Purpose. To understand the developmental processes in the differentiating bovine retina, topographic accumulation of rhodopsin mRNA in staged fetal and adult retinas was analyzed.

Methods. Isolated retinas were spread on a nylon membrane with the photoreceptor cells facing the membrane and dissected into 25-mm square tissue segments, sometimes with as many as 150 segments/eye. Subsequent to disruption of the tissue in each segment, rhodopsin and β-actin mRNA levels were quantitated with a solution hybridization assay. Slight variations in RNA extraction efficiency and retinal segment size were corrected using β-actin mRNA as an internal standard.

Results. Analysis of multiple fetal and adult bovine retinas revealed a relatively static central-to-peripheral gradient of rhodopsin mRNA level that appears at the time of transcriptional induction (6 to 6.5 months of gestation) and persists into adulthood. After induction of rhodopsin mRNA expression, increase of rhodopsin mRNA levels was detected simultaneously in all retinal segments. Furthermore, the rate of increase in rhodopsin mRNA levels in peripheral and central regions was identical.

Conclusions. Fetal induction of rhodopsin mRNA expression occurs simultaneously in all photoreceptor cells across the retina, but the levels are set according to a topographically predetermined pattern. This suggests that regulation of accumulation of rhodopsin mRNA during development is determined according to spatial coordinates before gene induction, most likely in a nonphotoreceptor retinal cell type. Invest Ophthalmol Vis Sci. 1995; 36:2008-2019.

Mechanisms governing developmental processes in the differentiating mammalian retina are not fully understood. A key question regards the relative importance of genetic lineage versus environmental cues in retinal cell fate. Early studies suggested a major and critical role for the retinal cellular microenvironment. More recent studies favor this viewpoint and provide experimental evidence that retinal progenitor cells are pluripotent. However, apparent lineage-specific effects have been reported as well. The simplest model to explain environmental cues regulating retinal maturation suggests that diffusible factors, cell-cell contact, or both, direct cell-fate pathways. Yet, precisely how such signals specifically induce expression of any retinal gene remains obscure.

Developmental aspects of mammalian retinal gene expression have been studied in several mammalian species. The postnatally maturing rodent retina shows a sequential appearance of rod photoreceptor specific mRNA. In contrast, for the in utero maturing bovine retina, onset of all rod photoreceptor specific mRNA accumulation occurs at the same stage. Regulation of opsin gene expression was determined to be primarily transcriptional in nature, and several regulatory cis-elements have been identified for rhodopsin, α-transducin, and arrestin. On a cellular level, several groups report a central to peripheral gradient for the birth of retinal cell types, rod outer segment development and interphotoreceptor matrix maturation. On the level of gene regulation, limited information on developmental topography is available. Transgenic experiments in mice revealed that a wild type topographic progress of expression of the reporter gene was only observed with a construct driven by the rhodopsin promoter region of more than 2 kb, but no more precise mapping was attempted.
To study the relationship between developmental topography and gene expression in the maturing retina, we chose the bovine fetus as a model because several characteristics are of critical value: bovine retinas are large (adult retinal area, 2500 mm²), thus allowing accurate and detailed topographic resolution; the gestation period in the cow is protracted (9 months), thus allowing detailed temporal resolution; fetal bovine retinas are readily available. In addition to the equivalent gestational periods for cows and humans, the progression of retinal development is similar in the two species in that rod photoreceptor differentiation is essentially completed by birth,\textsuperscript{25,31--33} Previous analyses of total bovine retinal RNA revealed that accumulation of most, if not all, rod photoreceptor specific mRNA begins at approximately 6 months gestation,\textsuperscript{16} at which time photoreceptor outer segment formation also begins.\textsuperscript{35} Interestingly, the accumulation of cone photoreceptor-specific gene transcripts also coincides with the onset of rod photoreceptor gene expression\textsuperscript{34} even though a difference of 1.5 to 2 months exists between birth of cone photoreceptor cells and the onset of cone specific gene expression because cones are generated significantly earlier than rods.\textsuperscript{25,34}

To formulate a working model for developmentally regulated gene expression in the mammalian (bovine) retina, we tested the hypothesis that a transacting factor(s), or morphogen, initiates gene expression at one or multiple foci in the central retina and spreads radially outward during development, analogous to the pattern of cell genesis. The activity of such a morphogen is consistent with the apparently coordinate transcriptional induction of rod and cone specific phototransduction genes and with the spatio-temporal pattern of retinal cell genesis, in which initiation in the area centralis proceeds outward in a central-toPeripheral pattern of maturation.\textsuperscript{25,33} Accordingly, we determined the topographic pattern of rhodopsin mRNA expression during the final trimester of fetal development using a sensitive solution hybridization assay. We found that rhodopsin mRNA begins accumulation at 6 to 6.5 months gestation and is highest in a central area of the retina just superior to the optic nerve head. The resultant central-to-peripheral gradient of the mRNA levels was maintained throughout all fetal stages into adulthood. Because there is no corresponding gradient in photoreceptor cell density, it appears that a range of rhodopsin mRNA levels is maintained in the bovine retina that are defined in specific photoreceptor cells by their topographic retinal location. Our findings are not compatible with morphogen hypothesis, and they show that rhodopsin mRNA expression proceeds in a pattern distinct from cell genesis. Rejection of the hypothesis and possible alternatives will be discussed.\textsuperscript{37,38}

MATERIALS AND METHODS

Tissue and RNA Preparation

Bovine eyes from adult animals and bovine fetuses varying in development from 5 months to full term were collected at a local slaughterhouse. Immediately after fetal heart puncture, eyes were enucleated, the anterior segment and vitreous were removed, and the eye cups were frozen in glycerol storage buffer\textsuperscript{18} on dry ice. Gestational age was determined by the crown-rump length.\textsuperscript{25,30} Eye cups have been stored in this manner up to 1 year at --80°C with no apparent loss in RNA levels. For analysis, the frozen eye cup was thawed on ice and filled with phosphate-buffered saline, and the retina was removed gently from the eye cup with a fine brush. The retina with the photoreceptor cells facing down was then transferred onto a Zeta-Probe nylon membrane (BioRad, Richmond, CA) soaked with phosphate-buffered saline. Short radial incisions at the periphery allowed the retina to flatten. According to a 5-mm lattice, the retina was cut into 25 mm² segments (Fig. 1). Retinal segments were transferred to microcentrifuge tubes and the tissue disrupted immediately in 100 µl lysis buffer (5 M guanidine thiocyanate, 100 mM ethylenediaminetetra-acetoc acid (EDTA))\textsuperscript{37,38} by two 30-second bursts of sonication (Microson Sonicator; Heat Systems—Ultrasonic, Farmingdale, NY). These tissue extracts were used directly in the tissue lysate solution hybridization assay outlined below.

Total retinal RNA was isolated by the one-step guanidine thiocyanate—CsCl method of Chirgwin et al.\textsuperscript{39} Retinal tissue was homogenized in 4 M guanidine thiocyanate; 0.1 M sodium acetate, pH 5.0; and 5 mM EDTA with a polytron (Polytron Homogenizer; Brinkman Instruments, Westbury, NY). The homogenate was loaded on 5.7 M CsCl cushion (RI 1.933) and centrifuged overnight at 33,000 rpm. The solution was then carefully removed by aspiration so as not to contaminate the RNA pellet. The RNA pellet was dissolved in sodium dodecyl sulfate (0.1% SDS, 5 mM EDTA, 10 mM Tris—HCl, pH 7.4), extracted with phenol—chloroform, and precipitated with ethanol. Total RNA was redissolved in water, its concentration determined spectrophotometrically, and then stored at --80°C. Retinal—RNA was stable for more than 1 year.

cDNAs and Probe Preparation

J. Nathans supplied the 1.06-kb bovine rhodopsin cDNA clone BD-20,\textsuperscript{40} and D. Morris supplied the 1.3-kb bovine β-actin cDNA clone pBA11.3.\textsuperscript{41} These cDNAs were subcloned into the BlueScript KS vector (Stratagene, La Jolla, CA). Anti-sense cRNA probes were synthesized in a 20-µl reaction consisting of 40 mM Tris—HCl, pH 7.5; 6 mM MgCl₂; 2 mM spermidine; 10 mM NaCl; 10 mM dithiothreitol; 0.5 mM

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adenosine triphosphate; 0.5 mM cytidine triphosphate; 0.5 mM guanosine triphosphate; 12 μM uridine triphosphate (UTP); Inhibit-Ace 0.5 U (5Prime-3Prime, Boulder, CO); 50 μCi α32P-UTP; 25 units of T7 or T3 RNA polymerase (Stratagene) for 30 minutes at room temperature. Then 1 μl of RNAase free DNAase I (10 U/μl; Stratagene) was added and incubated for 20 minutes at 37°C. The DNAase I was inactivated by the addition of 20 μl 0.4% SDS and 20 mM EDTA. The synthesized probe was separated from unincorporated nucleotides by passage over Chromaspin-100 spin columns (Clontech, Palo Alto, CA). Specific activities of the resultant probes were 0.5 to 1 × 10^6 cpm/μg RNA. The cRNA probes were diluted to 100 pg cRNA/μl (rhodopsin) or 50 pg cRNA/μl (bovine β-actin) in 1 M guanidine thiocyanate just before use.

**Tissue Lysate Solution Hybridization Assay**

Sense cRNA was synthesized from cDNA using the Megascript transcription kit (Ambion, Austin, TX). The reaction mixture was subjected to DNAase I digestion, spin-column purification as described, and phenol-chloroform extraction, followed by ethanol precipitation. Quality analysis of sense cRNA was carried out by gel electrophoresis, and the yield was quantitated spectrophotometrically. Quantitative calibration curves were made by preparing a dilution series in 1 M guanidine thiocyanate from 0 to 200 pg cRNA in 40-μg increments for rhodopsin or 0 to 100 pg bovine β-actin cRNA in 20-μg increments for bovine β-actin.

To account for variations in the amount of retinal tissue in each extract, fluorophotometric DNA determinations were carried out using Hoechst 33258 (Sigma, St. Louis, MO) according to Labarca and Paigen. A calibration curve of 0 to 6 μg of calf thymus DNA was made and used to calibrate 3 μl of retinal extract in duplicate. The DNA concentration in each retinal sample was determined by linear regression analysis.

For the solution hybridization assay, polystyrene 96-well microtiter plates (Flow Lab, Horsham, PA) were preincubated with 200 μl 1 M guanidine thiocyanate containing 100 μg/ml Torula yeast RNA (Sigma) for 2 hours at room temperature. Just before use, the plates were dried on absorbent paper. Thirty-microliter dilutions containing 5, 2.5, or 0 μl of tissue lysate for β-actin and 2.5, 1.25, or 0 μl of tissue lysate for rhodopsin mRNA were transferred to microtiter
plates. The final concentration of lysis buffer was kept at 1 M guanidine thiocyanate. Each plate was loaded with duplicate samples of 10 retinal samples for either bovine β-actin or rhodopsin mRNA, along with two corresponding calibration curves. Ten microliters of 32P-antisense cRNA probe was added to each well, and the reaction mixtures were overlaid with 40 μl of light mineral oil (Sigma) and incubated at 60°C overnight. After hybridization, 60 μl of RNase mix in TES buffer (10 mM Tris–HCl; 5 mM EDTA; 300 mM NaCl; pH 7.4; 85 U/ml RNAase A (USB, Cleveland, OH); 250 U/ml T1 RNAase (Gibco-BRL, Bethesda, MD)) was added, and was followed by an incubation at 37°C for 1 hour. Trichloroacetic acid (TCA) precipitation was carried out in 96-well filtration plates (Millipore, Bedford, MA) preincubated with 150 μl 4°C TCA-block solution (10% TCA; 2% Sodium pyrophosphate; 200 μg/ml Torula Yeast RNA) for 2 hours and dried on absorbent paper just before use. Seventy-five microliters of the hybridization reaction RNase mixture was added to 55 μl of TCA-precipitation solution (30% TCA; 6% sodium pyrophosphate; 0.2 mg/ml torula yeast RNA) followed by an incubation at 0°C for 30 minutes. Gentle vacuum was then applied to the 96-well filtration plates, and the filtration membranes were washed four times with 300 μl 5% TCA. The bottom of each filtration plate was then removed to allow the membrane to air dry. Quantitation of the precipitated radioactivity was carried out on an AMBIS image analyzer (AMBIS, San Diego, CA) for 5 hours.

Solution hybridization analysis was performed as described using isolated total retinal RNA with the following differences: the hybridization and dilution buffers consisted of 40% formamide, 0.5 M NaCl, 4 mM EDTA, 10 mM Tris–HCl, pH 7.6; and retinal RNA concentrations in hybridization wells ranged from 0 to 0.2 μg RNA/well and 0 to 1 μg RNA/well for rhodopsin and bovine β-actin, respectively.

Data Analysis and Calculations
Rhodopsin and bovine β-actin mRNA concentrations were calculated by comparison to a linear regression curve for the corresponding calibration standards. Values lower than twice the background, as determined by the y-axis intercept of the calibration curve, were considered unreliable and rejected. Fewer than 2% of the measurements on tissue segments had to be rejected. The average of the four calculated mRNA concentrations, expressed in pg mRNA/mm² retina, was determined for each tissue segment. The ratio of rhodopsin mRNA levels to bovine β-actin mRNA levels or DNA levels was then calculated, and these values were plotted using the Surfer graphics package (Golden Software, Boulder, CO). Aberrant values were detected by calculating the average values of the surrounding data in question. If the value in question was larger than the sum of the average plus two times the standard deviation, it was replaced by the average of the surrounding values. This smoothing function was repeated until no more changes occurred. Less than 1% of the initial data failed this criterion and had to be adjusted. Virtually all the adjusted values were located at the edges of the retinas, where uncertainty about the individual measurement values was highest.

RESULTS
Our primary aim was to generate a topographic map of rhodopsin mRNA levels in the adult and fetal bovine retina. To obtain reliable, quantitative estimates of mRNAs from as many as 150 regular tissue segments from the same retina, an RNA protection assay was used. This assay was amenable to automated quantitation and allowed handling of large sample numbers. Although documentation of RNAse protection assays have been described, key aspects for this retinal assay required confirmation. We had to satisfy several conditions essential for reliable and quantitative binding assays: separation of RNA bound probe and free probe must be efficiently achieved; saturation of the RNA transcripts with added probe required incubation until a dynamic equilibrium between RNA transcript and probe should be attained; concentration of added probe should be sufficient to saturate the RNA transcripts; and reliable discrimination between specific hybridization and nonspecific binding of the probe should be accomplished. Separation of bound and unbound probe depends on the known resistance of RNA–RNA hybrids to digestion with single-strand specific ribonucleases. Single-strand rhodopsin 32P RNA probe in the presence of as much as 20 μg carrier RNA in a standard digestion (5 μ RNase A + 15 μ T1 RNAse/well) was >97% degraded in 30 to 45 minutes, whereas the RNA-RNA hybrids were resistant for more than 2 hours at 37°C. RNAse digestion, therefore, was routinely run for 60 minutes at 37°C. Conditions for a dynamic equilibrium were established by overnight incubations of fixed amounts of sense rhodopsin RNA and retinal tissue extracts with increasing amounts of anti-sense 32P RNA probe, followed by RNase digestion and TCA precipitation. Two hundred picograms of sense RNA and 1 ng antisense RNA probe routinely gave a saturated signal. The amount of total retinal RNA in tissue extracts never exceeded 0.5 μg/well (200 pg rhodopsin mRNA maximally). Because hybridization reactions in guanidine thiocyanate are nearly complete in a few hours, overnight incubations were run to ensure complete hybridization. To discriminate between specific and nonspecific hybridization, sense 32P RNA probe was incubated with either retinal RNA ex-
tracts or in vitro synthesized sense RNA up to 200 pg. This reproducibly yielded only background signals and did not correlate with the amount of retinal extract or sense RNA used. We concluded that the hybridization assay described here complied with the criteria for a quantitative RNase protection assay and would allow sensitive topographic measurements of mRNA in the developing bovine retina.

The reproducibility of the solution hybridization assay was evaluated by assaying the same retinal extracts several times independently over an interval of several months. The retina from a left fetal eye of 6.6 months gestation was divided into 51 segments according to the dissection strategy depicted in Figure 1. The tissue segments were then immediately disrupted in lysis buffer, and rhodopsin mRNA concentrations were determined independently in triplicate over a period of 3 months. Despite slight variations in assay conditions, results were consistently reproducible (Fig. 2). In addition, we found that retinal RNA stored as tissue lysate at −20°C was stable in 5 M guanidine thiocyanate for at least 3 months.

To correct for any variation in cell density and sample size, particularly evident for segments at tissue edges, the use of an internal standard was required. Commonly, mRNA levels of structural or housekeeping genes, or DNA levels, are used as indicators of cell number and tissue mass. We compared DNA content with β-actin mRNA levels in the same tissue segments and found parallel results (data not shown). We chose to use β-actin mRNA concentration as an internal standard for normalizing all mRNA measurements. This was preferred to DNA concentration because an mRNA standard also takes into account relative efficiencies of RNA isolation from each tissue segment. Rod photoreceptor cells in the bovine retina represent approximately 50% to 70% of the total cell population; hence, β-actin mRNA measurements should primarily reflect photoreceptor cell density. As an independent test of whether β-actin mRNA is an accurate measure of photoreceptor cell density, we analyzed an adult human retina because photoreceptor cell density in the human retina has been mapped carefully. We compared the topographic patterns of rhodopsin mRNA levels in the adult human retina either as a ratio to β-actin mRNA or as a ratio to photoreceptor cell density (Fig. 3). The consistency between the two confirms the usefulness of β-actin as a quantitative internal standard.

**FIGURE 2.** Reproducibility of the solution hybridization assay for retinal RNA in tissue sections. Topographic rhodopsin mRNA levels in a 6.63-month fetal retina were analyzed in triplicate using the solution hybridization assay during a 3-month period. Conditions of the three analyses varied slightly. (A) Aliquots of retinal tissue extracts of 25-mm² square segments were diluted fourfold to eightfold and incubated with 1 ng of 32P-labeled antisense rhodopsin cRNA probe. (B) Aliquots of retinal tissue extracts of 25-mm² sections were diluted twofold to fourfold and hybridized to 1 ng of 32P-labeled antisense rhodopsin cRNA probe. (C) Aliquots of retinal tissue extracts of 25-mm² sections were diluted 8- to 16-fold and incubated with 0.5 ng of 32P-labeled antisense rhodopsin cRNA probe. (D) The average rhodopsin mRNA density (pg/mm²) of experiments A, B, and C was determined and the ratio to β-actin mRNA plotted. Contour plot is shown on the left, and three-dimensional surface plot is shown on the right panel.
assay for photoreceptor cells in a given retinal segment.

Global Parameters of Fetal Development of Bovine Retina

We followed retinal area, total RNA, total DNA, and length of photoreceptor inner plus outer segment length from 4 to 5 months gestation to adult. Retinal area was estimated by measuring the diameter of the eye at its largest width, just posterior of the ora serrata, using half the calculated spherical surface area. Ninety-four eyes from fetuses ranging from 3 to 7.8 months gestation were measured, as were 31 from adult donors (estimated to be 3 years of age or older). Figure 4 depicts the continuous increase noted in retinal area with increasing gestational age. Total retinal RNA was quantitated from 23 retinas ranging from 4 to 8.7 months gestation and from 12 adult retinas. Total retinal DNA was determined by summing DNA levels measured in individual segments prepared for tissue lysate solution hybridization assay. Retinal area and total RNA exhibited parallel developmental increases, whereas retinal DNA followed a distinctive pattern and reached a plateau at approximately 6.5 months gestation (Fig. 4). Because DNA levels reflect the balance between DNA synthesis and degradation, it is an indicator for the balance between cell division and cell death. Constant DNA levels after 6.5 months gestation suggested, therefore, that total cell number in bovine retina is constant after this stage and that further physical growth of the retina must reflect an increase in retina cell size and spacing, not in cell number. Because the discrimination between inner and outer segments was difficult, especially at early stages, preliminary assessment of the timing of outer segment formation was carried out by measuring inner plus outer segment lengths in fetal and adult retinas. From each retina, 5 to 10 samples were analyzed, and the average photoreceptor length of these samples per retina were plotted in relation to gestational age (Fig. 4B) (Lockwood, personal communication, 1993). At 6.1 months gestation, a rapid increase in
FIGURE 4. General developmental parameters of growth and development of the fetal bovine retina. During fetal development, the increase in retinal area (A), sum of inner and outer segment length (B), total RNA (A), and total DNA (B) were determined. Total RNA and retinal surface area show a steady and parallel increase during development into adult levels (A). In contrast, the DNA levels, as determined by summation of the DNA levels measured in the individual retinal segments, reached a plateau at 6.1 months gestation, suggesting that this is the end of retinal cell mitotic activity. The cessation of mitosis generally coincides with an onset of the growth of photoreceptor cell inner plus outer segment length. This increase is primarily caused by growth of the photoreceptor outer segments. For inner and outer segment measurements, fetal and adult retinas were fixed in 4% Karnovsky fixative and embedded in paraffin. Sections were analyzed from 5 to 10 evenly distributed samples. After alignment of the sections, the total length of inner and outer segments were measured. IS = inner segment; OS = outer segment.

Rhodopsin mRNA expression in bovine retina appeared highest in an area of six segments superior to the optic nerve head. The location of this area with peak values persisted throughout fetal development into the adult retina. Based on this consensus area of highest expression, the data were grouped into three regions: a central region corresponding to this area of highest expression and two other regions concentrically located around it—middle and peripheral—of equal retinal area. Summing the topographic data into these larger domains allowed a direct kinetic analysis of the developmental increase of rhodopsin mRNA as a function of retinal location. The results (Fig. 6) revealed that a gradient of rhodopsin mRNA expression exists at all developmental stages after transcriptional induction between 6 and 6.3 months. This suggests that the radial pattern of transcription does not begin initially in the central retina, but that all rod photoreceptor cells begin
FIGURE 5. Topography of rhodopsin mRNA levels during bovine fetal development. Topographic analysis of the ratio of rhodopsin mRNA to β-actin mRNA was carried out on preinduction retinas (6.08 months gestation) and followed into adulthood. The results are presented in contour plots (left hand panels) and surface plots (right hand panels). A scale setting was fixed for adult retina and was used for all graphs, resulting in a visual comparison of the absolute increase of rhodopsin mRNA levels during development. Notice the peak area in the 6.48-month retina is located superionasally, whereas it is transferred to a more superiortemporal position in the 8.37-month and adult retina (see also Table 1).

rhodopsin mRNA expression approximately coincidentally and that the resultant gradient of mRNA is the result of positionally determined transcript levels within the retina.

DISCUSSION

The goal of this study was to investigate the topographic patterns of late differentiation in mammalian retina. We chose the bovine retina as a model for these studies because of its size and protracted gestation period. To document expression patterns, a sensitive RNA assay was developed that allowed accurate mRNA quantification and manipulation of a large number of samples. Under the conditions described, reproducible detection levels for bovine β-actin mRNA and rhodopsin mRNA were achieved at 9 pg/sample and 12 pg/sample, respectively. This level of sensitivity allowed measurements on 25-mm² retina per segment and resulted in a resolution of between 50 segments in younger fetal retina and 150 segments in the adult retina. We observed a flat topographic pattern of rhodopsin mRNA in the 6.1-month gestation retina (Fig. 5E), reflecting basal expression levels that changed dramatically during approximately the next 2-week period. The 6.5-month gestation retina displayed central levels of rhodopsin mRNA 40% to 50% of the adult, with a steep central-to-peripheral gradient (Fig. 5E). The rhodopsin mRNA peak was located just superionasal to the optic disk. With increasing fetal age, levels of rhodopsin mRNA in all locations increased, but the initial gradient pattern with a peak superior of the optic nerve head persisted into adulthood. To test whether there was a radial progression of gene expression during development, we decided to cluster data points into concentric groups to simplify the analysis. We plotted the amount of rhodopsin mRNA/mm² relative to their adult counterparts from the transcript-rich central domain (center) and the transcript-poor peripheral (periphery).
Figure 6. Kinetic analysis of the central, middle, and peripheral increase of rhodopsin mRNA levels during bovine fetal development. Fetal and adult bovine retinas were analyzed topographically, and each retina was divided into three large domains: central, middle, and peripheral. The average rhodopsin mRNA level relative to β-actin mRNA in each subdivision was calculated and plotted as a percentage of the corresponding domain in adult retinas, which is set at 100%. Notice that the relative increase rate in each section is identical from 6 to 6.8 months gestation. Induction of expression of rhodopsin mRNA occurs simultaneously in all rod photoreceptor cells across the retina. From 7 months to adult, the increase in rhodopsin mRNA levels in the peripheral domain lags slightly behind the center and middle domains. The retinas were divided into quadrants, with the center at the optic nerve head and a superioinferior axis running through the major superior blood vessel. The average value of rhodopsin mRNA levels in each quadrant was calculated, and the quadrant with the highest value was set at 100%. After induction, the highest level of expression is consistently located in the superior part of the retina. With continuing retinal development, however, a shift of the quadrant with highest expression occurs from supersonal to supertemporal after 7 months gestation. The number in the upper row represents the rhodopsin mRNA over β-actin mRNA ratio, and the number in the bottom row represents the value relative to the peak value.

Results shown in Figure 6 reveal that at the earliest stage exhibiting transcript levels induced above the basal level, all retinal regions experienced an increase in rhodopsin mRNA; the central retina contained approximately 2 times that found in the periphery, but the periphery contained approximately 2.5 times that found in the same region 2 weeks earlier. This pattern continues throughout the remainder of fetal development with all regions increasing dramatically and in parallel (Fig. 6). Thus, the initial central-to-peripheral gradient of transcript levels is maintained, even in the adult. We conclude, therefore, that contrary to our initial expectation of a central-to-peripheral progression of initiation of rhodopsin transcription, it is more accurate to depict this developmental pattern as one in which the gradient is established over the entire retina as soon as transcriptional induction of the rhodopsin gene can be detected. From results reported, here it appears that rod cells are preprogrammed, depending on topographic retinal location, to possess a given amount of rhodopsin transcript. This, in turn, suggests that events before transcriptional induction of cell type-specific genes at approximately 6 months gestation may at a later point define this pattern of retinotopic gene expression. Recently, formation of retinotopic maps was described for the lateral geniculate nucleus. This map was developed and refined through analysis of bursts of action potential from ganglion cells. Although no evidence exists to relate such maps to later photoreceptor gene expression, a preexisting pattern of nonphotoreceptor cells, synapses, or both, could form the blueprint for rhodopsin gene expression because it is established before the induction of photoreceptor outer segments. Transmission of differentiation signals from ganglion cells to the photoreceptor cells could be mediated through synaptic connections between the two cell types; in the
mouse, synaptogenesis at the photoreceptor terminals precedes rhodopsin gene expression.53,54

Our data are most consistent with an induction signal that activates all the photoreceptor cells in the retina simultaneously and leads to transcript levels dependent on topographic retinal position, highest in the central retina and lowest in the periphery. Such a developmental pattern does not seem to support the presence of an extracellular morphogen that activates photoreceptor cells sequentially through diffusion or cell-to-cell contact. Our observations are supported by the lack of a mosaic pattern of photoreceptor cell degeneration in chimeras of mutated transgenic with wild type photoreceptor cells.54 There is one caveat to this conclusion: The maximal temporal resolution (1 to 2 weeks because of uncertainty in fetal aging) might be insufficient to visualize the kinetics of a rapidly spreading morphogen activity; therefore, we cannot completely reject the spreading morphogen hypothesis. However, the persistence of a gradient in transcript level in the adult retina unequivocally demonstrates the topographic dependency of rhodopsin mRNA levels. If microenvironmental cues dictated this transcriptional induction event in a manner analogous to cell fate determination,9,11,13 the resultant topographic gradient could be most directly explained by a central-to-peripheral concentration gradient of the putative inducer. However, unless that gradient is maintained throughout the last trimester and in the adult, it should soon be lost as the inducing agent equilibrates throughout the retina. This assumes, of course, that all rods are capable of responding equivalently and that there is no gradient of rod cell number. Photoreceptor cell density determinations demonstrate the absence of such a gradient in developing and adult bovine retina (Lockwood, personal communication, 1993). Hence, the observed gradient of accumulation of rhodopsin mRNA is inconsistent with most aspects of a spreading morphogen scenario.

Studies of cellular topography in the early retina have been reported continuously over the past 20 years, the majority studying retinal ganglion cells and synaptic connection (reviewed in Rager and Rager55). Depending on the species studied, gradients of cell types or synaptic connections usually are skewed distinctly with respect to the retinal center or optic nerve head and often shift during development. Although occurring later in development, bovine rhodopsin mRNA levels exhibit similar features. The area of highest transcript level is always within the superior half of the retina at all stages (Fig. 5, Table 1). Initiation of rhodopsin mRNA accumulation occurred just suprionasal to the optic disk in the 6.5-month gestation retina (Fig. 5 E), which, with increasing fetal age, expanded and stretched along a temporonasal axis. This leads to an apparent shift of highest expression toward the superiotemporal quadrant (Fig. 5, Table 1). Again, these results contradict the simple model of a diffusion or cell-to-cell contact which, once initiated, would be expected to proceed outwardly and more or less equivalently in all directions. Our data suggest that the mode of transmission of the transcriptional induction involves a more complex process, one that might be regulated, as suggested, according to a pattern determined earlier in development. Skewed patterns of development of visual pigment expression also have been demonstrated for cone photoreceptor cells in primate56 and mouse retina,56 strengthening the notion that regulation of cell differentiation may be distinct from pathways dictating cell fate. Thus, our data are most consistent with the suggestion that regulation of rhodopsin gene expression progresses according to a preexisting retinotopic map.

Key Words

development, fetal bovine retina, induction, rhodopsin mRNA, topographic gene expression

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2. Turner DL, Cepko CL. A common progenitor for neu-

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ST = superiotemporal quadrant; SN = superionasal quadrant; IT = inferiotemporal quadrant; IN = inferionasal quadrant.


Retinotopographically Determined Expression of Rhodopsin mRNA