identify RAR/RXR activities in the 5′ flanking region of the IRBP gene, retinoblastoma and CV1 cells were transfected with IRBP or thymidine kinase promoter-reporter genes and cotransfected with RARα and/or RXRγ expression vectors. No significant effect was observed on reporter gene activity in the presence or absence of retinoic acid. Transcriptional regulation of some photoreceptor genes, such as opsin and arrestin, by retinoic acid has been observed in developing mice (44; Dr. Ursula Dräger, personal communication). To determine whether the IRBP gene can also be regulated by retinoic acid, experiments were conducted in the same manner as before. In a series of experiments, dark-adapted C57BL/6 mice at postnatal day 6 were injected with all-*trans* retinoic acid (50 nmol/g of body wt.) or with solvent alone as a control, and transcription of IRBP, opsin, arrestin, and G3PDH genes was analyzed by Northern blotting 12 h after injection. Results showed that although there was a significant induction effect on both the opsin and arrestin genes, no effect was observed on the IRBP gene.

Unlike the primate retina, the mouse retina has no specialized regions such as a macula. Thus, the retina is fairly uniform in photoreceptor composition and density. The immunocytochemical data obtained from the superior central region of the retina in this study are therefore likely to be representative of the retina as a whole. Although unlikely, it is possible that the reduced IRBP protein levels in the region examined in the vitamin A-deprived mice were due to a localized decrease in IRBP mRNA levels. This effect would need to be either localized to a small area of the retina or counterbalanced by an increase in IRBP mRNA levels in other areas of the retina in order to explain the lack of reduction in IRBP mRNA levels in whole retinas of vitamin A-deprived mice. Not only is such a scenario unlikely, but it does not address the lack of correlation between IRBP protein and mRNA levels.

Our results on retinol deficiency cannot distinguish whether synthesis or degradation of IRBP protein is affected. This could only be resolved by analyses that determine the rates of translation and protein turnover. Lower steady-state levels of IRBP in the −A mice did not appear to be caused by a decrease in photoreceptor cell number, since the ERG sensitivity and IRBP level recovered completely after vitamin A repletion. In addition to decreasing IRBP levels, retinol deficiency also decreases rhodopsin and outer segment size (19, 20), which may regulate IRBP indirectly.

Animals are subject to temporary changes in environment, such as darkness (16), or in nutritional

status, such as the retinol deficiency of this study (19, 20). These animals show reversible changes in either IRBP mRNA or its protein content, but not both. Thus it is evident that transcription and translation of IRBP are regulated independently. This type of regulation is distinctively different from that of other photoreceptor proteins such as rhodopsin, α-transducin, or arrestin in that the conditions of light or dark lead to correlated changes in both mRNA and protein content (17, 18). This difference in regulation suggests that IRBP may have other functions in addition to its role in the visual process. A knockout of the IRBP gene causing a permanent change in its transcription in mice is being developed to understand its function (46).

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