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Differential regulation of melatonin synthesis genes and phototransduction genes in embryonic chicken retina and cultured retinal precursor cells

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Purpose: Photoreceptor differentiation involves the activation of two specific sets of genes; those encoding the proteins of the phototransduction cascade and those encoding the enzymes of the melatonin synthesis pathway, arylalkylamine N-acetyltransferase (AANAT) and hydroxyindole O-methyltransferase (HIOMT). The purpose of the present study was to examine the conditions of AANAT and HIOMT gene activation, relative to that of selected phototransduction markers (α -transducin and opsins), in both in vivo and in vitro differentiating photoreceptors of the chicken retina.

Methods: Neural retina RNA was obtained between embryonic day 7 (E7) and posthatch day 8 (P8) and analyzed on northern blots with cDNA probes to AANAT, HIOMT, visinin, α -transducin, rhodopsin, and the four cone opsins. Cell cultures were prepared from E7 chicken neural retina and incubated for two to four days in vitro, either in basal medium or in serum-supplemented medium or in medium containing an insulin-based supplement. RNA from the cultured cells was analyzed on northern blots as above. Real time RT-PCR was used to confirm in vitro changes in HIOMT and red opsin mRNA levels. The cultured cells were transfected with promoter-reporter plasmids for direct analysis of HIOMT promoter regulation by the dual luciferase method.

Results: The different mRNAs composing the photoreceptor phenotype appeared at E7 (visinin), E10 (α -transducin), E14 (HIOMT), E15 (rhodopsin, red opsin, and green opsin), E16 (AANAT), E17 (blue opsin), and E18 (violet opsin). In the early differentiating cones of the central retina, HIOMT mRNA appeared two days earlier than red opsin and green opsin mRNAs (E12 rather than E14). In cultured embryonic neural retina cells, basal medium was sufficient to activate α -transducin gene transcription, an insulin-based supplement was sufficient to activate HIOMT gene transcription, whereas serum was required for red opsin gene transcription after two days in vitro. All serum batches were able to activate red opsin gene transcription, whereas some of them failed to activate HIOMT gene transcription. Activation of the HIOMT gene promoter by an insulin-based supplement and by serum was confirmed after transfection of chicken embryonic neural retina cells with promoter-reporter plasmids.

Conclusions: Activation of the melatonin synthesis genes in vivo takes place in a time window very close to that of early opsins. However, a 24-48 h lead of HIOMT gene expression over early opsins was clearly observed. Our in vitro experiments indicate that different exogenous signals are required to activate the different genes encoding photoreceptor specific functions. Significantly, marker genes for light sensitivity (red opsin) and for melatonin synthesis (HIOMT) appear to be activated in response to different signals.

In addition to the role they play in vision by affecting the phototransduction cascade, retinal photoreceptors specifically convert the photoperiodic component of ambient light into a day/night rhythm of melatonin synthesis. Melatonin is produced with a large amplitude peak at night and regulates several aspects of retinal physiology, including disk shedding from rod outer segments and dopamine release from amacrine cells [1,2]. Melatonin is synthesized from serotonin in two enzymatic steps catalyzed by arylalkylamine N-acetyltransferase (AANAT, EC 2.3.1.87) and by hydroxyindole O-methyltransferase (HIOMT, EC 2.1.1.4). Photoreceptor specific expression of AANAT and HIOMT has been documented by immunocytochemical and in situ hybridization studies in the chicken pineal and retina [3-10]. Based on these observa-

tions, functional differentiation of photoreceptors in embryonic life would appear to rely on the activation of at least two specific sets of genes: those of the phototransduction cascade and those of the melatonin synthesis pathway.

The relative timing of expression of these two sets of genes during photoreceptor differentiation has not been examined and it is not known whether they are activated in response to similar or different developmental signals. A number of histological studies have shown that photoreceptor differentiation in the chicken retina is a protracted phenomenon, starting when precursors exit mitosis around embryonic day 6 (E6) and extending over two weeks, with stepwise appearance of the different cellular components that characterize the photoreceptor phenotype [11]. A similar picture was derived from gene expression studies, showing that photoreceptor precursors express visinin as soon as they exit mitosis at E6, whereas activation of the opsin genes takes place only eight to ten days later, as observed by in situ hybridization [12]. Studies in

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mammalian species appear to confirm the stepwise activation of phototransduction genes, as exemplified by the sequential expression of transducin, rhodopsin, phosphodiesterase 6, and arrestin over a period of several days or weeks [13-15]. A developmental study of a larger number of photoreceptor markers in the chicken retina would be required to examine whether there are any intermediate steps in photoreceptor specific gene activation between visinin expression at E6 and the activation of the opsin genes around E15. In this context, special interest in the melatonin synthesis genes is warranted because information on the timing of expression of AANAT and HIOMT in the embryonic chicken retina is relatively scarce [16-18], which makes it difficult to compare with the developmental expression of the phototransduction genes. To obtain a better description of these two aspects of photoreceptor differentiation, we simultaneously analyzed the mRNAs encoding HIOMT, AANAT, visinin, α -transducin, rhodopsin, and the four cone opsins in the developing chicken retina. The results indicate that α -transducin and HIOMT expressions start two to four days before opsin expression, thus suggesting intermediate steps in photoreceptor differentiation.

The next question we wished to examine was whether the phototransduction genes and the melatonin synthesis genes were activated by similar signals or by different signals during photoreceptor differentiation. To address this question, we took advantage of a dispersed cell culture of chicken retinal precursors that was previously shown to accelerate the differentiation of red cones [19,20]. In this system, we observed

that different culture conditions were required to support HIOMT, α -transducin, and red opsin gene transcription.

METHODS

Animals: Fertilized chicken eggs (*Gallus domesticus*) were purchased from Rumolo Cie (Quincay, France) and were incubated at 37 °C in an atmosphere of 70% humidity. Embryos were staged according to the method of Hamburger and Hamilton [21]. Newly-hatched chicks were transferred to a hot room maintained at 27 °C, with food and water ad libitum.

Cell culture: Primary retinal cell cultures were prepared as described by Adler [22]. Embryonic day 7 (E7) neural retinas, free of pigment epithelial cells, were dissociated and cultured at 37 °C in 6 well or 24 well polyornithine-coated plates (3×10^6 cells/well and 6×10^5 cells/well, respectively), in an atmosphere of 5% CO₂ in air. Basal culture medium was M199 (Sigma, Saint Quentin Fallavier, France) supplemented with 100 U/mL penicillin/streptomycin, 2 mM glutamine, and 0.5% bovine serum albumin (BSA). Serum-supplemented medium contained 0.1% BSA and either 10% newborn calf serum (NCS; Invitrogen, Cergy Pontoise, France) or 10% fetal calf serum (FCS; Invitrogen). ITS-supplemented medium contained 0.5% BSA and 1X ITS (1.5 μ M insulin, 69 nM transferrin and 38 nM selenium, Invitrogen).

cDNA probes: The cDNA probes were produced by PCR amplification of either plasmid clones or total retinal cDNA, with the primer pairs listed in Table 1, purified by agarose gel electrophoresis and silica gel adsorption (QIAquick, QIAGEN, Courtaboeuf, France or GENECLAN, Qbiogene, Illkirch, France) and verified by DNA sequencing (Big Dye, Perkin Elmer, Courtaboeuf, France). The chicken HIOMT probe was produced by PCR amplification of a 518-bp fragment from a previously described Blue-pGEM clone [23]. The chicken AANAT probe was produced by PCR amplification of a 1388-bp fragment from a previously described pBK-CMV clone [10]. The chicken visinin probe was produced by PCR amplification of a 444-bp fragment from total retinal cDNA. The chicken α -transducin probe was a 479-bp fragment of 89% identical sequence for the rod and cone forms (positions 574 to 1052 of the coding region in the cone form), produced by PCR amplification from total retinal cDNA. The presence of a mix of rod and cone forms in the PCR product was verified by DNA sequencing. Hybridization of this probe on northern blots of total retinal RNA sometimes showed two bands around 2 to 2.5 kb, in agreement with the reported sizes of rod and cone forms of α -transducin mRNA [24]. More generally, however, the two forms of α -transducin mRNA were not resolved on our northern blots due to the large quantity of material loaded on the gels. Because the α -transducin probe also showed 82% sequence identity with Gi2 α -subunit, we verified the absence of cross-hybridization on northern blots of total brain RNA. The different chicken opsins probes were produced by PCR amplification of pGEM-T-cloned fragments of the following sizes: rhodopsin 475 bp, red opsin 300 bp, green opsin 475 bp, blue opsin 429 bp, violet opsin 392 bp. Each opsin probe had less than 70% sequence identity with any other opsin and hybridized only with the mRNA of expected size on

TABLE 1. PRIMERS

Primer name	Sequence	Accession number
HIOMT	F: 5' - TTACTGCATTGACCTTTCCC - 3' R: 5' - CCTCCCAGAACAGCATCATA - 3'	X62309
Red opsin	F: 5' - CACGAAGAGGAGGACACCAC - 3' R: 5' - CGAGATCTGGTTGATGACGCT - 3'	X57490
Rhodopsin	F: 5' - AAGGCCAAGACTTCTACGTGC - 3' R: 5' - ATGATCCAGGAGAACGCGACC - 3'	D00702
Green opsin	F: 5' - TCCTCATCTCCACTGGTCTGC - 3' R: 5' - ACGTAGGACTCGTTGTGGTAG - 3'	M92038
Blue opsin	F: 5' - TGTTACAGACTATGGCGGCCT - 3' R: 5' - TGTACCGACTCCATCCGAAGA - 3'	M92037
Violet opsin	F: 5' - CATCGGACGACGACTTCTACC - 3' R: 5' - GATGTAACGCTCGAAGGCCAA - 3'	M92039
Visinin	F: 5' - GTTACACGGAGGAGGAGCTGA - 3' R: 5' - CCTCGCGATCTTATCGTTCT - 3'	M84729
α -transducin	F: 5' - AAGGACCTCAACTTCAGGATGT - 3' R: 5' - CAGTCTTGAGGTTCTCCTTG - 3'	NM_204690
GAPDH	F: 5' - AGGCGAGATGGTGAAAGTCG - 3' R: 5' - TCTGCCCATTTGATGTTGCT - 3'	NM_204305

Forward (F) and reverse (R) primers used in this study. All primers are for chicken sequence.

northern blots: rhodopsin at 1.6 kb (major band) and 3 kb (minor band); red opsin at 1.6 kb; green opsin at 2.8 kb; blue opsin at 3.1 kb, and violet opsin at 3.7 kb [25].

Northern blot analysis: Neural retinas were obtained at the beginning of day time, from at least 10 animals for each experimental point, dissected free of the pigment epithelium and rapidly frozen in liquid nitrogen as pools. For center to periphery gradient studies, a 4 to 9 mm piece of retina was dissected at the tip of the pecten (area centralis), and another 36 mm piece of retina was dissected from the nasal periphery. Total RNA was extracted as previously described [26]. Tissues were sonicated in a suitable volume (20-50 μ l/mg of tissue) of ice-cold LiCl/urea solution (3 M LiCl, 6 M urea). The homogenates were incubated overnight at 4 °C. Total RNA was pelleted by centrifugation at 15000x g for 30 min. Pellets were washed once with 1 volume of LiCl/urea solution and were then resuspended in TE/SDS buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8, 0.5% sodium dodecyl sulfate). Total RNA was then purified by extraction with phenol/chloroform and precipitated with ice-cold ethanol. RNA concentrations were measured by the optical density at 260 nm. Total RNA was denatured with 48% formamide and 6.4% formaldehyde at 95 °C for 5 min, and loaded (15 μ g per lane) on 1% agarose/0.7 M formaldehyde gels containing 0.7 μ g/ml ethidium bromide. After electrophoresis, the RNA was transferred overnight onto nitrocellulose membrane in 10X NaCl/Citrate buffer (1.5 M NaCl, 0.15 M sodium citrate, pH 7). Nonspecific sites were blocked by incubating the membrane for 2 h at 42 °C in the high-stringency hybridization solution (40% formamide, 10% dextran sulfate, 4X NaCl/Cit, 0.02% polyvinylpyrrolidone, 0.02% BSA fraction V, 0.02% Ficoll 400, 20 mM Tris-HCl, pH 7.4, and 0.3 mg/ml denatured salmon sperm DNA).

The cDNA probes described above were labeled to similar specific radioactivities by random priming [27] with [α -³²P]dCTP (3000 Ci/mmol). The chicken 18S rRNA probe was a 21-mer antisense oligonucleotide corresponding to bp 956-936 (GenBank accession number M59389) and was labeled with [α -³²P]dCTP (3000 Ci/mmol) using Terminal Deoxynucleotidyl Transferase (Amersham Biosciences, Orsay, France). The prehybridized membranes were incubated overnight at 42 °C with the radiolabelled probes diluted at 1 μ Ci/ml in high-stringency hybridization solution. Membranes were then washed for 1 h at room temperature in 2X NaCl/Citrate, 0.1% SDS and for 2 h at 52 °C in 0.1X NaCl/Citrate, 0.1% SDS and exposed on the screen of a PhosphorImager (Storm 820, Amersham Biosciences).

Real time RT-PCR: Total RNA (1 μ g) from cultured retinal cells was reverse transcribed with M-MLV (Promega, Charbonnières, France) and random hexamers (Amersham Biosciences) at 37 °C for 2 h and the reaction was terminated by a 1/5 dilution with double-distilled water. Real time PCR was performed on a LightCycler (Roche Applied Science, Meylan, France) according to the manufacturer's instructions. A typical reaction was performed in 10 μ l, consisting of 5 μ l of a 1/5 diluted RT reaction and 5 μ l of SYBR Green Master

Mix (Roche Applied Science), containing the specific primer pairs (final 0.5 μ M each). The PCR temperature cycle was 95 °C 10 min, followed by 45 cycles of 95 °C 10 s, 65 °C 10 s, 72 °C 20 s, with SYBR Green fluorescence recording at the end of each elongation segment. The PCR reaction was followed by a melting curve analysis: 95 °C 10 s, 60 °C 10 s, linear increase to 95 °C at 0.1 °C/s, with continuous SYBR Green fluorescence recording. The primer pairs used in this study for HIOMT (S518, AS1035), red opsin (S47, AS488) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, S7, AS260; Table 1) were validated as follows: They gave a single PCR product as verified by melting curve analysis, agarose gel electrophoresis, and DNA sequencing, and the distribution of the PCR sigmoids was linear (r was 0.99 to 1) over 5 logs of template concentration with an efficiency of 1.85 to 1.98. The critical cycle of each sigmoid PCR curve was calculated by the LightCycler Software 3.5® (Roche Applied Science) as the PCR cycle corresponding to the maximum of the second derivative. Total cDNA from each cell culture sample was analyzed by real time PCR for HIOMT, red opsin, and GAPDH. Critical cycle values of GAPDH PCR reactions were used for correction of the RNA load in the RT reactions. After this correction, critical cycle differences for HIOMT or for red opsin, between samples cultured in basal medium and samples cultured in either ITS- or NCS-supplemented medium were converted into fold increase over control.

Promoter-reporter analysis: Embryonic retinal cells were seeded at a density of 3×10^6 cells per well in 6 well plates, one day before transfection. Cells were transfected with promoter-reporter constructs (2 μ g/well) containing either 3 kbp or 225 bp of the chicken HIOMT promoter, upstream of the firefly luciferase reporter gene, as previously described [28], or with the promoterless plasmid pGL3-Basic (Promega). To correct for variations in transfection efficiencies, each experimental group was cotransfected with a plasmid containing the Renilla luciferase reporter gene, under control of the SV40 promoter and enhancer (pRL-SV40, Promega; 1.5 μ g/well). Transfections were performed following the CaPO₄ method. Briefly, plasmid DNA was diluted in 75 μ l of 250 mM CaCl₂ and was added dropwise to 75 μ l of 2X HeBS (280 mM NaCl, 1.4 mM Na₂HPO₄, 50 mM HEPES, pH 7.1). Cells were incubated overnight with the precipitate (150 μ l/well in 2 ml of culture medium) and were cultured for 24 h in fresh medium before being assayed for luciferase activities. Transfections and assays were performed in triplicate for each experimental group. Basal medium alone (M199 with 0.1% BSA) could not be used in these experiments because it generated a major CaPO₄ precipitate that was lethal to the cells. Instead, culture and transfections were performed in basal medium supplemented either with 10% NCS, or with 10% FCS from a batch that did not induce northern blot-detectable levels of HIOMT mRNA (this batch is thereafter termed inactive FCS). Firefly and Renilla luciferase activities, were assayed using the Dual-Glo luciferase assay system (Promega). Light emission was measured on a Beckman LS 6500 counter, on the luminometer program (Beckman Instruments, Gagny, France). The differ-

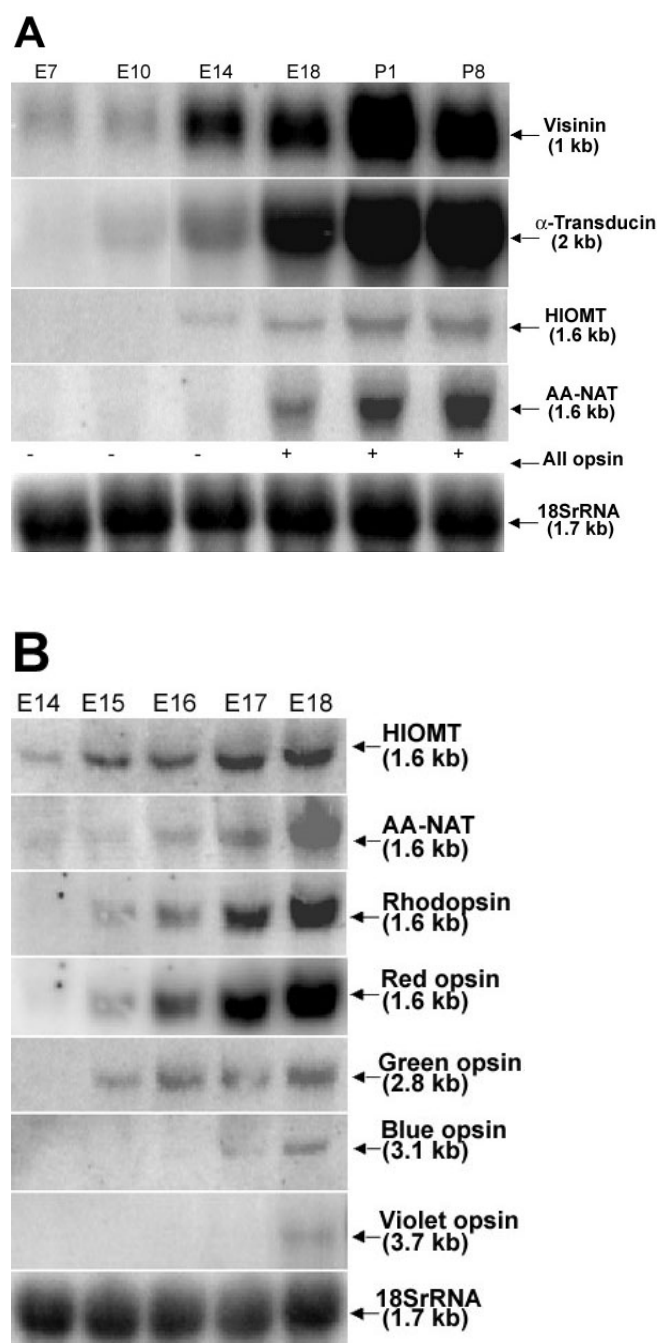


Figure 1. Development of mRNA levels for melatonin-synthesizing enzymes and phototransduction proteins. Total RNA (15 μ g) was extracted from 10 to 30 neural retinas at the indicated developmental stages and analyzed on northern blots with the indicated 32 P-labeled cDNA probes. A 32 P-labeled 18S rRNA oligonucleotide was used to verify equal RNA loading in the different lanes. **A:** After analysis on a broad time scale, the successive appearances of visinin, α -transducin, HIOMT, and AANAT mRNAs are illustrated. For clarity, the detection of all opsin mRNAs is summarized by (-) or (+). The results are representative of two independent experiments analyzed on four northern blots. **B:** Analysis on a higher resolution time scale allows a closer comparison of the different timing of gene activation for the melatonin-synthesizing enzymes and for the five opsins. The results are representative of one experiment analyzed on two northern blots.

ences in transfection efficiency between the experimental groups were normalized by calculating the ratio between the firefly and Renilla luciferase activities.

RESULTS

Developmental expression of melatoninergic and phototransduction genes in the embryonic chicken retina: Developmental activation of the genes encoding melatonin-synthesizing enzymes and phototransduction proteins was examined by northern blot, at 3-7 days intervals between embryonic day 7 (E7) and posthatch day 8 (P8) and at 1 day intervals between E14 and E18 (Figure 1). Analysis on the wide time scale indicated that visinin mRNA was present from E7 onwards, α -transducin mRNA appeared at E10; HIOMT mRNA at E14, while AANAT and all opsins mRNAs were detected from E18 onwards (Figure 1A). Minor mRNA bands (about 10% of the signal) were observed for HIOMT (at 4 kb) and for rhodopsin (at 3 kb), in agreement with previous reports [20,25,29]. The intensity of these minor bands increased in parallel with the major signal (not illustrated). Analysis on a more resolutive time scale allowed us to accurately define the order of appearance of the mRNAs encoding the melatonin-synthesizing enzymes, relative to the mRNAs encoding the different opsins. HIOMT gene expression at E14 was confirmed, while AANAT mRNA became clearly detectable at E16, with only a faint trace at E15 (Figure 1B). In agreement with a previous *in situ* hybridization study [12], rhodopsin, red opsin, and green opsin mRNAs became detectable at E15, while blue opsin mRNA appeared at E17 and violet opsin mRNA only at E18 (Figure 1B). The antecedence of HIOMT gene transcription over early cone opsins (red and green) was confirmed in three independent experiments.

To further analyze this time difference in gene expression, we examined the spatial distribution of HIOMT mRNA in the developing retina, based on the notion that cone photoreceptors differentiate in a center to periphery gradient [12]. When the area centralis and the nasal periphery of the retina

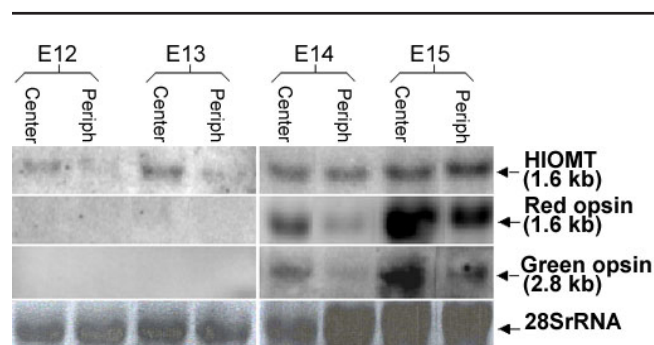


Figure 2. Gradients of HIOMT and opsin mRNAs in the embryonic chicken retina. Center to periphery gradients of HIOMT, red opsin, and green opsin mRNAs in the embryonic chicken retina are presented. At the indicated developmental stages, the area centralis and the nasal periphery of 10 to 16 neural retinas were dissected separately. Total RNA (15 μ g) was analyzed by northern blot and probed with the indicated 32 P-labeled cDNAs. 28S rRNA was visualized by BrEt-induced fluorescence.

were dissected separately, northern blot analysis allowed us to detect HIOMT mRNA in the central retina as early as E12, whereas it became detectable in the peripheral retina at E14 (Figure 2). The previously described center to periphery gradient of red opsin and green opsin expression [12] could be observed at E14 and E15 (Figure 2). Thus, the data clearly indicated a 48 h lead of HIOMT over red opsin and green opsin in the early-differentiating photoreceptors of the area

centralis. The relative precocity of HIOMT gene transcription (Figure 3) would appear to make it a valuable marker to examine the early events controlling melatoninergic differentiation of photoreceptors.

Differential regulation of HIOMT, α -transducin, and red opsin gene expression in retinal precursors in vitro: In agreement with previous reports [19,20], retinal precursors dissociated at E7 and cultured for 2-3 days in serum-supplemented

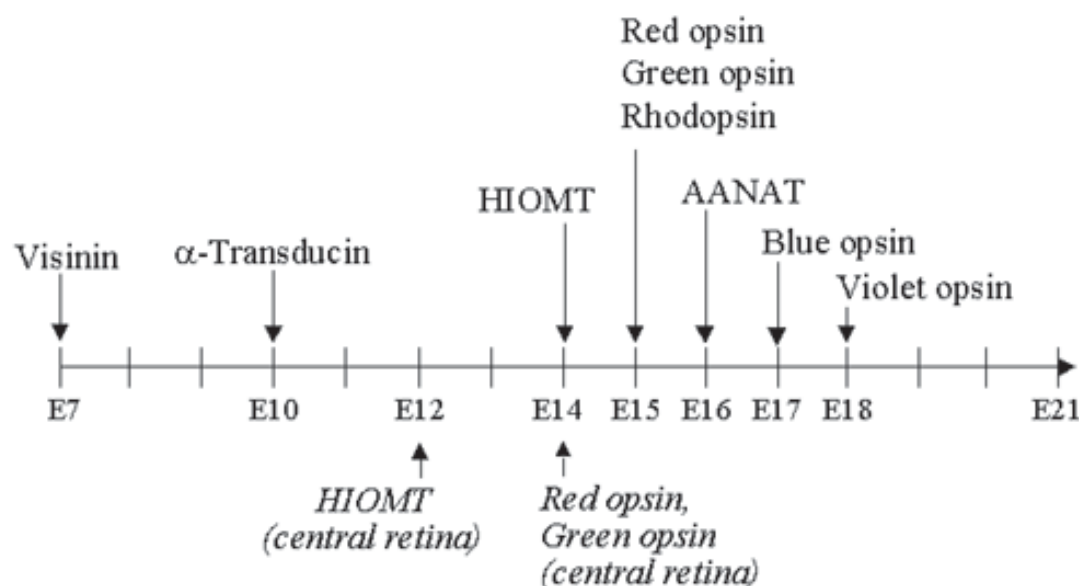


Figure 3. Schematic representation of gene activation steps in the developing chicken retina. This graph summarizes the data presented in Figure 1 and Figure 2. On a time scale spanning embryonic day 7 (E7) to E21 (hatching), arrows point to the dates of appearance of the indicated mRNAs, as detected by northern blot analysis. The dates refer to analyses performed on total retina, except when otherwise indicated (italics).

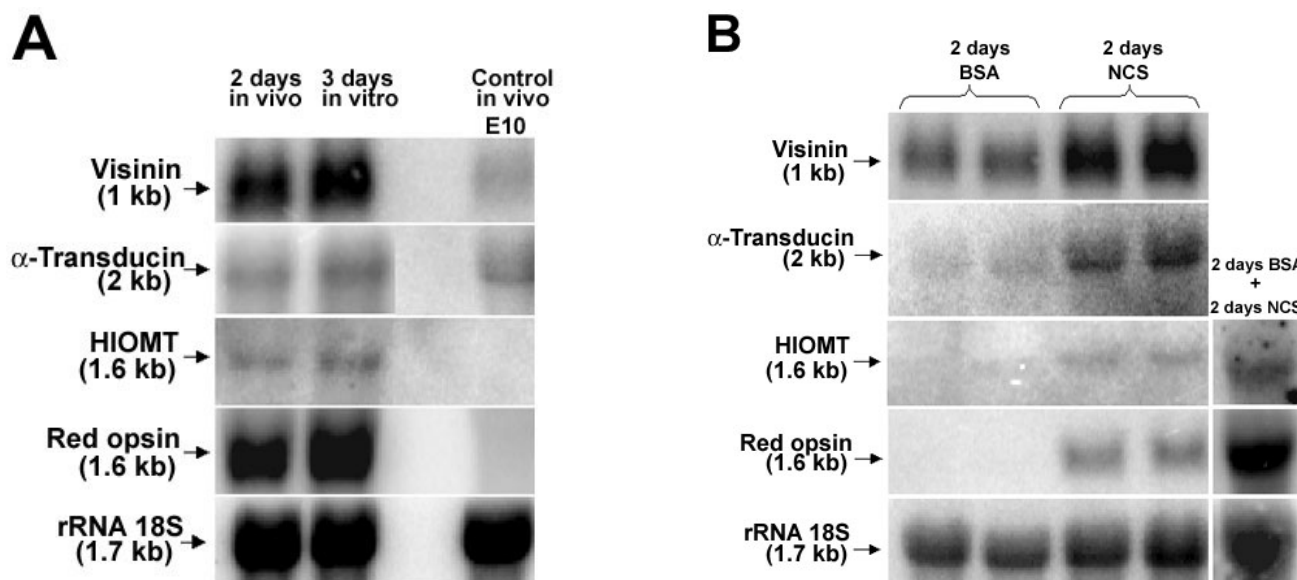


Figure 4. Effect of media serum on mRNA levels in cultured retinal cells. Visinin, α -transducin, HIOMT, and red opsin mRNA levels in retinal cells cultured with or without serum were assessed. Total RNA (15 μ g per lane) extracted from approximately 10^7 cultured cells or from a pool of 10 embryonic (E10) retinas, was analyzed on northern blot with the indicated 32 P-labeled cDNA probes. A 32 P-labeled 18S rRNA oligonucleotide was used to verify equal RNA loading in the different lanes. **A:** E7 retinal cells were cultured for two to three days in serum-supplemented medium. Their profile of gene expression is compared with that of neural retinas from E10 embryos. The results are representative of at least eight experiments. **B:** Duplicate cultures of E7 retinal cells were kept for two days in either basal medium (0.5% bovine serum albumin) or serum-supplemented medium (10% newborn calf serum). A batch of cells cultured for two days in basal medium was transferred to serum-supplemented medium for two additional days.

medium showed a precocious activation of red opsin gene transcription and an increase in visinin mRNA levels, when compared to in vivo E10 retinas (Figure 4A). With all due caution when comparing in vitro and in vivo situations that do not necessarily reflect the same embryonic stages, our data appeared to confirm that red cones undergo an accelerated differentiation in vitro. Our experiments also confirmed that retinal precursor cells failed to differentiate into other cones or into rods, as indicated by the absence of rhodopsin, green, blue or violet opsins mRNAs (data not shown). In addition, we observed that the cultured cells expressed other components of the photoreceptor phenotype (i.e., the mRNA transcripts of α -transducin and HIOMT genes; Figure 4A). AANAT mRNA was hardly detectable on northern blots after two or three days of culture (data not shown) and therefore was not further investigated.

The different mRNA transcripts composing the photoreceptor phenotype were differentially affected when cells were cultured in serum-free (basal) medium. Red opsin and HIOMT transcripts remained undetectable after two days of culture in basal medium (Figure 4B). In contrast, visinin mRNA was still present at a concentration about half that observed in serum-supplemented cultures (Figure 4B). Moreover, α -transducin gene transcription was clearly activated in cells cultured in basal medium, albeit less efficiently than in the presence of serum (Figure 4B). Cell counts indicated 60% plating efficiency after two days of culture in both basal and serum-supplemented media. Although cell viability dropped rapidly in basal medium (30% after three days and about 10% after four days), cells cultured for two days in basal medium remained capable to activate red opsin and HIOMT gene transcription when transferred to serum-supplemented medium for two additional days (Figure 4B). Together, the data seemed to indicate that photoreceptor precursors cultured for two days in basal medium were viable and actively differentiating (activation of the α -transducin gene), but were lacking a serum factor to activate red opsin and HIOMT gene transcription.

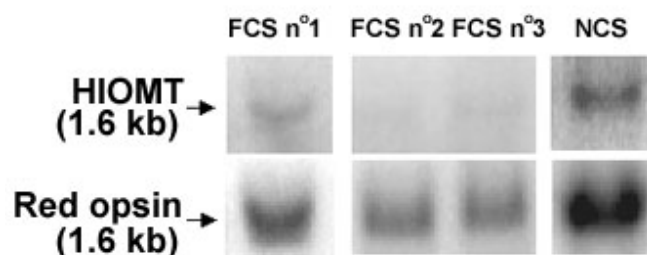


Figure 5. Sensitivity of HIOMT gene transcription to different lots of serum. E7 retinal cells were cultured for three days in medium supplemented with different lots of fetal calf serum (FCS) or with newborn calf serum (NCS). Total RNA (15 μ g per lane) extracted from approximately 10 million cells was analyzed on northern blot with the indicated 32 P-labeled cDNA probes. HIOMT gene transcription was efficiently activated by one lot of FCS out of three, whereas red opsin gene transcription was activated by all lots of FCS. This variability was not observed for NCS, which consistently activated transcription of both HIOMT and red opsin.

Further experiments indicated that different batches of fetal calf serum (FCS) varied strongly in their ability to activate HIOMT gene transcription, although they all proved capable to activate red opsin gene transcription (Figure 5). This was the first hint at a differential regulation of these two genes during photoreceptor differentiation in vitro. In contrast to the variability of FCS batches, newborn calf serum (NCS) consistently activated both HIOMT and red opsin gene transcription (Figure 5).

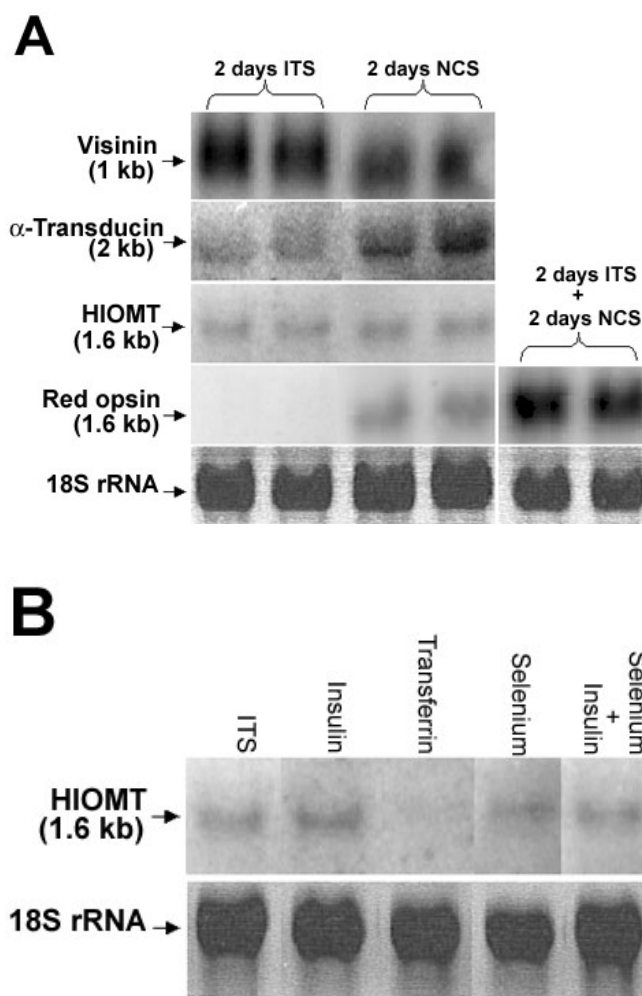


Figure 6. Effect of insulin-based culture supplement on HIOMT and red opsin gene regulation. The effect of an insulin-based culture supplement on HIOMT and red opsin gene regulation was assessed. Total RNA (15 μ g per lane) extracted from approximately 10^7 cells was analyzed on northern blot with the indicated 32 P-labeled cDNA probes. 18S rRNA BrEt-induced fluorescence was used to verify equal RNA loading in the lanes. **A:** Duplicate cultures of E7 retinal cells were kept for 2 days in medium supplemented with either insulin-transferrin-selenium (ITS) or newborn calf serum (NCS). Two batches of cells cultured for two days in ITS-supplemented medium were transferred to NCS-supplemented medium for two additional days. **B:** E7 retinal cells were cultured for three days in medium containing each component of the ITS supplement, as indicated (insulin 1.5 μ M, selenium 38 nM, transferrin 69 nM).

To further compare HIOMT and red opsin gene transcription *in vitro*, retinal precursors were cultured in a chemically-defined medium, where serum was replaced by an insulin-based culture supplement (insulin, transferrin, selenium: ITS). After two days of culture in the presence of ITS, HIOMT gene transcription was strongly activated, whereas red opsin mRNA remained undetectable (Figure 6A). Photoreceptor precursors survival in ITS-supplemented medium could be verified by the presence of visinin and α -transducin mRNAs (Figure 6A). In addition, cells cultured for two days in ITS-supplemented medium retained the ability to activate red opsin gene transcription, if further-cultured for two additional days in the presence of NCS (Figure 6A). When the components of the ITS culture supplement were tested separately, transferrin had no effect, whereas insulin (1.5 μ M) and selenium (38 nM) both activated HIOMT gene transcription, without apparent additivity (Figure 6B). The effect of insulin was not observed at 15 nM concentration (data not shown).

Real time RT-PCR was used to confirm the effects of ITS and NCS on HIOMT and red opsin mRNA levels. Due to its higher sensitivity, real time RT-PCR detected small amounts of both HIOMT and red opsin mRNAs in cells cultured in basal medium, which allowed for quantitation of the relative increase in mRNA levels evoked by either ITS or NCS. In a

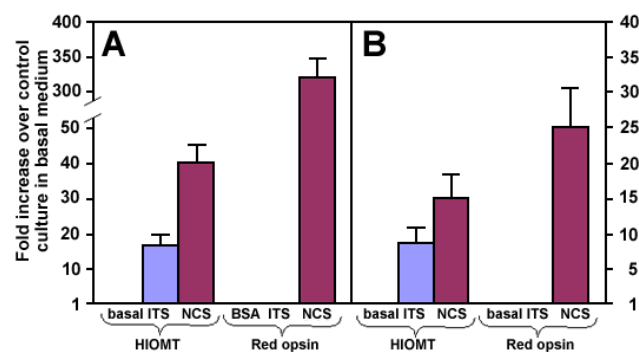


Figure 7. Effect of insulin-based culture supplement or serum on HIOMT and red opsin gene regulation. Real time RT-PCR analysis was conducted to assess the effects of serum or an insulin-based supplement in culture media on HIOMT and red opsin mRNA levels. E7 retinal cells were cultured for two days in either basal medium containing 0.5% bovine serum albumin (BSA) or medium supplemented with insulin-transferrin-selenium (ITS) or medium supplemented with newborn calf serum (NCS). Total RNA (about 1 μ g) was reverse transcribed and cDNA aliquots (a tenth of the RT reaction) were analyzed successively by real time PCR with primer pairs specific for HIOMT, red opsin, and GAPDH. For both HIOMT PCR curves and red opsin PCR curves, the differences in critical cycle number observed between the different culture conditions were converted into fold increase over control culture in basal medium, after correction of the RNA load in each sample by the critical cycle number of GAPDH PCR (only marginal corrections were required). A first series of 5 duplicate cultures (A) and a second series of eight duplicate cultures (B) were analyzed. Bars represent means for $n=10$ (A) and $n=16$ (B); error bars represent the SEM.

first series of five consecutive experiments, this quantitation revealed a 17 fold increase in HIOMT mRNA levels with ITS and a 40 fold increase with NCS, compared to no supplement (Figure 7A). In the same experiments, red opsin mRNA levels showed a 300 fold increase with NCS and no significant increase with ITS (Figure 7A). Another series of eight experiments confirmed this observation, albeit with less dramatic changes in mRNA levels: HIOMT mRNA concentration was increased 9 fold by ITS and 15 fold by NCS, while red opsin mRNA concentration was increased 25 fold by NCS and not increased by ITS (Figure 7B).

Accumulation of HIOMT mRNA in response to NCS or ITS appeared to reflect transcriptional activation rather than mRNA stabilization, because both culture conditions also activated promoter-reporter constructs consisting of the luciferase reporter under control of either a 3 kbp or a 225 bp version of the chicken HIOMT promoter (Figure 8). Due to constraints on transfection efficiency (see Methods), basal conditions in these experiments did not correspond to unsupplemented medium, but to medium supplemented with a batch of inactive FCS (i.e., a batch of FCS that did not induce northern blot-detectable levels of HIOMT mRNA). By comparison with a

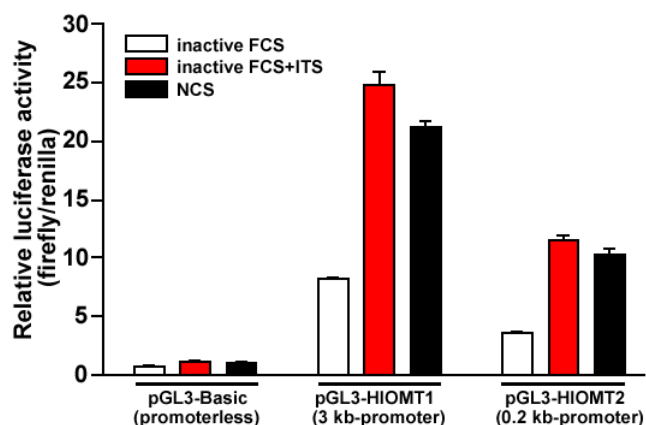


Figure 8. Effect of insulin-based culture supplement or serum on HIOMT promoter activity. Activation of the chicken HIOMT promoter by newborn calf serum (NCS) and by the insulin-transferrin-selenium culture supplement (ITS) was assessed. E7 retinal cells were cultured in medium supplemented with either 10% newborn calf serum (NCS) or 10% inactive fetal calf serum (i.e., FCS that did not induce northern blot-detectable levels of HIOMT mRNA expression) or with 10% inactive FCS plus insulin-transferrin-selenium (ITS). The cells were transfected with plasmids containing the firefly luciferase reporter gene alone (pGL3-Basic) or under control of either 3 kbp or 225 bp of the chicken HIOMT promoter (pGL3-HIOMT1 and pGL3-HIOMT2, respectively). To correct for variations in transfection efficiencies, all experimental groups were cotransfected with a second plasmid (pRL-SV40) containing the renilla luciferase reporter gene under control of the SV40 promoter. The levels of luciferase activities were measured 24 h post-transfection. The ratios of firefly/renilla luciferase activities were calculated and were expressed relative to pGL3-Basic. Histograms represent the mean (3 in each experimental group) from one representative experiment; error bars represent the SEM. Similar results were obtained in four independent experiments.

promoterless plasmid, some HIOMT promoter activity was clearly observed in the presence of inactive FCS. However, transcription of the promoterless plasmid did not change in the presence of NCS or ITS, whereas both supplements increased HIOMT promoter activity about 3 fold (Figure 8).

DISCUSSION

The present study describes the timing of gene activation for core proteins of the phototransduction cascade (opsins and α -transducin) and for the enzymes of the melatonin synthesis pathway (AANAT and HIOMT), in the developing chicken retina. In addition, our *in vitro* experiments allowed us to define culture conditions that differentially stimulated α -transducin, HIOMT, and red opsin gene transcription in chicken retina precursor cells. Although AANAT gene transcription could not be analyzed in our short-term cultures, other studies have documented the regulation of this gene by light and circadian oscillators after longer culture times [30,31]. It should also be mentioned that a minor proportion of the AANAT mRNA detected *in vivo* might be contributed by retinal ganglion cells, because this cell type was recently shown to express low levels of AANAT [32].

Our *in vivo* experiments indicated that transcriptional activation of the melatonin synthesis genes takes place in a time window very close to that of early opsins gene activation. However, the antecedence of HIOMT gene expression was clear, especially when the center to periphery gradient of differentiation was taken into account. It is tempting to speculate on a possible relationship between this early onset of HIOMT gene transcription and the detoxifying role played by this enzyme in the bis-retinyl arylalkylamine scenario recently proposed by Klein [33] to explain the evolution of photoreceptors and pinealocytes.

The timings of expression of HIOMT and α -transducin genes *in vivo*, between visinin expression at E6 and early opsins at E15 [12], are of special interest because they define intermediate steps in photoreceptor differentiation. Previous studies in mammals have also shown that transcriptional activation of the genes encoding beta-PDE6, opsins, α -, β -, and γ -transducin, arrestin and recoverin occur at several days intervals during retinogenesis [13-15]. In the chicken retina, a more complete study, including a larger number of photoreceptor markers (phosphodiesterase 6, arrestin, phosducin, peripherin, etc.) would still be needed to precisely delineate all the successive steps of specific gene activation. Nevertheless, our data strengthen the view that photoreceptor differentiation, in terms of specific gene activation, is a stepwise process that extends over several days, as previously noted for morphological traits such as the production of outer segments or the establishment of synaptic contacts [11]. As previously stated by Adler [34], this protracted differentiation might represent the execution of an endogenously-timed program initiated at the exit of mitosis, or the successive actions of exogenous signals required to activate each functional gene composing the photoreceptor phenotype. Our *in vitro* experiments suggest that both hypotheses apply, depending on which functional gene is being considered.

The photoreceptor precursor cells placed in culture at E7 appeared to be fated in as much as they continued to express visinin mRNA and were able to activate α -transducin transcription, in the absence of any culture supplement. However, they were unable to activate HIOMT and red opsin gene transcription after two days in basal medium, at least not to a level that would be sufficient for detection by northern blot. The sustained and even increased expression of visinin mRNA in culture is in agreement with previous reports [20]. The activation of α -transducin gene transcription in basal medium is new information, which adds support to previous studies indicating that photoreceptor precursors can achieve a certain degree of phenotypic differentiation in a cell-autonomous manner [35]. The absence of northern blot-detectable red opsin mRNA after culture in basal medium contrasts with previous reports indicating that red opsin-positive cells can be detected by *in situ* hybridization after four days of culture in serum-free medium [20]. The sensitivity cut-off of northern blot analysis and the shorter culture duration used in our experiments (two days rather than four days) may be responsible for these contrasted results. Indeed, our real time PCR analysis confirmed the presence of small amounts of red opsin mRNA in cells cultured in basal medium, but it also showed that serum causes a dramatic increase in the concentration of this transcript (up to 300 fold). This large increase in red opsin mRNA levels, which appeared to be required for detection on northern blot, would represent a more advanced degree of phenotypic differentiation achieved in response to serum factors. Like red opsin, HIOMT mRNA levels were increased by serum to northern blot-detectable levels. This observation generalizes the regulation of HIOMT gene transcription by serum that we previously observed in embryonic chicken pineal gland and in human retinoblastoma Y79 cells [26,36].

One unexpected result of the present study is that all batches of serum were able to increase red opsin mRNA levels, whereas only some of them activated HIOMT gene transcription. This is reminiscent of another study on chicken retina precursor cells, indicating that multiple lots of serum had to be screened to obtain a high proportion of photoreceptors and high levels of AANAT activity [31]. These observations stress the sensitivity of retinal precursor cells to serum composition and should be taken into account in further studies utilizing this culture system. In the present study, the variability among serum batches was meaningful, because it was the first hint at a differential regulation of HIOMT and red opsin gene transcription. This was further supported by the use of an insulin-based culture supplement which activated HIOMT gene transcription without increasing red opsin mRNA levels. The high concentration of insulin required for HIOMT gene activation suggests a nonspecific effect. However, this response was also observed with selenium, which has been characterized as an insulinomimetic in several model systems [37,38] and whose effects have been related to activation of both the PI3K and the MAP kinase pathways, downstream of the insulin receptor [39]. Further studies using insulin-like growth factors and other peptides known to activate the same intracellular signaling pathways should help elucidate the mechanism of

HIOMT gene activation. This would improve our knowledge of signal-dependent gene activation in developing chicken photoreceptors, which was first evidenced with the CNTF-dependent activation of green opsin gene expression [20]. Also of interest is the fact that ITS and NCS activated transcription of promoter-reporter constructs containing both long and short versions of the HIOMT promoter. Although no single consensus insulin-response element has been defined, examination of the HIOMT promoter sequence revealed several regions sharing up to 73% identity with DNA elements previously shown to mediate insulin effects in other gene promoters [40]. Further studies on truncated or mutated versions of the HIOMT promoter should lead to the identification of the cis-regulatory elements involved in this response. Comparative studies with other promoters known to be activated in retinal precursor cells, like the IRBP promoter [41], would also be of interest to elucidate the signaling mechanisms governing functional differentiation of photoreceptors. Ultimately, a comparison with red opsin, transducin, and visinin promoters should be most informative.

Together, the different culture conditions used in the present study have allowed us to selectively activate the transcription of either α -transducin (basal medium), HIOMT (ITS), or red opsin (some lots of FCS), in chicken retinal precursor cells. Although our short-term cultures do not rule out the possibility that photoreceptor precursors might differentiate in a cell-autonomous manner if they were cultured long enough in basal medium, they do indicate that different exogenous signals are involved in activating the different genes encoding photoreceptor specific functions. Significantly, marker genes for light sensitivity (red opsin) and for melatonin synthesis (HIOMT) appear to be activated in response to different signals.

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