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Opsin Expression in the Rat Retina Is Developmentally Regulated by Transcriptional Activation

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The gene for rhodopsin, the primary light sensor of the visual system, is specifically expressed in the rod photoreceptor cells of the retina. We show here that in the rat, opsin RNA first accumulates to detectable levels at postnatal day 2 (PN2) and that nascent transcripts can be detected at PN1; this is the time when peak numbers of photoreceptor cells are generated by the final division of their neuroepithelial precursors. Accumulated opsin RNA then increases to reach the adult level, 0.06% of total retinal RNA, at about PN10. The transcription rate of the opsin gene increases to a similar extent over the same time course between PN3 and adulthood, suggesting that transcriptional activation is responsible for the increase in opsin expression. We used the antibody RET-P1 to show that rhodopsin protein is also detectable at PN2 and that the number of cells expressing the protein increases with time in a central-to-peripheral gradient in the retina. This increase in the number of differentiating photoreceptors in the tissue appears to account for much of the increase in opsin gene transcription and RNA accumulation. In situ hybridization to opsin RNA shows that it is restricted to the photoreceptor layer from the time it can first be detected, at PN7. Later in development, when RET-P1 staining shifts to the photoreceptor outer segments, opsin RNA becomes localized to the inner segments, suggesting that the distributions of opsin protein and RNA are related.

Cell type determination in the mammalian nervous system is still a poorly understood process in which the existence and relative importance of preprogrammed developmental decisions and instructive or permissive environmental signals have yet to be elucidated. Because much neuronal differentiation is not visible until after the final mitosis of multipotential precursor cells, the role of cell division in activating the genes underlying cell type-specific functions is unclear. Mitosis is probably not essential for every stage of molecular differentiation, since after the final division of a retinal neuroblast, the daughter cells can become very different cell types, such as a rod photoreceptor and a Muller glial cell (43). One difficulty encountered in studying these questions in many areas of the nervous system is that the extensive cellular heterogeneity results in a very low proportion of any particular cell type. The retina, in contrast, contains a small number of cell types arranged in clearly defined layers, is accessible, and undergoes much of its development postnatally; these qualities make it a useful model system. The anatomy and physiology of the retina have been studied intensively (for a review, see reference 27), and more recently, many of its cell type-specific products have been identified by monoclonal antibodies (for reviews, see references 4 and 5) and, in the case of photoreceptors (6, 22, 32, 35) and Muller glia (46), by cloned DNA probes. In adult rodents, rod photoreceptors constitute approximately 90% of all retinal cells and are therefore particularly amenable to study.

Photoreceptors are responsible for the initial step in visual processing, converting the signal of photon absorption into synaptic transmission. A number of the proteins involved in the phototransduction cascade have been characterized. Light is absorbed by the visual pigment protein rhodopsin, and the subsequent conformational change leads to the activation of a cyclic GMP phosphodiesterase through a specific G-protein intermediate, transducin (for a review, see reference 42). The interphotoreceptor retinol-binding protein (IRBP) is also involved in the visual cycle. This protein is synthesized by photoreceptors and secreted into the extracellular space between photoreceptors and the retinal pigment epithelium (26). It carries the retinal chromophore between photoreceptors and the retinal pigment epithelium, where the bleached all-*trans* isomer is enzymatically converted back to the active 11-*cis* isomer (31). Other molecules specific to photoreceptors, such as rhodopsin kinase and the light-regulated cation channel, have been less well characterized biochemically.

In rodents, the peak of rod photoreceptor birth is at postnatal day 1 (PN1) to PN2 (12, 39, 52); cells in the central retina are born before those in the peripheral retina. The precursor cell that generates rods can also give rise to a bipolar, amacrine, or Muller glial cell on its final division (43). As rod differentiation proceeds, mitochondria and rough endoplasmic reticulum collect at the distal pole of the cell to form an inner segment at about PN4 (23). From a distal cilium, an outer segment begins to form at PN6 to PN7 and reaches its mature form by PN14 (48). Spectrophotometric detection of the retinal chromophore is possible after PN6 to PN7 in the mouse (11), but by using immunocytochemistry, opsin can be detected on a few rat retinal cells as early as PN1 (24). Other components of the visual transduction apparatus are present by the end of the first postnatal week, since an antibody sensitive to light-induced phosphorylation of the opsin molecule can detect this modification at PN8 (24). IRBP has been shown to appear at embryonic day 17 and increase during and after the time of inner segment formation in mice (11), and the protein encoded by c-*src* appears in the processes of rods and cones, as well as in ganglion and amacrine cells, at the end of mitosis in the chick retina (41), correlating with an increase in c-*src* mRNA (45). While some morphological differentiation and regionalized opsin expression can occur in monolayer culture (1, 2), full outer segment maturation may require interactions with

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other cells, such as Muller glia (2a). The pigment epithelium may also play a role in retinal development (47).

To understand the molecular mechanisms governing rod photoreceptor differentiation, it is first necessary to define the developmental stage at which particular properties appear and the level at which their expression is controlled. Here we examine one of the earliest stages in photoreceptor differentiation, the commitment to opsin gene expression. We show that opsin RNA levels increase over the first 10 days postnatally, and we used nuclear runoff transcription assays to show a similar increase in transcription initiation at the opsin gene in comparison with transcription initiation at ribosomal RNA and β-tubulin gene controls, suggesting that opsin expression is regulated at the transcriptional level. Immunological staining of retinal tissue with an antibody to opsin indicates that much of the early phase of the increase in transcription is consistent with an increase in the number of cells differentiating into rod photoreceptors. Opsin transcripts are shown to be localized to the photoreceptors and to become localized to their inner segments when opsin protein becomes restricted to the outer segments; mRNA location may be a primary determinant of protein cellular and subcellular localization.

MATERIALS AND METHODS

Clones. The original bovine opsin cDNA clone, cBR26.1, was the gift of Meredith Applebury; it was a 2.65-kilobase-pair (kb) *Eco*RI fragment cloned into pBR329, including 1 kb of 5′ and 500 base pairs (bp) of 3′ noncoding sequence. A 326-bp *Eco*RI fragment of IRBP cDNA cloned into pUC13, clone B-23, was obtained from David Bridges. A chicken β-tubulin 1.7-kb cDNA cloned into the *Pst*I site of pBR322 (16), a mouse 28S rRNA 4.8-kb clone in the *Sal*I and *Eco*RI sites of pBR322 (original source, N. Arnheim), and a *Drosophila* $tRNA^{arg}$ 500-bp clone in the *Hin*dIII site of pBR322 (40) were generously provided by David Clayton.

RNA preparation. Tissues frozen in liquid nitrogen were homogenized in 5 M guanidine thiocyanate–25 mM sodium citrate–0.5% sarcosyl–2 mM EDTA–5% β-mercaptoethanol by using a Polytron (Brinkman Instruments). After 5 min at 50°C, 0.1 g of CsCl was added per ml and 3.5 ml of the extract was centrifuged through a 1-ml 5.7 M CsCl–0.1 M EDTA cushion for 16 h at 20°C and 200,000 × *g*. The pellet was suspended in 7 M urea–2% sarcosyl, extracted with phenol-chloroform-isoamyl alcohol (24:24:1, vol/vol), and ethanol precipitated. Poly(A)$^+$ RNA was selected on oligo(dT) cellulose (Collaborative Research, Inc.), and Northern (RNA) blot analysis was done as described by Maniatis et al. (33) after size fractionation on formaldehyde-agarose gels.

Dot blots. RNA samples for blotting were suspended in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA at a concentration of 1 to 2 mg/ml; the exact concentration was determined from the A_{260}. Control samples were treated with DNase-free RNase at 50 μg/ml for 30 min at 37°C. All samples were then adjusted to 100 μl with water; 300 μl of 10× SSC–6.15 M formaldehyde was added (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and the samples were incubated for 15 min at 65°C before they were loaded onto nitrocellulose prewet with 10× SSC in the blotting apparatus (Schleicher & Schuell, Inc.) and washed through with 10× SSC. DNA samples were incubated for 30 to 60 min at 65°C with 0.3 M NaOH and neutralized with 1 volume of 2 M NH_4OAc (pH 7.0) before they were loaded.

Preparation of probes and filter hybridization. The rOPps plasmid (Fig. 1) was linearized with *Hin*dIII for sense transcription and with *Eco*RI for antisense transcription; T7 RNA polymerase was used in the sense direction, and T3 polymerase was used in the antisense direction. Transcription was carried out for 1 h at 37°C in a 20-μl volume of 40 mM Tris hydrochloride (pH 7.5)–6 mM $MgCl_2$–2 mM spermidine–10 mM NaCl–10 mM dithiothreitol–1 U of RNasin per μl–0.5 mM ATP, GTP, and CTP–24 μM UTP–10 μg of plasmid DNA per ml–2.5 mCi of [α-^{32}P]UTP per ml (3,000 Ci/mmol; New England Nuclear Corp.)–0.8 U of polymerase per μl. The sample was then adjusted to a final volume of 300 μl containing 0.1% sodium dodecyl sulfate and 0.1 mg of yeast tRNA per ml. The sample was extracted with phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol and precipitated once with 0.3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol, once with 3 M LiCl overnight at 0°C, and once with 0.3 M NH_4OAc and 2.8 volumes of ethanol. The extent of incorporation and the specific activity of the probe were evaluated by trichloroacetic acid precipitation of part of the sample, and the size of the probe was determined by polyacrylamide gel electrophoresis; the majority of transcripts were full length.

Filters were prehybridized at 55°C in 50% formamide–50 mM sodium phosphate (pH 6.5)–5× SSC–0.1% sodium dodecyl sulfate–1× Denhardt solution–1 mM EDTA–200 μg of salmon sperm DNA per ml–100 μg of yeast tRNA per ml for 1 to 2 h and hybridized in a fresh lot of the same solution with the addition of 2×10^6 to 1×10^7 trichloroacetic acid-precipitable cpm/ml of probe. Filters were washed at a final stringency of 0.1× SSC–0.1% sodium dodecyl sulfate at 65°C, including, in some cases, a 15-min RNase A treatment (1 μg/ml, 2× SSC, room temperature) and exposed to X-ray film (Kodak XAR-5). Dot blots were then cut into individual spots, which were counted in a liquid scintillation counter.

Nuclear runoff transcription assay. A total of 20 to 25 rats were used per experiment; the total yield was 2×10^8 to 8×10^8 retinal nuclei and 0.5 to 10 ng of RNA labeled to 3×10^9 to 5×10^9 cpm/μg. The procedure used was that of Derman et al. (19) as modified by Clayton et al. (14) with the following changes. Retinas were dissected into cold phos-

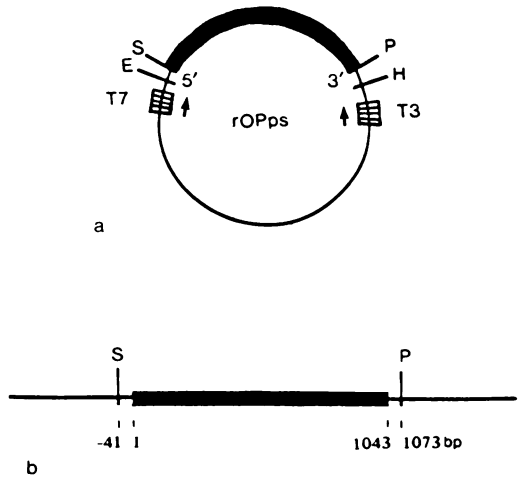


FIG. 1. (a) Plasmid rOPps, used to synthesize RNA probes complementary to the opsin gene. Symbols: ▬, subcloned sequence; ▤, T3 and T7 promoter sequences; →, direction of transcription. The vector, pT3/T7-18, was obtained from Bethesda Research Laboratories. (b) Subcloned region in relation to opsin coding sequence (▬). P, *Pst*I; S, *Sma*I; H, *Hin*dIII; E, *Eco*RI.

phate-buffered saline (PBS), washed twice in cold PBS and once in cold RSB (10 mM NaCl–10 mM Tris hydrochloride (pH 7.4)–3.5 mM $MgCl_2$–14 mM β-mercaptoethanol), and suspended in cold RSB containing 0.2% Triton X-100. After 3 min on ice, KCl was added to 150 mM and the tissue was gently homogenized with 4 to 8 strokes of a Teflon pestle. Nuclei were pelleted at 4°C for 4 min at 800 × *g* and were frozen in 40% glycerol–50 mM Tris hydrochloride (pH 8.0)–5 mM $MgCl_2$–0.1 mM EDTA. After the nuclei were labeled (14), incorporation was measured by trichloroacetic acid precipitation and filtration. Then, labeled RNA purified as described in reference 14 (5×10^6 to 2×10^7 cpm/ml) was hybridized to 5-μg samples of cloned cDNAs blotted onto nitrocellulose as described above. A 1/100 dilution of the probe was used for a separate 28S RNA filter, and in some experiments, a 1/10 or 1/100 dilution was used for the tRNA filter. A spot of the vector pT3/T7-18 was always included as a control for nonspecific hybridization. A high-stringency wash in 0.2× SSC–0.1% sodium dodecyl sulfate (30 min, 65°C) was included after the washes in RNase and proteinase K. Filters were scanned on a densitometer, using the Drexel UNIX-based microcomputer image analysis system; at least three exposures were included for every film, and only those samples within the linear range of optical density, determined from a set of standard spots, were included in the quantitation.

In situ hybridization. The procedure used was that of Cox et al. (17) with slight modifications. Eye cups were prefixed for 1 h in 4% paraformaldehyde in PBS, sunk in 30% sucrose in PBS overnight, and frozen in Tissue-Tek (Miles) compound. Sections (15 μm) were mounted on slides coated with polylysine (molecular weight, >150,000; 50 μg/ml in 10 mM Tris hydrochloride [pH 8.0]) and briefly postfixed to the slides in 4% paraformaldehyde in PBS. The pretreatment with proteinase K was reduced to 15 min to better preserve tissue morphology and was followed by a 10-min postfixation in 4% paraformaldehyde in PBS. Acetic anhydride treatment was included as described. Probes were transcribed as described above by using α-^{35}S-labeled UTP (1,000 Ci/mmol) and hydrolyzed at 60°C in 40 mM $NaHCO_3$–60 mM Na_2CO_3 (pH 10.2) for 40 min, which reduced them to 150 to 200 bp to improve their penetration. Sections were hybridized at 50°C overnight under mineral oil in 50% formamide–0.3 M NaCl–20 mM Tris hydrochloride (pH 8.0)–5 mM EDTA–1× Denhardt solution–500 μg of yeast tRNA per ml–10 mM dithiothreitol–10% dextran sulfate–10^7 cpm of RNA probe per ml. Washes were done in 20 μg of RNase A per ml of 0.5 M NaCl–10 mM Tris hydrochloride (pH 8.0, 30 min, 37°C) in 0.5 M NaCl–10 mM Tris hydrochloride (pH 8.0, 30 min, 37°C) and in 0.6 M NaCl–1 mM EDTA–20 mM Tris hydrochloride (pH 7.4)–50% formamide (three times, 1 h, 37°C). Slides were then dehydrated, dipped in Kodak NTB-2 emulsion diluted 1:1 with 0.6 M ammonium acetate, and exposed for 7 to 10 days at 4°C. Counterstaining of cells was done with 0.1% toluidine blue–1% sodium borate. Grains were counted by using an eyepiece grid (Morrell) in a Zeiss microscope at a magnification of ×630. Radial sections of retina were chosen at random in areas where the cellular morphology was good enough to distinguish all layers, and an area of 15 by 150 μm was counted in each layer.

Immunocytochemistry. Eye cups were prepared as described above and 15-μm frozen sections were mounted on subbed slides. Sections were preincubated for 30 min in 5% normal goat serum in PBS and then incubated for 2 h in RET-P1 in 5% normal goat serum. RET-P1 is a monoclonal antibody that reacts with an epitope at the N terminus of rhodopsin; it does not cross-react with cone opsins (4, 5, 21). Sections were washed in PBS and incubated for 1 h in rhodamine-conjugated goat anti-mouse immunoglobulin G in 5% normal goat serum. After another wash in PBS, sections were mounted in PBS-glycerol (1:1, vol/vol) and viewed in a Zeiss microscope equipped with epifluorescence illumination.

RESULTS

A 1.1-kb *Sma*I-*Pst*I fragment from the bovine opsin cDNA clone cBR26.1 was subcloned into the vector pT3/T7-18 to allow the synthesis of asymmetric RNA probes (Fig. 1). This construct (rOPps) includes the entire opsin-coding sequence, extending from 41 bp 5′ of the initiator codon to 30 bp 3′ of the terminator codon. An antisense probe transcribed from rOPps recognized a single band of 1.5 kb on Northern blots of poly(A)$^+$ RNA from adult or PN7 rat retina (Fig. 2, lanes a and d). However, the control coding strand probe and the vector sequences included within the antisense probe did not hybridize to retinal RNA (Fig. 2, lanes b and c). The amount of opsin mRNA increased five- to sixfold between PN7 and adulthood, but there was no gross change in its size. Related genes, such as those coding for the cone opsins, were not recognized at the stringency used (0.1× SSC, 65°C) in either Northern blots (Fig. 2) or Southern blots (data not shown).

The time course of opsin RNA accumulation throughout development was evaluated by filter hybridization; total RNA extracted from rat retinas at a series of ages was

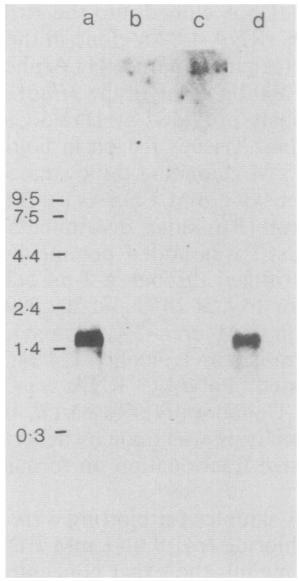


FIG. 2. Northern blot hybridization showing that the probe used is specific for opsin mRNA. Lanes a, b, and c contain 3 μg of poly(A)$^+$ adult retinal RNA. The RNA was probed with an antisense RNA strand transcribed from rOPps (lane a), a sense strand transcribed from rOPps (lane b), or a mixture of transcripts made in both directions from the vector pT3/T7-18, linearized at the same sites used to generate the sense and antisense probes, representing the portions of these probes not derived from opsin (lane c). Lane d is from a separate experiment and contains 4 μg of PN7 poly(A)$^+$ RNA, which was probed with an antisense rOPps transcript. Lanes b, c, and d were exposed for approximately five times as long as lane a. Molecular size standards (in kilobases) are from an RNA ladder (Bethesda Research Laboratories).

blotted onto nitrocellulose and probed with the antisense rOPps transcript. All RNA samples were used at three different concentrations, each in duplicate, and a control sample at the highest concentration was treated with RNase to correct for possible hybridization to contaminating DNA. Individual spots were then counted in a scintillation counter to measure the extent of hybridization. Opsin RNA was barely detectable over background at PN2 but increased sharply over the next 10 days to reach the adult level at PN10 to PN12, just before the time of eye opening (Fig. 3). By comparison with the signal bound to known quantities of rOPps plasmid DNA on the same filter, opsin-homologous sequences were found to account for approximately 0.06% of total retinal RNA in the adult (Fig. 3b); this represents a 300-fold increase over the level at PN2. The level of 28S ribosomal RNA, as measured by the same procedure, declined slightly over this period (data not shown), but this change was probably not sufficient to account for all the increase in opsin transcripts as a proportion of total RNA, since the same increase was not seen for other genes transcribed by polymerase II, such as the tubulin gene and c-*yes*.

The rate at which the opsin gene is transcribed was measured by pulse-labeling the nascent transcripts of isolated retinal nuclei and hybridizing them to filters with spots of plasmid DNA in excess of the probe. IRBP cDNA was also included in this experiment, and since an absolute measure of transcription rate cannot be obtained, opsin gene transcription was measured relative to the transcription of β-tubulin, 28S rRNA, and $tRNA^{Arg}$ cDNA controls. When used to detect 28S rRNA, the probe was diluted 1/100, and when used to detect tRNA, the probe was diluted 1/10 or 1/100 or was left undiluted, as necessary. Figure 4a shows a typical series of autoradiographs, and Fig. 4b shows the result of densitometric scanning of films from triplicate experiments at each age. In comparison with genes transcribed by polymerase I (28S RNA), polymerase II (tubulin), and polymerase III (tRNA), the opsin gene showed an increase in transcription rate (30-fold with respect to 28S RNA) from birth to adulthood, with the sharpest rise between PN3 and PN9. The IRBP gene also showed an increase in transcription rate, which occurred largely between PN6 and PN9 (Fig. 4b). However, tubulin, which is not specific for photoreceptors, showed little change after birth when compared with 28S RNA. The transcription rate of the tRNA gene fluctuated with age, but there was no general trend of increase or decrease. These measurements are unlikely to be affected by changes in cell number; the bulk of rod formation is over by PN4, and although Muller and bipolar cells are born postnatally, their numbers are too small to cause a significant dilution of photoreceptor gene transcription (52).

The cellular specificity of opsin transcription was analyzed by in situ hybridization to retinal sections with ^{35}S-labeled RNA probes. At PN7, the earliest age at which a specific signal could be obtained, the photoreceptor layer showed a clear accumulation of silver grains, representing hybridization to opsin transcripts, while the other cellular and synaptic layers showed only background hybridization; these observations were quantified by grain counting (Fig. 5a). Silver grains were evenly distributed over the entire outer nuclear layer at this age, but at PN10, most grains became localized over the inner segment layer, where they remained in the adult retina (Fig. 5b and c). The coding strand probe was used as a control in all experiments and never gave hybridization above the background level. At PN4, neither the antisense nor the sense probe hybridized to any cell layer at a level clearly above background.

The appearance of opsin protein was correlated with the expression of its gene by fluorescence immunocytochemistry using the monoclonal antibody RET-P1, the binding of which to photoreceptors is inhibited by purified opsin (21). At PN2 (Fig. 6a), staining was observed on a few scattered cells in the outer neuroblastic layer. At PN5 (Fig. 6b and c), many more of the cell bodies in this layer were labeled, and the labeling showed a clear developmental gradient; in the central retina (Fig. 6b), more cells were stained than in the peripheral retina (Fig. 6c). By PN9, all the cells in the outer nuclear layer appeared to be labeled and there was a band of labeling over the photoreceptor outer segments (Fig. 6d); at PN13, there was a further shift of fluorescence intensity to the outer segments (Fig. 6e).

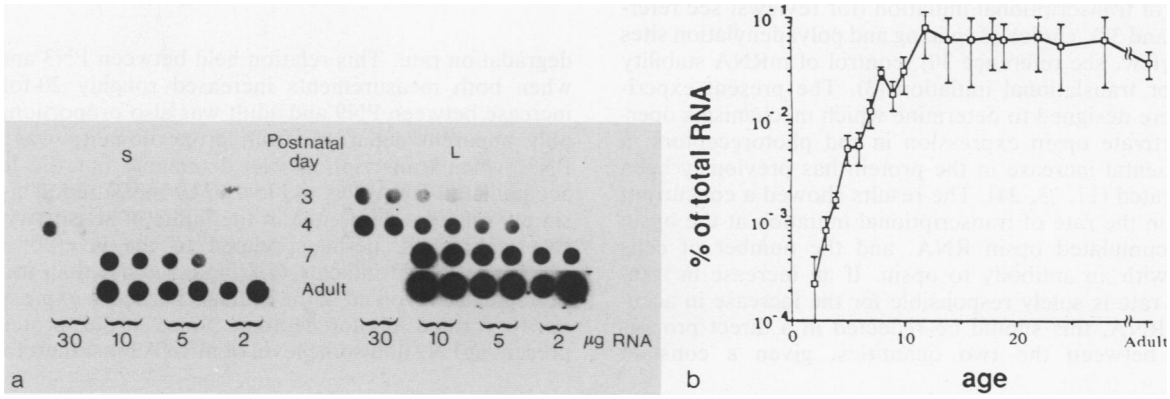


FIG. 3. (a) Examples of the intensity of hybridization seen in dot blots of total retinal RNA probed with an antisense rOPps transcript. Samples of 30, 10, and 5 μg are shown for PN3 and PN4 animals, and samples of 10, 5, and 2 μg are shown for PN7 and adult animals. The same film was exposed longer for the blots on the right (L) than for those on the left (S). RNase-treated 30-μg (PN3 and PN4) or 10-μg (PN7 and adult) samples gave no visible signal at either exposure (data not shown). (b) Results of quantitation of similar blots at all ages examined. The counts per microgram bound to an RNase-treated control were subtracted from the mean counts per microgram bound, and the percentage of total RNA was calculated by comparison with the counts per picogram bound to rOPps plasmid DNA samples. Error is equal to the standard deviation.

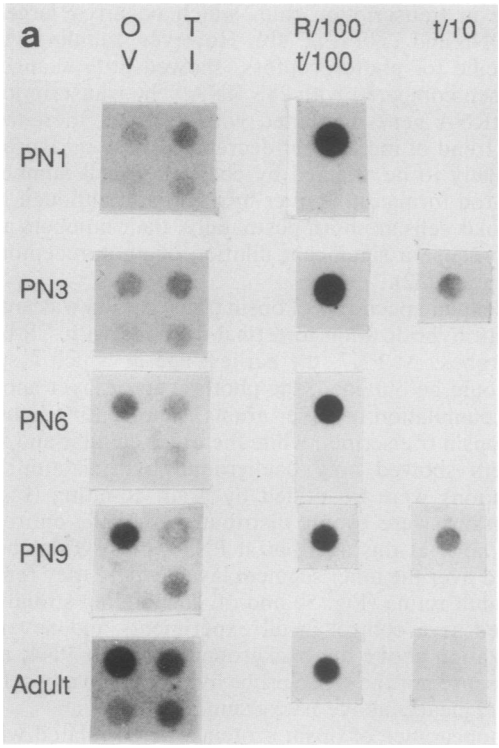
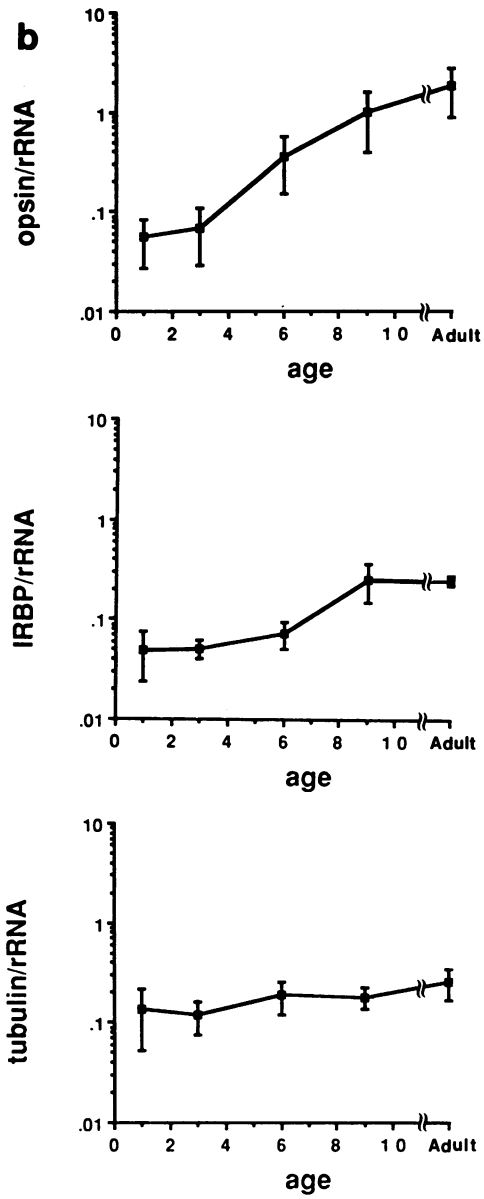


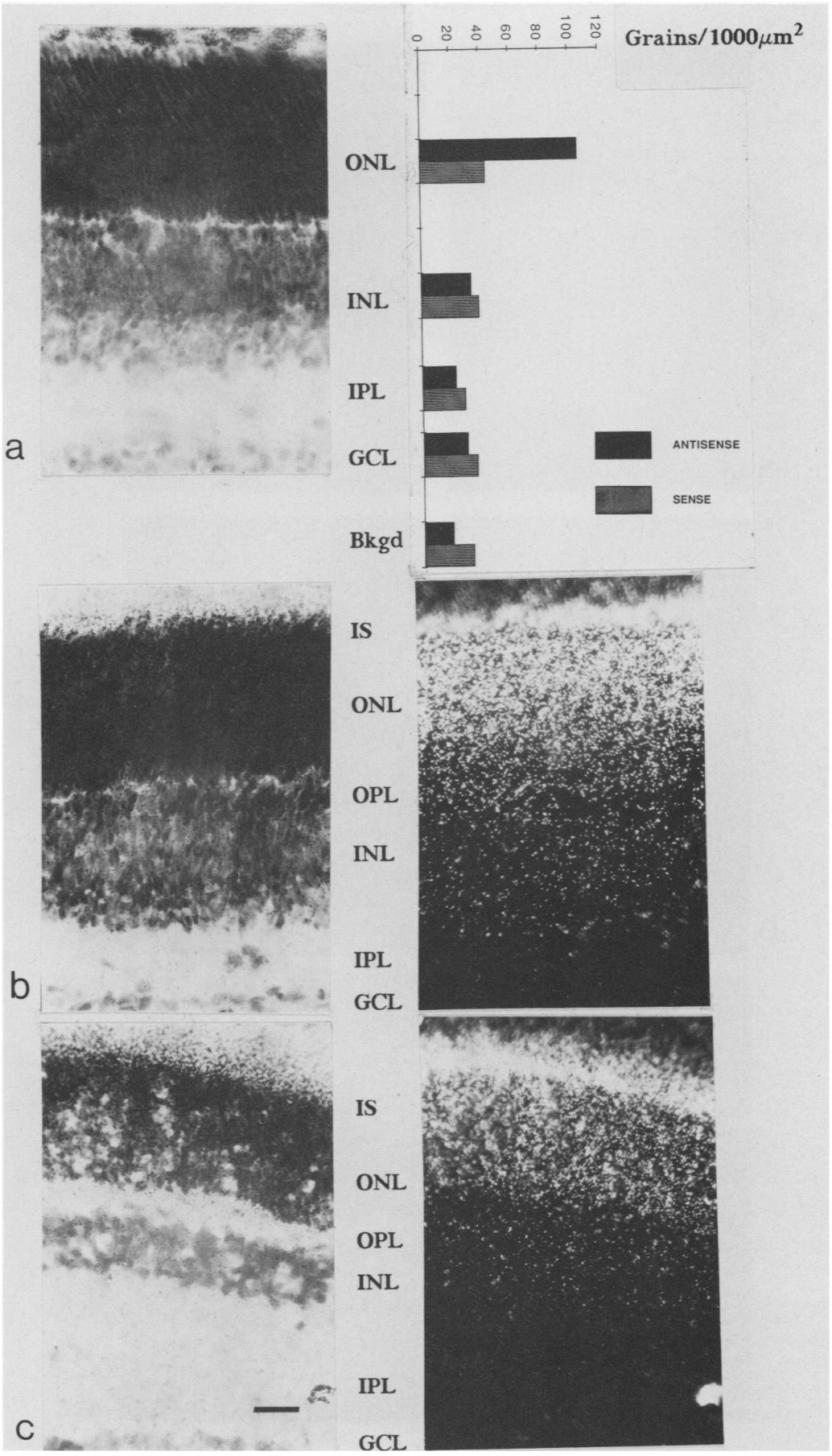
FIG. 4. Developmental assays of transcription rate. (a) Typical series of filters showing the increase in hybridization to opsin cDNA compared with that to other plasmids. O, Opsin; T, β-tubulin; V, pT3/T7-18, a control for nonspecific hybridization; I, IRBP; R/100, 28S RNA, probe diluted 1:100; t/100, $tRNA^{Arg}$, probe diluted 1:100; t/10, $tRNA^{Arg}$, probe diluted 1:10. tRNA probe dilutions of 1:100 are shown for PN1 and PN6, and dilutions of 1:10 are shown for PN3, PN9, and adult samples. (b) Results of densitometric analysis of several exposures of three sets of filters. The ratios of opsin to 28S rRNA (top), IRBP to 28S rRNA (middle), and tubulin to 28S rRNA (bottom) are shown. The optical densities actually measured were used to calculate these ratios; the true rRNA transcription rate would be 100 times greater. Error is equal to the standard deviation.



DISCUSSION

Developmental regulation of gene expression can occur at the level of transcriptional initiation (for reviews, see references 34 and 38), choice of splicing and polyadenylation sites (for a review, see reference 30), control of mRNA stability (7, 13), or translational initiation (3). The present experiments were designed to determine which mechanisms operate to activate opsin expression in rod photoreceptors; a developmental increase in the protein has previously been demonstrated (11, 23, 24). The results showed a concurrent increase in the rate of transcriptional initiation at the opsin gene, accumulated opsin RNA, and the number of cells staining with an antibody to opsin. If an increase in transcription rate is solely responsible for the increase in accumulated RNA, this should be reflected in a direct proportionality between the two quantities, given a constant degradation rate. This relation held between PN3 and PN9, when both measurements increased roughly 20-fold; the increase between PN9 and adult was also proportional. The only apparent departure from proportionality was before PN3, when transcription was detectable but the level of accumulated RNA was too low to be measured. This could simply reflect a difference in the limits of sensitivity of the two techniques, perhaps related to the level of rRNA, although it could indicate that factors other than transcription rate are involved in the initiation of opsin expression on newly formed photoreceptors. Since opsin protein was present at PN2 and some level of mRNA must therefore also

FIG. 5. In situ hybridization to opsin RNA. (a) Section of PN7 retina labeled with antisense opsin RNA and results of counting silver grains over cellular and synaptic layers of randomly chosen regions of PN7 retina labeled with antisense and sense probes. Eight regions were counted for each probe. The photoreceptor layer shows more than twice the background level of grain accumulation. PN10 (b) and adult (c) retinas were also labeled with antisense opsin RNA. Bright-field photomicrographs are on the left, and dark-field photomicrographs are on the right. IS, Inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; Bkgd, background on slide. Bar, 20 μm.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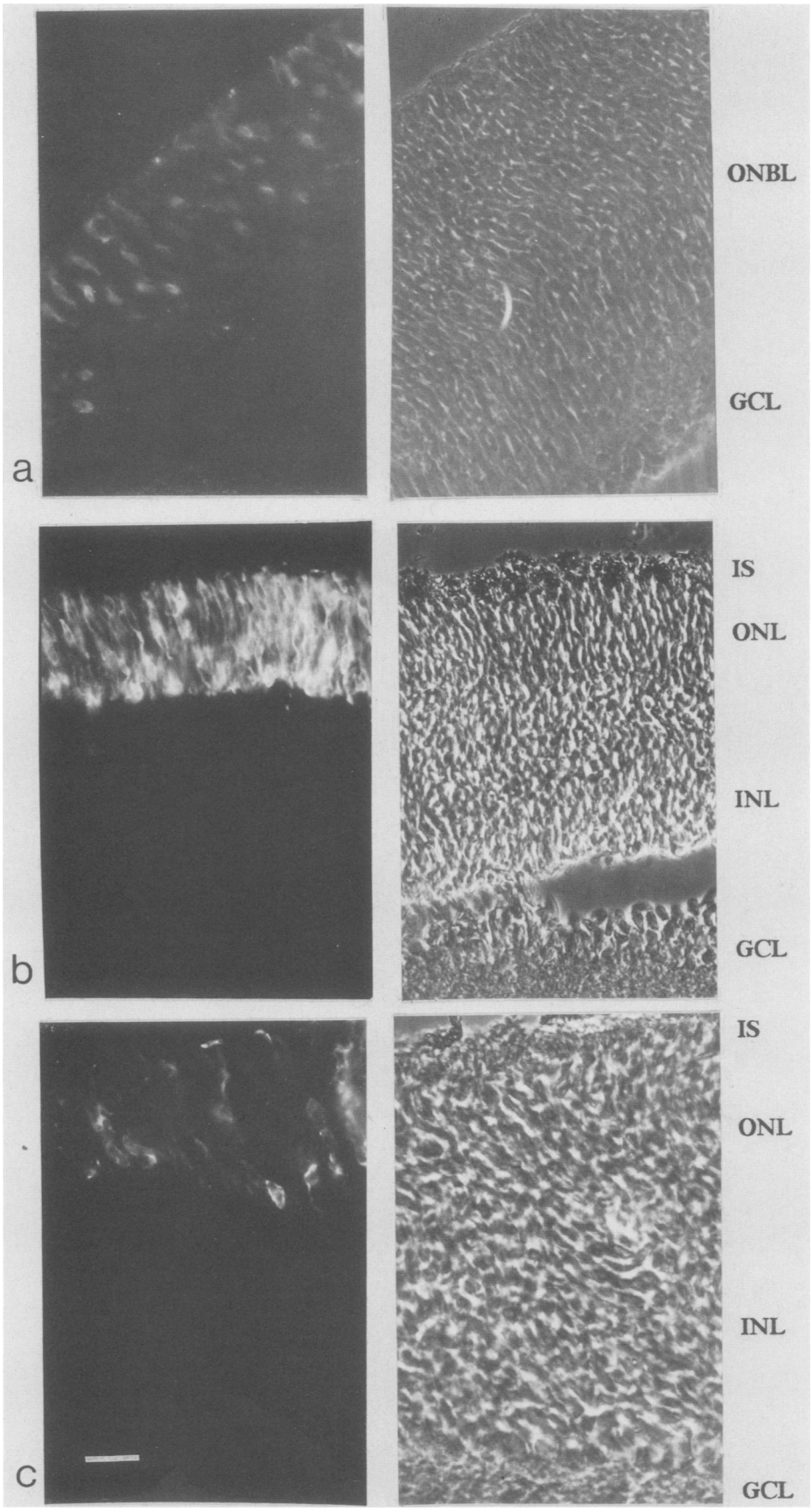
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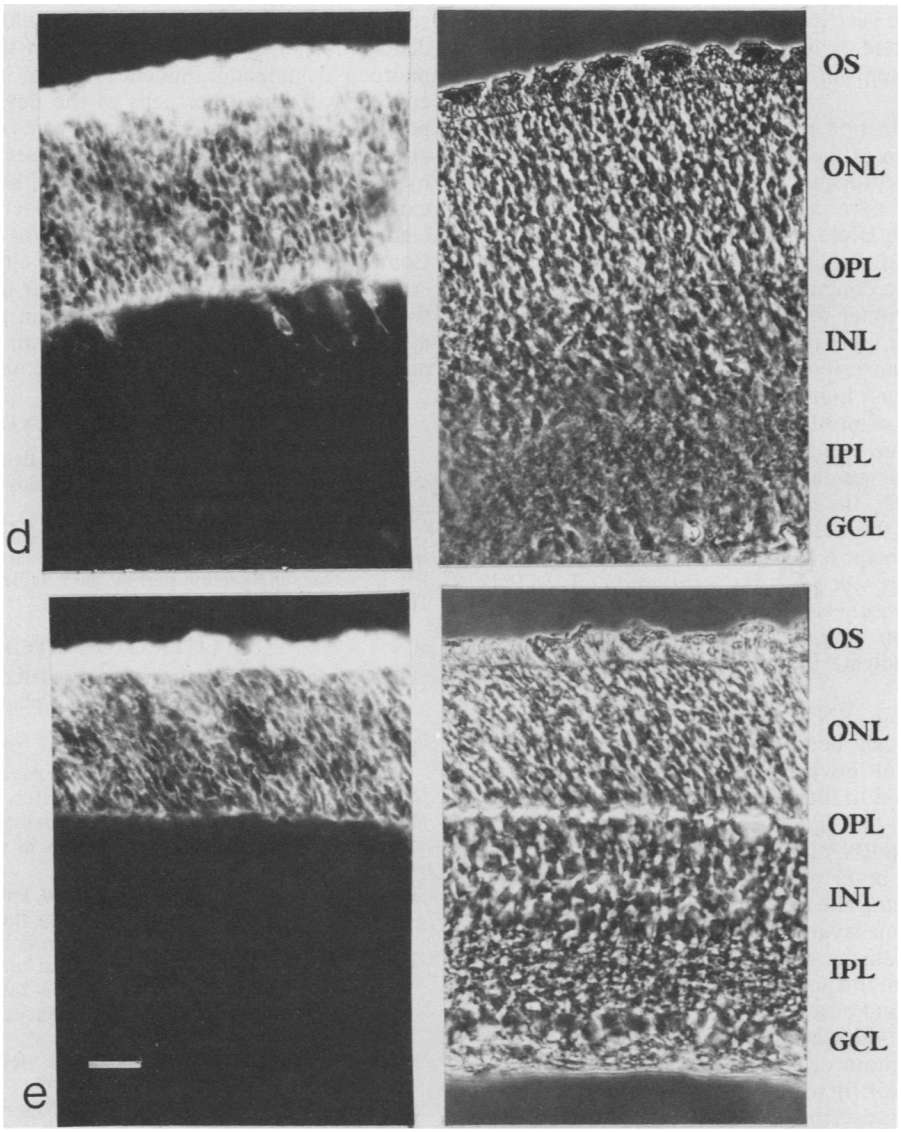

FIG. 6. Tissue sections stained with antibody RET-P1. Rhodamine epifluorescence micrographs are on the left, and phase-contrast micrographs are on the right. (a) PN2 retina; (b) PN5 central retina; (c) PN5 peripheral retina; (d) PN9 retina; (e) PN13 retina. ONBL, Outer neuroblast layer; GCL, ganglion cell layer; IS, inner segment; ONL, outer nuclear layer; INL, inner nuclear layer; OS, outer segment; OPL, outer plexiform layer; IPL, inner plexiform layer. Bar, 20 μm.

be present, it seems likely that transcriptional activation in increasing numbers of cells, followed by or concurrent with an increase in transcription rate in each cell, can account for all the observations. Opsin RNA levels appeared to fall off slightly after a peak at PN12 to PN18; such a plateau might be expected, because the rate of disk synthesis is greatest at this time (28).

Although the data obtained for opsin are relative to total RNA or rRNA and therefore depend on the behavior of other genes, the trend observed was the same for all independent measurements and was clearly different from expression of the tubulin gene. Cleveland and others (10, 15, 37) have shown that in cultured mammalian cells, β-tubulin is autoregulated by selective destabilization of its mRNA rather than by changes in transcription rate. Filter hybridization measurements of tubulin RNA as a proportion of total RNA in the retina during development show that it decreases 20-fold between PN1 and adult rats (unpublished observations). However, its transcription rate is constant compared with that of rRNA (Fig. 4), and this suggests that RNA degradation may be the regulated step in this system as well. The IRBP gene may exhibit transcriptional activation at a slightly later stage than the opsin gene does, but further experiments would be necessary to confirm this, since only a fivefold increase in the transcription rate of the IRBP gene was measured, rather than the 30-fold change seen for the opsin gene.

Under the conditions used in these experiments, cone opsins would not have been detected. In humans, it is possible to detect cone opsin genes by low-stringency hybridization to a rhodopsin probe (36). In addition, antibodies against some regions of the rhodopsin molecule cross-react with cone opsins (25). Since the peak of cone photoreceptor birth is at embryonic day 14 (52), more than a week earlier than the peak of rod photoreceptor birth, it would be of great interest to compare the mechanisms regulating rod and cone

opsin expression. The very low proportion of cones in the rat retina would necessitate either a large increase in sensitivity or the use of a more appropriate species to carry out such an analysis.

The in situ hybridization results shown here support the idea that transcription of the opsin gene is activated specifically in the photoreceptors and not in any other cell type. At PN7, opsin RNA was approximately 0.01% of total RNA, or 17% of its adult level, while at PN10, it had almost reached its adult level (Fig. 3). In situ hybridization confirmed this increase and showed a concurrent concentration of the RNA in the photoreceptor inner segments. Residual hybridization over the outer nuclear layer probably reflects the presence of immature nuclear transcripts. Staining with RET-P1 similarly showed an increase in intensity between PN5 and PN13 and a concentration of protein in the outer segments. The distribution of the stain in a central-to-peripheral gradient at PN5 follows a similar gradient of photoreceptor birth rate (12, 52). Unfortunately, the in situ technique is not sensitive enough to compare the cellular distribution of RNA and protein at very early ages; a central-to-peripheral gradient of RNA at PN5 or its presence in a few scattered cells at PN2 would support the hypothesis that opsin expression depends only on transcription rate, while a more general RNA distribution would indicate the involvement of later regulatory steps.

Restriction of opsin RNA to the photoreceptor inner segments has previously been demonstrated by Brann and Young (9) for the adult bovine retina. In PN7 rats, hybridization is not restricted to the inner segments, although they are already present at this age as a clearly defined layer (23). Instead, the change in RNA location seems to correlate with the development of outer segments and the transport of opsin protein into them. The concentration of opsin message over the inner segment layer is interesting to compare with the distribution of transducin and IRBP RNA. Transducin RNA is also specific to the inner segment (9), and the protein is found in the inner and outer segments, the amount in each varying with the light-dark cycle (8). However, IRBP RNA is present over the photoreceptor cell bodies (44), and the protein is secreted (26). It would be of interest to examine the distribution of transcripts of other rod photoreceptor genes, such as those coding for tubulin, a cytoplasmic protein found in the cilium, cell body, and axon, and SVP38, a synaptic vesicle protein (20), to determine whether RNA distribution bears any relationship to eventual protein distribution. Restricted distributions of the RNAs for some cytoskeletal proteins have been reported to correlate with the locations of their protein products (29). Much of the translational apparatus of the rod cell is concentrated in the inner segment (51), but this could simply reflect the high concentration of protein in the outer segments and their constant degradation and renewal; in adult rats, the transcription rate of the opsin gene was eight times that of the tubulin and IRBP genes (Fig. 4). Further data on these issues might shed some light on the question of protein localization in polarized cells in general.

The observation that opsin expression is transcriptionally regulated raises the question of how the active state of the gene can be selectively established in only one of the two daughters of a dividing cell, allowing a single precursor to generate two different cell types (43). One possibility is the use of replication to introduce modifications of chromatin structure, for example, by retention of factors or modified bases on the parent strand and loss of the ability to reproduce them on the newly formed strand (for a review, see reference 49). Other mechanisms could involve an uneven distribution of cytoplasmic factors in the precursor cell or microenvironmental influences, such as signals from or contact with the other cells of the developing retina. It is possible that early determinative events could be detected as alterations in chromatin structure revealed by nuclease-hypersensitive sites (18, 50). It would be of great interest to examine the regulation of gene products that appear much later in rod photoreceptor development than opsin does (5). Comparison of the times at which changes in chromatin structure and transcription rate occur for these genes with the corresponding times for the opsin gene would provide insight into the number and mechanism of molecular events necessary to produce a differentiated neuron.

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