Precocious IRBP Gene Expression During Mouse Development

Gregory I. Liou, Mier Wang, and Suraporn Matragoon

Purpose. To determine if the time course for the onset of gene and protein expression for interphotoreceptor binding protein (IRBP) precedes that of opsin in the developing mouse retina.

Methods. Relative mRNA levels of the IRBP and opsin genes were determined in prenatal and postnatal retinal RNA with RNase protection analysis (RPA). To determine if IRBP and opsin protein expressions are differentially regulated, dissociated retinal cells from postnatal (P) days 2 and 3 mice, that were injected with BrdU, were then double-labeled with antibodies against BrdU and either opsin or IRBP.

Results. With RPA, IRBP mRNA was detected on embryonic (E) day 11 at the time of cone formation, whereas opsin mRNA was not detected until PO. It took until P3 for opsin expression to reach significant levels, whereas rods already appear during embryonic development. IRBP transcription preceded that of opsin because it rapidly increased from E13 to an early postnatal day. By P20, the expression levels of IRBP and opsin achieved constancy. Double antibody labeling revealed positive staining for both IRBP and BrdU as soon as 2 hours after injection, but it took until 40 hours for double positive staining for opsin and BrdU.

Conclusion. Because only IRBP protein expression was observed before the last mitosis of the photoreceptor precursor cells, IRBP could be essential for retinal development. Invest Ophthalmol Vis Sci. 1994;35:1083-1088.

In mouse retinal development, the cone cells, which represent 3% to 5% of the photoreceptor population, first appear on E10 and their generation is completed on E17. On the other hand, the predominant rod cells do not appear until E13, and their generation continues until P7. IRBP, the major glycoprotein found in the interphotoreceptor matrix, which is present throughout retinal development, is synthesized by both the rod and cone cells, and it is then secreted into the interphotoreceptor matrix for binding to retinoids and fatty acids. IRBP is thought to serve as a vehicle for the translocation of the retinoids during the rhodopsin-opsin cycle.

The timing of IRBP protein expression with development was characterized by rocket immunoelectrophoresis and rocket immunoblotting in mouse retina. With these techniques, IRBP expression was already detected on E17, an early stage of development just subsequent to when the rods and cones first appear on E13 and E10, respectively. This early gene expression of IRBP could indicate that IRBP is required for the positioning of retinoids and fatty acids needed for subsequent photoreceptor inner and outer segment formation, even before its involvement in the rhodopsin cycle. The identification of when IRBP first actually appears could be open to question because the aforementioned techniques have possible limitations —antibody sensitivity, electrophoresis efficiency dependent on diffusion or blotting, and rate of protein accumulation. Consistent with these potential drawbacks, a marked slower increase in protein expression of IRBP than its mRNA was reported in the developing rat. To resolve these possible drawbacks, we employed more sensitive methods that permit accurate time course quantitation of gene and protein expres-
sion. With our approach, it is possible for the first time to determine with certainty the importance of IRBP expression to development of the mouse retina.

MATERIALS AND METHODS

The plasmid pBluescript II KS CAT was made from pTZ CAT (K.L. Chow, Yeshiva University, New York, NY) and pBluescript II KS (Stratagene, La Jolla, CA). RNA isolation solution, RNA STAT-60, was from DuPont-New England Nuclear (Reston, VA). Restriction enzymes were from New England Biolabs (Beverly, MA). RNA isolation solution, RNA STAT-60, was from Tel-Test “B” Inc. (Friendswood, TX). BrdU, goat anti-mouse IgG coupled to biotin, streptavidin coupled to Texas red, and goat anti-mouse IgG coupled to fluorescein were from Oncogene Science, Inc. (Manhasset, NY).

All animals were handled according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mouse stocks (inbred C57BL/6) were kept in a 12 hours light/12 hours dark cycle. Mating cages were set up by placing two females with a stud male in the late afternoon. The day on which the copulation plug was found in the vagina was denoted day 0. Mice were sacrificed by CO2 inhalation and cervical dislocation. The identification of the stages of the embryos was confirmed by counting the number of somites, measuring crown-rump length and by examining the paw morphology. Whole eyes were dissected from either fetuses or newborn mice and dropped into liquid nitrogen for storage at — 180°C. Eyes isolated from either fetuses or newborn mice and dropped into liquid nitrogen for storage at — 180°C.

Frozen eyes were homogenized, and total RNA was isolated with the reagent RNA STAT-60. RPA was performed as described, except that total RNA, rather than mRNA, was used. Antisense RNA probes for the IRBP, opsin and β-actin genes were synthesized using T3 or T7 polymerase on the respective cDNA templates cloned in pBluescript. The sizes of the antisense RNAs for IRBP, opsin and β-actin were 259, 163, and 300 bases, respectively. The sizes of the RNase protected fragments for IRBP, opsin, and β-actin were 245, 108, and 250 bases, respectively.

P2 or P3 mice were injected with BrdU and neural retinal cells were dissociated into single-cell suspensions with trypsin, fixed, immunoreacted, and stained with fluorescence according to established procedures. Monoclonal antibody against turtle opsin (kindly provided by Dr. V. Gaur) recognizes bovine opsin amino acid sequence 190 to 197, allowing it to bind to the external surface of rod outer segments. Monoclonal antibody against bovine IRBP (kindly provided by Dr. L. Donoso) recognizes amino acids 361 to 367 of the native human IRBP sequence and binds to mouse IRBP.

RESULTS

RPA was used to determine the time course for the expression of IRBP and opsin mRNAs during development. The results shown in Figure 1 indicate that IRBP and opsin gene expression during prenatal and postnatal development displayed distinctly different expression patterns. As a positive control for quantity and quality of the RNA at each time point, β-actin expression was also determined. IRBP mRNA was barely detected at E11 or E12 but was evident at E13. If the autoradiograms were overexposed, the IRBP mRNA was evident already at E11 to 12. During late embryonic and early postnatal stages, IRBP mRNA increased rapidly to become prevalent in the retina. In contrast, opsin mRNA could only be detected later at P0, provided the autoradiograms were overexposed. Only at P6 was there a substantial increase in opsin mRNA. To validate that IRBP mRNA on E11 did not originate from any contaminating brain tissues, we analyzed its total RNA on E11 for IRBP mRNA expression. The result, shown in Figure 1, clearly indicates that there was no IRBP mRNA. The autoradiography bands were analyzed by densitometry and normalized to β-actin expression. Each ratio was then normalized to the IRBP expression level on P27, whose value was taken as 100%.

The normalized values for IRBP and opsin transcriptions are shown as a function of development in Figure 2. Expression of the IRBP gene already begins at E11 and then rapidly increases after E13, whereas with opsin it begins at P0 and rapidly increases after P3. Both genes showed nearly constant levels of mRNA by P20.

Watanabe and Raff injected P1 and P2 rats with the thymidine analog BrdU to determine whether opsin is synthesized in the postmitotic photoreceptor cells. To do this, the retinal cells were dissociated after BrdU injection and then double-labeled with antibodies against opsin and BrdU. Two hours after injection, thousands of cells were opsin positive. However, none of them were BrdU positive. Only after 48 hours were opsin-positive and BrdU-positive cells detected. Because the average retinal cell generation time in the P2 rat is 28 hours, this result suggests that opsin is expressed only in postmitotic rod cells.

Accordingly, we chose to compare directly the time course of IRBP and opsin protein expression during the last cell cycle before terminal differentiation. We used the method of Watanabe and Raff in developing mice at P2 or P3. This time interval was chosen because during this time, rod cells are still being formed from their precursors. Furthermore, both opsin and IRBP protein expression is easily detectable at these stages. Two hours after BrdU injection, the retinal cells were dissociated and then incubated with either anti-IRBP or anti-opsin and anti-BrdU antibod-
FIGURE 1. RNase protection quantitation of mouse IRBP opsin and β-actin mRNA levels during development. Total RNA of either 40 μg (E11 and E12), 15 μg (E13 to E15), 20 μg (E16 to E19), or 10 μg (P0 to P27, and E11 brain) was used for each sample. Quantitation of mouse IRBP mRNA was conducted twice, as indicated by IRBP 1 and IRBP 2. IRBP 3 and Opsin 2 indicate overexposures of the autoradiographies.

FIGURE 2. Densitometric comparison of mouse IRBP and opsin gene expression during development. At either stage, about 10% (i.e., 12 out of 141 cells, Table 1) of the examined cells were opsin positive. However, none of them were BrdU positive. On the other hand, between 15% and 30% (i.e., 24 out of 138 cells and 52 out of 178 cells, Table 1) of the examined cells were IRBP positive. About 6% (i.e., 8 out of 138 cells, Table 1) of the examined cells were already both BrdU positive and IRBP positive at this time. Figure 3 shows the result of one of the analyses as an example. In this figure, only two cells that were strongly stained with the IRBP and BrdU antibodies are considered IRBP and BrdU double-labeled. The weakly IRBP-immunostained cells in Figure 3b may either be rods expressing IRBP at very low levels or nonphotoreceptor cells showing background staining and are not considered IRBP positive. In any case, these cells are not considered double-labeled because they were not stained with BrdU antibody. Because the S phase in P1 mice lasts 16 hours in a cell cycle of 30 hours, this result suggests that IRBP may be synthesized in cells that are still proliferating. This notion is substantiated by evaluating the ratio between cells positive for IRBP and BrdU divided by proliferating cells (i.e., IRBP positive and BrdU positive/total BrdU positive), which is shown in Table 1. Because its value is 40%, 40% of the proliferating cells synthesize IRBP. To determine the minimum lag time between the last S phase of a precursor cell and the first detectable expression of opsin,
TABLE 1. Numbers of IRBP+, Opsin+, BrdU+, and IRBP or Opsin, BrdU Double-Labeled Photoreceptor Cells at Different Time Intervals Following BrdU Injection in Developing Mice

<table>
<thead>
<tr>
<th>Age</th>
<th>Time (hr)</th>
<th>Number of Cells Examined</th>
<th>Number of IRBP+ Cells</th>
<th>Number of BrdU+ Cells</th>
<th>Number of Double-Labeled Cells (%)</th>
<th>Number of Opsin+ Cells</th>
<th>Number of BrdU+ Cells</th>
<th>Number of Double-Labeled Cells (%)</th>
<th>Double-Labeled/Total BrdU+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2</td>
<td>2</td>
<td>138</td>
<td>24</td>
<td>12</td>
<td>8 (5.8)</td>
<td>141</td>
<td>12</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>P3</td>
<td>24</td>
<td>178</td>
<td>52</td>
<td>14</td>
<td>10 (5.6)</td>
<td>152</td>
<td>10</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>P4</td>
<td>40</td>
<td>165</td>
<td>44</td>
<td>38</td>
<td>20 (12.0)</td>
<td>220</td>
<td>48</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>P5</td>
<td>48</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = not determined.

BrdU was injected into P2 or P3 mice. The retinal cells were dissociated 2, 24, 40, and 48 hours later and double-labeled as described. A few BrdU-positive and IRBP-positive cells were first observed 2 hours after BrdU injection (about 6%, Table 1) and a few more double-labeled cells were observed 24 and 40 hours later (12% to 14%, Table 1). However, the value of the ratio of IRBP-positive and BrdU-positive cells to total BrdU positive cells seems to be unchanged. BrdU-positive and opsin-positive cells were not observed until 40 hours after BrdU injection (about 4% of the examined cells, Table 1), suggesting that opsin was expressed only in postmitotic cells. A few more double-labeled cells were observed 48 hours later (12%, Table 1). The value of the ratio, (opsin-positive and BrdU-positive cells/total BrdU-positive cells) only appears to increase 40 to 48 hours after BrdU injection.

DISCUSSION

We determined in mice the expression levels of the opsin and IRBP genes during retinal development and found that they are differentially regulated. IRBP mRNA was already detected at E11 when cones are formed, which was before rods appear. On E13 when rods are formed, IRBP mRNA is significantly in-

FIGURE 3. Two IRBP-positive and BrdU-positive cells that developed from retinal cells isolated from a P2 mouse 2 hours after BrdU injection. Cells were obtained by trypsinization, immunostained with anti-IRBP and anti-BrdU monoclonal antibodies, and photographed. The cells were photographed using (a) phase-contrast, (b) Texas red for IRBP, and (c) fluorescein for BrdU. Bar, 10.0 μm.
increased. In contrast, the rod-specific opsin mRNA was only barely detectable at P0, and it did not increase significantly until after P3. Our findings on the onset of opsin transcription in developing mouse agree well with those of Treisman et al in rat, and Al-Ubaidi et al in mouse. In both studies, opsin mRNA was always detected as early as P1. However, our findings do not agree with those of Bowes et al. In their study on rd and control mouse retinas, opsin mRNA was not detected until P5. The different onset time of opsin transcription in these studies may reflect the sensitivity of different detection methods that may be attributed to methods of hybridization (northern, RPA, or slot blot) hybridization probes used (RNA probe or DNA probe).

Under control of the IRBP promoter, expression of the CAT reporter gene in transgenic mice was first detected at low levels on E13, and it reached significant levels on E17. This experimental paradigm was not employed at earlier embryonic stages. We now find that IRBP may actually be expressed at E10 when the cone cells are born. However, precise analysis of IRBP expression before E11 is difficult because of the small number of cones formed at E10 and the difficulties involved in the dissection of ocular tissues at this early developmental stage. To circumvent these problems, we took advantage of the fact that the formation of the rod cells in mice extends from E13 to P7, with a growth peak at P0 to P1. Accordingly, BrdU injection and double labeling experiments were performed to determine whether IRBP is expressed in either the proliferating or postmitotic cells. Because the same method was used to analyze the expression of opsin in the developing rat retina, we used opsin in our experiment as a point of reference.

We found that opsin-positive and BrdU-positive cells were not detected until 40 hours after BrdU injection, which is similar to what was observed in developing rats. In contrast, IRBP-positive and BrdU-positive cells were always detected 2 hours after injection. Because the S phase in the P2 rat retinal cells lasts about 12 hours, and in the P1 mouse retinal cells it lasts 16 hours, synthesis of IRBP should already begin in the S phase. Retinas were not examined until 2 hours after BrdU injection, but there are 12 to 16 hours in the S phase. Therefore, even if 100% of the BrdU-positive cells that left the S phase after injection were labeled with IRBP, the value of the ratio of IRBP and BrdU-double labeled cells to total BrdU-positive cells IRBP positive would only lie between 16.6% (2 hours/12 hours) and 12.5% (2 hours/16 hours). However, its value at 2 hours after BrdU injection was constantly 40% (Table 1). This result also indicates that during this time, approximately 40% of the proliferating cells synthesize IRBP. This quotient seems to be unchanged during later intervals. At later intervals the "total BrdU-positive cells" would include both proliferating and emerging postmitotic cells, and this constancy further suggests that the same proliferating cells synthesizing IRBP continue to do so after mitosis. For the opsin-synthesizing cells, the "total BrdU-positive cells" obtained 40 hours and 48 hours after BrdU injection includes both proliferating and postmitotic cells. Because the value of the ratio (opsin-positive and BrdU-positive cells/total BrdU-positive cells) increased during this later interval, the number of opsin-positive cells also increases along with the number of postmitotic cells.

A disadvantage of this double-labeling method, using dissociated retinal cells, is its inability to distinguish morphologically between different cell types. However, the newly generated photoreceptor cells of P2 or P3 mice expressing opsin and IRBP are likely to be only rods: IRBP is known to be synthesized only by the rod and cone photoreceptors, and at either P2 or P3 the generation of rods continues from its peak level at P0 to P1 whereas the generation of cones has already terminated at E17.

The observed expression of IRBP at P2 or P3 in the double-labeling experiments was found to be comparable to that of opsin (Table 1) but lower than that of the corresponding mRNA levels at these stages (Fig. 2). This difference may reflect the slower increase in the expression of IRBP than that of its mRNA. A similar pattern of IRBP expression was observed previously in developing rats. This slower increase in the expression of IRBP than that of its mRNA during early development, coupled with the limited efficiency of immunoblot and immunodiffusion procedures, may explain why IRBP expression could not be detected before E11.

Using the human IRBP promoter to direct the expression of simian virus 40 (SV40) T antigen in transgenic mice, Al-Ubaidi et al reported an early formation of highly undifferentiated, bilateral retinal and brain tumors in these mice. In contrast, expression of the SV40 T antigen in transgenic mice under the control of the opsin promoter developed rod degeneration after postmitotic expression of the transgene. The earlier onset of IRBP expression in undifferentiated photoreceptor cells may explain the highly undifferentiated nature of bilateral retinal and brain tumors generated by the early expression of the SV40 T antigen in these transgenic mice.

Because the visual cycle is probably not functional throughout prenatal and early postnatal development in mice, the detection of IRBP gene expression at an early embryonic stage suggests its importance for development. One of its roles may be the extracellular transport of retinoids and fatty acids to the photoreceptor cells for the elaboration of outer segments and discs. Although IRBP has not directly been implicated
in retinal development, IRBP is the only identified interphotoreceptor retinoid carrier present in the interphotoreceptor matrix when photoreceptor cells are found. Therefore, IRBP's presence makes it an important candidate as a factor required for photoreceptor differentiation.

Key Words
gene expression, interphotoreceptor retinoid-binding protein, opsin, cell cycle, photoreceptor cell differentiation

Acknowledgments
The authors thank Dr. Larry Donoso and Dr. Vinod Gaur for gifts of the monoclonal antibodies to opsin and IRBP. They also thank Drs. Pamela A. Raymond, Peter Reinach, and Richard S. Nowakowski for their many helpful comments.

References