Transcription of Photoreceptor Genes during Fetal Retinal Development

EVIDENCE FOR POSITIVE AND NEGATIVE REGULATION*

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Rod photoreceptor outer segments are elaborated at approximately 6 months gestation in the cow coinciding with a dramatic increase in mRNAs encoding many visual transduction and associated proteins. Nuclear run-on determination of relative transcription rates demonstrates that gene expression follows three distinct patterns. Opsin, S-antigen, and transducin are all minimally detectable at 5.2 months gestation and increase throughout development. Only opsin demonstrates an additional sharp increase in transcription activity which resembles a positive gene-specific enhancer that is first effective between 6.3 and 7.4 months gestation. In contrast, interphotoreceptor retinoid binding protein (IRBP) transcription is already at 43% of its adult level at 5.2 months gestation. To further understand these differences, the relative contributions of initiation and elongation to nuclear run-on signals were examined using either Sarkosyl or ammonium sulfate. Transcriptional rates for S-antigen and transducin were not affected, however, opsin was reduced ~4-fold and IRBP was increased ~2-fold. Opsin is therefore likely to be initiated de novo during the run-on reaction and responds to a gene-specific positive regulator. The increase in IRBP transcription rate suggests the removal of an elongation inhibitory factor and supports the idea that a negative regulatory element may be involved in controlling IRBP expression.

Vertebrate retinal development involves the formation of highly organized layers of specialized cells from an apparently homogenous neuroepithelial cell layer (reviewed in Barnstable, 1991). Retroviral labeling experiments aimed at determining the origin of retinal cell types have demonstrated that a single progenitor cell in the mammalian retina can generate all the different classes of retinal cells (Turner and Cepko, 1987; Turner et al., 1990). The molecular mechanisms governing this and later events in retinal tissue maturation are not well understood; however, cell differentiation typically involves expression of specific genes in appropriate temporal and cell type patterns (reviewed in Maniatis et al., 1987; Martin, 1991). In this paper we have examined the developmental expression of bovine rod photoreceptor genes at the transcriptional level as a means to begin understanding the molecular mechanisms governing their expression during the later stages of retinal maturation.

The rod photoreceptor is responsible for detection of achromatic dim light and its transduction into a neurochemical signal. This is accomplished by a functionally linked group of proteins in the photoreceptor outer segments that comprise the visual transduction cycle (reviewed in Stryer, 1991; Hurley, 1992). We chose the bovine fetus as a model for retinal development since its gestational period is equal to that of humans, and its progression of morphological development is very similar to the human in which the rod photoreceptor development is essentially completed by birth (Abramov et al., 1982; Johnson et al., 1985; Hauswirth et al., 1992). The bovine fetus has the additional advantage relative to smaller laboratory animals in allowing greater temporal resolution of fetal stages. This advantage was noted in another species by LaVail et al. (1991), working in the fetal primate retina, who reported distinct waves of cell genesis that were not apparent in the fetal and neonatal mouse and concluded that the extended period of fetal development in the primate allowed better resolution of developmental stages.

Previous work has demonstrated that the mRNAs for bovine visual transduction genes begin accumulating coordinate between 5.5 and 6.5 months gestation as determined by Northern blot analysis (Timmers et al., 1993). This is a critical developmental time point morphologically because rod outer segments are first elaborated at approximately 6 months gestation in both cows and humans (Ozanics and Jakobiec, 1982; Hauswirth et al., 1992). A preliminary analysis of transcription rates for these genes revealed that this mRNA induction event was achieved at least in part at the level of transcription because relative rates of transcription also increased in a coordinate pattern from about 6 months gestation to adult (Timmers et al., 1993). However, the sensitivity of these experiments was not sufficient to confidently determine transcriptional rates in retinas from fetal animals younger than 6 months gestation. This work examines in greater detail the early events in photoreceptor gene expression, emphasizing fetal ages at or before the transcriptional induction event by using a nuclear run-on protocol specifically optimized for retinal nuclei. Transcriptional activity could be reproducibly detected from fetal retina as early as 4.5 months gestation. The transcriptional rates of the visual transduction genes opsin, the α subunit of transducin (α-Tr),1 and S-antigen

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1 The abbreviations used are: α-Tr, α-transducin; SAg, S-antigen; IRBP, interphotoreceptor retinoid-binding protein; kb, kilobase(s).
(SAG) were determined and analyzed relative to steady state mRNA levels. Additionally, RNase protection experiments were used to measure mRNA levels in the preinduction retina in order to accurately quantitate minor steady state amounts of transcripts that are not easily visualized by Northern blot analysis.

IRBP is produced by both rod (Hollyfield et al., 1985; van Veen et al., 1986) and cone (Porrello et al., 1991) photoreceptor cells and is believed to shuttle retinoids within the interphotoreceptor matrix between the photoreceptors and the retinal pigment epithelium (Lai et al., 1982). It is, therefore, a vital part of the visual system but not a primary member of the visual transduction cascade. In the mouse, IRBP protein (Carter-Dawson et al., 1986) and mRNA (Gonzalez-Fernandez and Healy, 1990) have been found at earlier developmental stages than those for the visual transduction genes. In the fetal cow IRBP can also be detected by enzyme-linked immunosorbant assay much earlier than rhodopsin (Hauswirth et al., 1992), however, Northern blot analysis of bovine IRBP mRNA demonstrates an induction event very similar to that observed for the other visual transduction genes. Therefore, transcriptional rates for IRBP were also determined with emphasis on times before its observed mRNA induction.

MATERIALS AND METHODS

Clones—Many cDNA clones were generously provided by a number of laboratories: J. Nathans provided a 1.1-kb rod opsin clone (Nathans and Hogness, 1983); J. B. Hurley a 700-base pair partial rod α-Tr cDNA; T. Shinohara a 1.6-kb SAG clone (Shinohara et al., 1987); C. L. Hatch a 905-base pair complete histone H2A.Z cDNA (Hatch and Bonner, 1988); D. Morris a 1.3-kb complete β-actin cDNA clone (Degen et al., 1983); J. Stein a 6.6-kb human genomic 28 S RNA clone (Wilson et al., 1978); T. Choi a 1.65-kb rat tubulin clone (Lemischka and Sharp, 1982). All cDNAs are from bovine tissue unless otherwise stated. All clones received were recloned into either pBluescript or pBlueScript vectors (Stratagene, La Jolla, CA) and authenticity checked by sequencing.

The IRBP gene was isolated from a bovine genomic λ library purchased from CloneTech (Palo Alto, CA). It was identified using an RNA probe transcribed from a bovine IRBP RNA transcript as template to amplify the exon 4 fragment with primers corresponding to a published sequence (Borst et al., 1986). Fragments for subcloning were identified by hybridization with a 1.1-kb rod opsin clone (Nathans and Hogness, 1983) for use in control studies.

Developmental Photoreceptor Gene Expression

RNA isolated from the retinal nuclear preparations used in the test for IRBP attenuation was purified as above using the supernatant from the nuclear preparation described below. An equal amount of guanidium solution (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.1 M 2-mercaptoethanol, 0.5% Sarkosyl) was added to the cell lysis supernatant and layered on a CsCl cushion as in Chirgwin et al. (1979).

Nuclear Run-on Analysis—Dot-blot membranes for run-on transcription analyses were prepared using 3 µg of plasmid DNA denatured by boiling for 10 min in 0.4 M NaOH + 10 mM EDTA and applied to Zeta Probe nylon membranes (BioRad) following the manufacturer’s protocol using a 96-well vacuum manifold (Bethesda Research Laboratories). Membranes were neutralized in 2 × SSPE (360 mM NaCl, 20 mM NaHPO4, 2 mM EDTA, pH 8) and dried in an 80 °C vacuum oven for 1 h. Blots were used up to 4 times each following stripping in 0.1 × SSPE + 0.5% SDS at 95 °C for 60 min between experiments. No signal remained on the blots after stripping as determined by a 4-day exposure to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA).

Transcription rates were determined using a modification of the protocol of Greenberg and Ziff (1984). All buffers and manipulations were done on ice or at 4 °C. Nuclei were isolated from whole retina by Dounce homogenization 5 times with a loose fitting pestle in 4 ml of lysis buffer (10 mM Tris, pH 8.5, 5 mM MgCl2, 10 mM NaCl, 60 mM KCl, 0.25 mM sucrose, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol, 1 mM diethiothreitol, and 0.5% Nonidet P-40) and pelleted at 1.000 × g for 3 min. Nuclei were resuspended in 1 ml lysis buffer, counted on a hemocytometer, and frozen on dry ice in aliquots of 3 × 106 nuclei/100 µl of storage buffer containing 3 µl of Inhibit-ACE (5 Prime to 3 Prime, Inc., Boulder, CO). This number of nuclei/reaction was determined to be optimal for maximal run-on signals from single copy genes. Nuclei remained transcriptionally active for approximately 2 months when stored at −80 °C.

The nuclear run-on RNA elongation reaction was performed by adding 500 µg of [α-32P]UTP (3000 mCi/mM, Amersham Corp.), 5 µl of Inhibit-ACE, and 150 µl of 2 × run-on buffer (10 mM Tris, pH 8.0, 50 mM KCl, 5 mM MgCl2, 1 mM ATP, 1 mM CTP, 1 mM GTP, 1% Sarkosyl) to 150 µl aliquots of nuclei, and incubated for 30 min at 30 °C. Labeled RNA was isolated by a modification of Chomczynski and Sacchi (1987). Briefly, 500 µl of guanidium solution (as used in the total RNA isolation protocol), 100 µl of 2 M NaOAc, pH 4, and 20 µl of 25 mg/ml tolula yeast RNA were added and the reaction sonicated using a Microsonic Cell Disruptor (Heat Systems Ultrasone Inc, Farmingdale, NY) until the solution was no longer viscous. The reaction was extracted twice with acid phenol/chlorofor (6:1 plus 2% v/v isoamyl alcohol) with ethanol precipitations between each extraction. The sample was resuspended in 100 µl of TE (10 mM Tris, pH 8.0, 1 mM EDTA) and RNA was purified using an incorporated labeled RNA by passage over a G-25 spin column (5 Prime to 3 Prime, Inc.). This procedure typically resulted in 1–4 × 104 counts/minute of labeled RNA. The entire reaction was added to 2 ml of hybridization mix (1 × Denhardt’s salts (Sambrook et al., 1989), 6 × SSPE, 0.5% SDS, 0.2 mg/ml tolula yeast RNA, 10% dextran sulfate, 40% formamide) and incubated for 48 h at 42 °C. Blots were washed three times each in 0.1 × SSPE, 0.5% SDS for 20 min at 65 °C and exposed for 4 days to a Molecular Dynamics PhosphorImager screen before scanning. All signal intensities were computed using Molecular Dynamics ImageQuant software.

Nuclei from run-on assays containing Sarskoyl in the elongation reaction were are above with the addition of 0.4% Sarskoyl (Sigma) to the 2 × run-on buffer. The ammonium sulfate experiments were performed as above with the 2 × elongation buffer substituted with 500 mM ammonium sulfate, pH 8.5, 100 mM Tris, pH 8, 180 mM KCl, 10 mM MgCl2, 1 mM ATP, 1 mM CTP, 1 mM GTP. Experiments containing α-amanitin were performed as above with 1 µg/ml α-amanitin ( Sigma) added to the nuclei for a 5-min incubation on ice before the normal elongation reaction.

RESULTS

RNAse Protection Analysis of mRNA Levels—Previous work has demonstrated that the levels of mRNA for rod photoreceptor visual transduction genes increased in a coordinate fashion at approximately 6 months gestation (Timmers et al., 1993). In order to accurately quantitate the low levels of steady state mRNAs prior to the induction event, an RNase protection analysis was performed using a probe derived from the 3' untranslated region of the mouse rhodopsin gene. The probe was subcloned into the pBluescript II vector (Stratagene) and used to transcribe antisense RNA using T7 RNA polymerase. The antisense RNA was hybridized to a dot-blot membrane containing cDNA templates from preinduction and induced retinas. The RNase protection reaction was performed as above with the 2 × elongation buffer substituted with 500 mM ammonium sulfate, pH 8.5, 100 mM Tris, pH 8, 180 mM KCl, 10 mM MgCl2, 1 mM ATP, 1 mM CTP, 1 mM GTP. Experiments containing α-amanitin were performed as above with 1 µg/ml α-amanitin ( Sigma) added to the nuclei for a 5-min incubation on ice before the normal elongation reaction.

TRANSCRIPTIONAL RATES

Table 1 summarizes the transcription rates for the various genes. The transcription rates for the rhodopsin gene (Rh) in the preinduction retina are approximately 10 times lower than those for the visual transduction genes and are of the same magnitude as those for the visual transduction genes. The transcription rates for the rhodopsin gene (Rh) in the preinduction retina are approximately 10 times lower than those for the visual transduction genes and are of the same magnitude as those for the visual transduction genes.

DISCUSSION

The data presented in this study demonstrate that the levels of mRNA for rod photoreceptor visual transduction genes increase in a coordinate fashion at approximately 6 months gestation (Timmers et al., 1993). In order to accurately quantitate the low levels of steady state mRNAs prior to the induction event, an RNase protection analysis was performed using a probe derived from the 3' untranslated region of the mouse rhodopsin gene. The probe was subcloned into the pBluescript II vector (Stratagene) and used to transcribe antisense RNA using T7 RNA polymerase. The antisense RNA was hybridized to a dot-blot membrane containing cDNA templates from preinduction and induced retinas. The RNase protection reaction was performed as above with the 2 × elongation buffer substituted with 500 mM ammonium sulfate, pH 8.5, 100 mM Tris, pH 8, 180 mM KCl, 10 mM MgCl2, 1 mM ATP, 1 mM CTP, 1 mM GTP. Experiments containing α-amanitin were performed as above with 1 µg/ml α-amanitin ( Sigma) added to the nuclei for a 5-min incubation on ice before the normal elongation reaction.
tion assay was used which has significantly greater sensitivity than Northern blot analysis (Sambrook et al., 1989). The results for a staged series of fetal retinas showed that while mRNAs for opsins, SAg, and IRBP were present in preincubation retina, their levels were only 1–10% of that found in the adult (Fig. 1). Steady state levels of each mRNA increased dramatically and followed the same relative pattern of accumulation after approximately 6 months gestation, the time of rod outer segment formation.

**Nuclear Run-on Analysis**—The mRNA levels for rod photoreceptor genes suggested that they may share common regulatory elements because they accumulated coordinately during fetal development (Timmers et al., 1993 and Fig. 1). However, these experiments measured steady state levels of message which may be influenced by differences in the half-lives of individual transcripts. To test the hypothesis that photoreceptor genes share common transcriptional regulatory elements, it is necessary to examine these genes at the level of transcriptional rate. Nuclear run-on reactions measure in vitro nascent nuclear RNA synthesis by endogenous RNA polymerases and are considered to be an accurate measure of transcriptional complexes initiated in vivo (reviewed in Darnell, 1982). This assay was optimized for bovine retinal nuclei and generated labeled nucleic acid sensitive to RNase A and 0.4 M NaOH but resistant to DNase I. RNA products migrated through a 6% denaturing polyacrylamide gel as a family of sizes ranging from >1000 to <100 nucleotides with no dominant product size (data not shown). Individual labeled run-on transcripts were detected by hybridization to cDNA dot-blots in order to quantitate the transcriptional activity of each specific gene. It was first necessary to confirm that this nuclear run-on reaction mimicked the known in vivo properties of photoreceptor genes. The tissue specificity of each target gene was therefore determined by comparison of the products of a run-on protocol using bovine brain cortex nuclei as a control tissue. Brain cortex is a neural tissue of similar embryonic origin as retina, however, photoreceptor-specific gene products have been found in the retina and pineal tissues only and no photoreceptor mRNAs have been demonstrated in the brain (Bowes and Farber, 1987; Kuo et al., 1986). Total incorporated label in the brain cortex run-on reaction (5 × 10⁷ cpm) was similar to that for retinal runon reactions using equal numbers of nuclei. Upon hybridization of brain cortex run-on RNA to target genes (Fig. 2A), only β-actin and tubulin cDNAs had detectable hybridization signals, whereas all genes tested had detectable signals from adult retina (Fig. 3), thus demonstrating that tissue specificity was maintained in the retinal run-on assay.

The retinal run-on signals were also shown to be products of polymerase II transcription. α-Amanitin at 1 μg/ml in the run-on reaction is an inhibitor of polymerase II but not polymerase I or III (Marzluff and Huang, 1981). In the presence of α-amanitin, no signals above background were seen for any photoreceptor gene (Fig. 2B). Retinal run-on reactions with α-amanitin incorporated [³²P]UTP at 67% of the value without α-amanitin. This α-amanitin-resistant activity was most likely due to polymerase I transcription since hybridization of treated and untreated reactions to 28S rRNA dot-blots resulted in the same signal intensity (Fig. 2B).

In order to make valid comparisons between the rates of transcription in retina of different fetal ages, it was necessary to determine each transcription rate relative to an internal control gene, preferably a polymerase II transcript of similar abundance that does not vary over the developmental ages used in this study. We noted that the rate of transcription of β-actin relative to 28S rRNA did not change as a function of gestational age in the bovine retina (data not shown). Furthermore, the rates of transcription of tubulin and histone H2A.Z relative to β-actin also showed no increase throughout gestation (Fig. 3, A and B). Thus, β-actin was chosen as the unaffected internal standard and served as a control in all transcription rate determinations.

Nuclear run-on experiments were carried out on retinas from a series of gestational ages (Fig. 3A) and transcriptional activities determined (Fig. 3C). The visual transduction genes

![Fig. 1. Steady state levels of photoreceptor mRNAs during fetal development determined by solution hybridization.](attachment:image)

![Fig. 2. A, nuclear run-on dot-blots of brain nuclei demonstrating the lack of rod photoreceptor gene transcription in this tissue. Nuclei were isolated from an adult bovine brain and assayed as described for retinal tissue. The specific cDNA targets are: His, Histone H2A.Z; Tub, tubulin; V, pBluescript vector; b-actin, β-actin. The 28S rRNA level was determined on a separate blot with 3-fold dilutions of target DNA, starting at 3 μg. The blot for 28S rRNA used the same run-on RNA hybridized at a 1:100 dilution due to the intensity of the 28S signal. b, retinal nuclear run-on blots in the presence of 1 μg/ml α-amanitin demonstrating the RNA polymerase II origin of the hybridization signals. Separate blots of 28S rRNA were hybridized at a 1:100 dilution and show that the signal for this polymerase I transcript was unchanged by this treatment.](attachment:image)
for SAg from 5.2 months to adult. Opsin was distinct from SAg and α-Tr because it demonstrated an additional sharp increase in transcription rate between 6.3 and 7.4 months gestation. In striking contrast, IRBP was already at 43% of its adult level at 5.2 months and exhibited a steady increase toward the adult level over the ensuing 4 months of development.

Test for Attenuation of the IRBP Gene—The transcription rates for opsin and IRBP can be compared directly to their respective mRNA levels when each value is expressed as a percent of its adult level (Fig. 4). The rate of transcription and the level of mRNA were similar for opsin throughout gestation suggesting that transcriptional efficiency may be the dominant and perhaps only level at which cytoplasmic message is regulated. Data for IRBP contrasts to that for opsin. The high rate of IRBP transcription at 5.2 months gestation (43% of adult) compared to its low level of mRNA (~10% of adult) could be indicative of post-transcriptional regulation. Alternately, the data also support regulation of mRNA levels by transcriptional attenuation, in which production of a full-length product at early fetal ages is negatively regulated until developmentally relieved at later ages. Attenuation of IRBP was tested by comparing the hybridization signal of a fourth exon probe with that of a first exon probe in another series of run-on reactions. A schematic of the intron/exon structure of IRBP is shown in Fig. 5A with the segments used as targets in the transcription assay indicated. The assays were performed on 4.5 month, 5.2 month, and adult animals (Fig. 5, B and C) and showed that although there was a difference in the transcription rate measured for the first versus the fourth exon at each age, that difference was maintained throughout development. Both ends of the IRBP gene were transcribed at ~50% of their adult level at 4.5 months gestation. Therefore, IRBP does not appear to be transcriptionally attenuated in a developmentally regulated fashion.

In order to test whether the discordance between IRBP transcription rates and mRNA levels was due to the fact that retina from different animals were used for each set of experiments, mRNA from the same nuclei used in the nuclear run-on experiments shown in Fig. 5 was isolated and the level of IRBP mRNA quantitated by an RNase protection assay. We measured mRNA levels of 5' (exon 1) and 3' (exon 4) ends of IRBP using regions of the same probes that were used as targets in the run-on transcription assay. This also allowed all demonstrated increased transcriptional activity as development proceeded but each followed one of three distinctive patterns. Opsin, SAg, and α-Tr transcription were at 1–8% of their adult levels at 5.2 months gestation and exhibited total increases of 31-fold for opsin, 17-fold for α-Tr, and 12-fold

Fig. 3. A, nuclear run-on dot-blot for control and photoreceptor genes of retina from 5.2, 6.3, 7.4, and 8.0 months gestation and adult animals. The 28 S rRNA blot was hybridized separately at a 1:100 dilution of the run-on RNA. Blots were exposed for 4 days to a PhosphorImager screen and the data analyzed using ImageQuant software. B, mean and standard deviation of the transcription rates for the control genes histone and tubulin as a ratio to β-actin during fetal development. Values were derived by determining the intensity of the signal for a specific cDNA less vector signal and reported as a ratio to the signal for β-actin less vector signal. Nuclear run-on experiments were performed three times on each fetal age and eight times in total on four different adult animals. C, mean and standard deviation for the relative transcription rates of the photoreceptor genes during fetal development as a ratio to β-actin. Abbreviations are as in Fig. 2.

Fig. 4. Comparison between transcription rates (solid lines) and steady state mRNA levels (dashed lines) as a percent of the respective adult values for opsin and IRBP. The data for transcription rates were taken from Fig. 3 and for mRNA levels from Fig. 1. Both sets of values were recalculated as a percent of the adult level in order to easily compare the data.
us to simultaneously test the specificity of the probes, since it was also possible that observed differences in the transcription experiments between the 5' and 3' ends of the gene were due to cross-hybridizing run-on RNA. The probes used in this protection assay are diagrammed in Fig. 6A and an autoradiograph of a gel with the protected fragments is shown in Fig. 6B. The expected fragment sizes were protected in each case, and each exon 1 probe detected no major cross-reactive product. A quantitative value for each protected product was calculated as a ratio to the β-actin signal in the same sample lane: lane 1, IRBP 3' end probe; lane 2, IRBP 5' end short (224 nucleotides); lane 3, IRBP 5' end long (440 nucleotides); and lane 4, IRBP 3' end probe. Lanes 5–7 have the β-actin, IRBP 5' end short, and IRBP 3' end probes in the same sample lane: lane 5, adult RNA; lane 6, 5.2 month RNA; and lane 7, 4.5 month RNA. Lanes 8–10 have the same β-actin and 3' end probes, plus the longer 5' end probe: lane 8, adult; lane 9, 5.2 month; lane 10, 4.5 month. Lane 11 is a HpaII-digested pBR322 DNA marker. The arrows indicate the protected product for each reaction and its expected size in nucleotides. No other major bands were present. C, intensity of the RNase-protected products in panel B relative to the internal β-actin signal as determined by quantitation using a PhosphorImager and ImageQuant software. The numbers are listed as a percentage of the adult value. The length of each protected fragment is shown in parentheses.

**Elongation Versus Initiation in the Nuclear Run-on Assay**—A nuclear run-on signal is believed to be primarily a measure of elongation of nascent RNA, however, there may be a contribution of nascent messages that were initiated in vitro. Elongation versus initiation can be resolved by the use of Sarkosyl or high ammonium sulfate either of which prevent...
initiation by inactivating any RNA Polymerase II not already associated with a gene in a transcription complex (Yu and Feigelson, 1972; Gariglio et al., 1974; Green et al., 1975; Tolunay et al., 1984; Hawley and Roeder, 1985). Relative transcription rates in the presence of either reagent for adult photoreceptor genes are shown in Fig. 7A. Transcription rates for SAg and α-Tr did not change, however, the rates for opsin and IRBP were affected significantly and in opposite directions. When Sarkosyl was added, opsin transcription was lowered approximately 4-fold from a mean of 6.6 to 1.7, and IRBP transcription was enhanced 2-3-fold from a mean of 2.1 to 5.5. Ammonium sulfate similarly reduced the opsin transcription rate approximately 3-fold from a mean of 6.6 to a mean of 2.4, however, no effect was observed on the transcriptional activity of the IRBP gene. Reduction in opsin run-on signal by either Sarkosyl or ammonium sulfate suggests that this gene is being initiated in vitro and that the high rate of transcription observed for opsin is not only due to active elongating transcription complexes in many nuclei but also due to an efficient mechanism initiating transcription during the run-on reaction.

The increase in transcriptional activity observed with Sarkosyl for the IRBP gene has several potential explanations. The consistently higher run-on signal for the first exon versus the fourth exon without Sarkosyl (Fig. 5) suggests that the Sarkosyl effect could be due to alleviation of an RNA polymerase pause. A similar effect has been noted in Drosophila where transcripts of hsp70, arrested after synthesis of 25 nucleotides in vivo, were extended downstream after addition of Sarkosyl to a nuclear run-on experiment (Rougvie and Lis, 1988). Gene regulation by polymerase pausing has also recently been observed in mammalian nuclei where polymerase II elongation of c-myc is blocked in HL60 cells that are differentiated (Strobl and Eick, 1992). In order to determine whether a similar situation was occurring for IRBP, 0.4% Sarkosyl was added to a run-on reaction and the levels of 5' versus 3' transcription determined in an adult retina. The relative rate of transcription for the 5' end increased from 1.8 in the untreated nuclei to 6.3 in the treated reaction. The 3' end increased from 0.3 without Sarkosyl to 1.2 with Sarkosyl (Fig. 7B). The increase in rate of transcription was therefore 3.5-4-fold for either end of the IRBP gene with no preferential enhancement of the 3' end. Thus, if a factor was removed by Sarkosyl that allowed for additional RNA elongation by polymerase II, it appears to exert its effect either uniformly throughout the gene or within the first exon in a manner not detected by the probes used in this study.

**DISCUSSION**

This work expands upon earlier studies that demonstrated an increase in the mRNAs and proteins for the rod photoreceptor visual transduction genes at approximately 6 months gestation (Hauswirth et al., 1992; Timmers et al., 1993). Rod outer segment discs are first elaborated at this time and are the site of accumulation of visual transduction proteins. Perhaps the triggering of this major morphological event is connected mechanistically with the induction of the genes encoding components of the outer segments. The apparently coordinate accumulation of photoreceptor messages suggests that these genes which encode proteins that function in the same pathway may share common regulatory elements.

Further analysis of rod photoreceptor gene transcription has been hampered both by the lack of an appropriate differentiated cell culture line and the absence of an in vitro transcription assay. An alternate method commonly employed to study transcriptional rates is the nuclear run-on assay (reviewed in Marzluff and Huang, 1981). This technique has been successfully used to study induction of gene expression in many tissue culture systems, as well as to study endogenous gene expression in developmentally staged tissues, such as Caenorhabditis elegans embryos (Schauer and Wood, 1990) and embryonic chicken lens (Alamany et al., 1989). After conditions were optimized by maximizing the amount of transcription detectable using a variety of nuclei isolation procedures and RNA extraction and hybridization protocols, the nuclear run-on assay proved to be a viable approach for studying bovine fetal retinal gene expression.

These studies revealed three distinct patterns of transcriptional activity for rod photoreceptor genes as a function of fetal retinal development. The visual transduction genes opsin, α-Tr, and SAg were at very low levels at 5.2 months gestation and began increasing in a similar fashion between 5.2 and 6.3 months gestation. Opsi, however, showed an additional increase in transcription rate between 6.3 and 7.4 months which we suggest may indicate the positive effect of a gene specific enhancer element for opsin that does not effect either SAg or α-Tr. This would be the first experimental evidence for a transcriptional element specifically enhancing opsin transcription. Such an element might be expected since 90% of the disc protein is rhodopsin (Hargrave and McDowell, 1992), and the levels of rhodopsin are 10-100-fold greater than the other visual transduction proteins (Kuhn, 1981). Furthermore, it might be expected that an opsin-specific element would begin exerting its effect at the time of outer segment elaboration since rhodopsin additionally acts as a structural protein in the photoreceptor disc, and large amounts of opsin protein would be needed at this time.

IRBP, made and secreted by photoreceptor cells, is believed to shuttle retinoids through the interphotoreceptor matrix (Lai et al., 1982) and is therefore connected functionally with visual transduction. However, IRBP is transcriptionally distinct from the visual transduction genes tested because its transcription rate is nearly at half of its adult level at a time when the photoreceptor genes are still at basal levels. Murine IRBP mRNA (Gonzalez-Fernandez and Healy, 1992) and protein (Carter-Dawson et al., 1986) as well as bovine IRBP protein (Hauswirth et al., 1992) have been found much earlier in retinal development than rhodopsin. Consequently, it has been suggested that this early appearance of IRBP occurring before components of visual transduction may imply another role for this protein, possibly as a carrier for morphogens.
necessary for final photoreceptor maturation (Gonzalez-Fernandez and Healy, 1992; Hauswirth et al., 1992). The high rate of IRBP transcription at early fetal ages coupled with the earlier detection of protein, underscores the distinction between regulatory mechanisms for the IRBP and opsin genes and further strengthens the view that preinduction IRBP may play a role in photoreceptor development separate from its role in vision.

Contrasting mechanisms of gene regulation were also revealed upon comparison of the levels of opsin and IRBP mRNA to their rates of transcription. Ospin transcription rates and mRNA levels parallel each other throughout development. Therefore, its transcriptional efficiency may be the major, if not sole, controlling factor in regulating opsin gene expression. These data agree with observations in the mouse where opsin mRNA and transcription rate also followed a similar pattern (Treisman and Barnstable, 1988) and suggest that mammals in general may regulate the amount of opsin protein at the level of RNA polymerase II transcriptional efficiency. IRBP, in contrast, had very different levels of mRNA relative to transcriptional activity in preinduction aged retina. This difference was not due to a developmentally regulated attenuation event within the body of the gene since both ends of this gene show approximately the same increase in rates of transcription between 4.5 months and the adult. In the absence of evidence for a transcriptional mechanism, the low level of IRBP mRNA is most likely due to post-transcriptional control, possibly related to low message stability in the preinduction retina as has been previously suggested (Timmers et al., 1993).

Further experimental distinction between mechanisms of photoreceptor gene regulation was provided by modified nuclear run-on experiments designed to separate elongation from initiation by inactivating any polymerase II not in an initiated transcription complex. Although transcription rates determined in this way may not necessarily reflect the in vivo situation because proteins are stripped from chromatin by both Sarkosyl and high salt, it has proven to be an informative system to further dissect transcriptional mechanisms. The opsin gene in the adult retina experienced an approximately 4-fold reduction in transcription rate with either Sarkosyl or ammonium sulfate while SAg and α-Tr were unaffected. It, therefore, appears that de novo initiation occurs only for opsin in the run-on reaction as might be expected for a strong opsin-specific positive regulatory element. Interestingly, the diminished rate for opsin transcription observed in the presence of Sarkosyl or ammonium sulfate is similar to the transcription rates for SAg and α-Tr under all conditions, suggesting that the primary regulatory difference between opsin and these genes is the presence of an opsin-specific positive regulatory element.

An enhancement of IRBP transcription was seen with the addition of Sarkosyl to the run-on reaction which we hypothesize is due to removal of a negative regulatory element affecting IRBP elongation. This effect was seen with Sarkosyl and only minimally present with the addition of ammonium sulfate. As noted above, these reagents were chosen because both high salt and Sarkosyl will prevent initiation, however, their ability to remove proteins from chromatin is markedly different. Sarkosyl has been shown to remove at least 80% of chromatin-associated proteins other than initiated RNA polymerase II (Green et al., 1975; Scheer, 1978). Ammonium sulfate removes much less chromatin-associated protein, with 250 mM removing less than 30% of total protein (Butterworth et al., 1971). Therefore, the greater stimulatory effect on IRBP transcription with Sarkosyl is consistent with its greater ability to remove chromatin-associated proteins. A similar observation had been noted in isolated mouse nuclei, where a general increase in RNA polymerase II transcription was observed upon treatment with either Sarkosyl or ammonium sulfate; however, the optimal stimulation seen with Sarkosyl was always greater than that observed for optimal ammonium sulfate (Green et al., 1975).

It is important to begin defining the cis elements responsible for the distinctive temporal patterns of retinal gene-specific expression revealed here. Transgenic mouse studies using the opsin promoter showed that ~500 bases upstream sequence is sufficient for tissue-specific expression (Lem et al., 1991; Zack et al., 1991). It is therefore possible that the enhancer element identified here will be located in this region of the opsin gene. Interestingly, a region in the rat opsin promoter that is 110 base pairs upstream (5′) from the start of transcription has been footprinted and appears to be of developmental importance (Morabito et al., 1991). Although the function of this region is not known, this sequence is conserved in the bovine rod opsin gene suggesting a possible role in transcription.

In summary, we extend previous studies demonstrating that the rod visual transcription genes, including IRBP, follow a parallel temporal pattern of mRNA accumulation, coinciding with the emergence of outer segments. On this level photoreceptor gene regulation was provided by modified nuclear run-on experiments designed to separate transcriptional mechanisms. The opsin gene in the adult retina experiences an approximately 4-fold reduction in transcription rate with either Sarkosyl or ammonium sulfate while SAg and α-Tr were unaffected. It, therefore, appears that de novo initiation occurs only for opsin in the run-on reaction as might be expected for a strong opsin-specific positive regulatory element. Interestingly, the diminished rate for opsin transcription observed in the presence of Sarkosyl or ammonium sulfate is similar to the transcription rates for SAg and α-Tr under all conditions, suggesting that the primary regulatory difference between opsin and these genes is the presence of an opsin-specific positive regulatory element.

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